REGULATION OF DOPAMINE RELEASE FROM RAT MESENCEPHALIC CELL CULTURES BY EXCITATORY AMINO ACIDS

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ABSTRACT

Excitatory amino acids (EAAs) are known to play an important role in the regulation of dopaminergic neurotransmission. This thesis examined effects of EAAs and modulators of EAA receptor activity on the release of [^{3}H]dopamine ([^{3}H]DA) from dissociated cell cultures of ventral mesencephalon. The results provide evidence that multiple EAA receptor subtypes are present on DA-releasing neurons and participate in regulating this release. NMDA, quisqualate and kainate, preferred agonists for respectively named EAA receptor subtypes, stimulated Ca^{2+}-dependent, tetrodotoxin (TTX)-insensitive [^{3}H]DA release, with 3 distinct profiles of sensitivity to antagonists and of ontogenic development in culture. Low concentrations of the endogenous EAAs, glutamate, homocysteate, homocysteine sulfinate, cysteate and cysteine sulfinate evoked Ca^{2+}-dependent [^{3}H]DA release through activation of NMDA receptors, although non-NMDA receptors mediated effects of these agonists at higher concentrations ($\geq$ 100 μM, depending upon the EAA tested). Aspartate induced [^{3}H]DA release that was mediated solely by the NMDA receptor.

Glycine potentiated NMDA-evoked [^{3}H]DA release when the test was made under nominally Mg^{2+}-free conditions, but caused strychnine-sensitive inhibition of the NMDA response in the presence of 1.2 mM Mg^{2+}. It is suggested that these results demonstrate how voltage-dependent blockade of the NMDA receptor by Mg^{2+} may regulate sensitivity of NMDA responses to convergent inputs.

Micromolar concentrations of the psychotomimetic agent, phencyclidine (PCP) blocked NMDA-evoked [^{3}H]DA release. At higher concentrations ($\geq$ 100 μM), PCP-related compounds stimulated TTX-insensitive [^{3}H]DA release. This

stimulatory effect was not due to actions of these compounds at the PCP/NMDA receptor, sigma binding site, dopamine uptake site, or cation channels for Ca^{2+} or Na^{+}, but may have been caused by K^{+} channel blockade.

Les acides aminés excitateurs (AAEs) jouent un rôle important dans la régulation de la neurotransmission dopaminergique. Dans cette thèse, nous avons étudié les effets des AAEs et de certains modulateurs de ces systèmes sur la libération de la dopamine tritiée ([^{3}H]DA) dans des cellules dissociées du mésencéphale ventral en culture. Les résultats montrent que différents sous-types de récepteurs aux AAEs sont présents sur les neurones dopaminergiques et que ces récepteurs contrôlent la libération de la DA. Le NMDA, le quisqualate et le kainate, les agonistes préférentiels pour chacun des trois sous-types de récepteurs aux AAEs, stimulent la libération de la [^{3}H]DA seulement en présence de calcium et selon un mécanisme résistant à la tétrodotoxine (TTX). De plus, trois profils distincts de sensibilité aux antagonistes et des développements ontogéniques des réponses en culture différents sont observés pour chacun des agonistes. Les AAEs endogènes, comme le glutamate, l'homocystéate, l'homocystéine sulfinate, le cystéate et le cystéine sulfinate, stimulent la libération de la [^{3}H]DA par un mécanisme nécessitant la présence de calcium en agissant à faibles concentrations sur les récepteurs NMDA et à des concentrations plus élevées ($\geq$ 100 μM, en fonction de l'AAE testé) sur les récepteurs non-NMDA. L'aspartate induit la libération de la [^{3}H]DA en agissant exclusivement sur le récepteur NMDA.

La glycine potentialise la libération de la [^{3}H]DA évoquée par le NMDA dans un milieu ne contenant pas de Mg^{2+}. Cependant, elle inhibe, par un mécanisme sensible à l'antagoniste strychnine, la réponse induite par le NMDA en présence de 1.2 mM Mg^{2+}. Ces résultats suggèrent que le blocage voltage-dépendant du récepteur NMDA par le Mg^{2+} peut moduler la sensibilité des réponses NMDA et ce, en fonction des différents stimuli reçues.

A des concentrations micromolaires, la phencyclidine (PCP), un agent psychotomimétique, bloque la libération de la [^{3}H]DA évoquée par le NMDA. Toutefois, aux concentrations plus élevées ($\geq$ 100 μM), les composés de type PCP stimulent la libération de la [^{3}H]DA selon un mécanisme insensible à la TTX . Cet effet stimulateur n'est pas lié à l'interaction de ces composés avec le récepteur PCP/NMDA, le site de fixation sigma, le site de recapture de la DA ou encore les canaux cationiques calciques ou sodiques, mais probablement à une action sur les canaux potassiques.

STATEMENT OF AUTHORSHIP

MANUSCRIPTS AND AUTHORSHIP[a]

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STATEMENT OF CONTRIBUTIONS

This thesis is comprised of five co-authored papers. All experimental work described in the papers was performed by H.M., with the following exceptions:

1. In section 3 (*J. Neurochem.* 52, 1300-1310 [1989]), mesencephalic cell culturing methods in the laboratory were developed jointly by H.M. and S.W.

2. In section 4 (*Synapse* 5, 271-280 [1990]), the dependence of quisqualate- and kainate-evoked [^{3}H]dopamine release on extracellular Na^+ and Cl^- was examined by J.K.-A., in fulfillment of requirements for an undergraduate project.

3. In sections 5, 6 and 7 (*J. Neurochem.* 55, 268-275 [1990]; *Can. J. Physiol. Pharmacol.* 68, 1200-1207 [1990]; *Molec. Pharmacol.* [submitted]), the majority of cell cultures were prepared with the assistance of I.C.

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[a]reprinted from the Guidelines Concerning Thesis Preparation, Faculty of Graduate Studies and Research, McGill University, March 1989.

To Dorien, Jim and Ursula, with love and gratitude...

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1. INTRODUCTION AND LITERATURE REVIEW

Dopamine (DA) is a neurotransmitter that plays a central role in many locomotor behaviors, memory and the rewarding properties of certain stimuli. Because of the functional importance of DA systems and the postulated role of disturbed DA activity in important neurological disorders (*e.g.* Parkinson's disease, schizophrenia), the physiological regulation of DA function (by neurotransmitters and neuromodulators) has become a critical area of pharmacological research. Excitatory amino acids (EAAs) are particularly interesting in this regard, as they provide the principal excitatory inputs to areas of the brain containing DA neuronal terminals or cell bodies. Also, there is considerable evidence that EAAs are important regulators of DA transmission and it has been proposed that dysfunction of EAA inputs may be a factor in neurodegenerative and affective disorders in which DA systems are affected. Still, little is known about the receptor mechanisms underlying interactions of EAAs with DA neurons, the cellular localization of these receptors and the neuromodulatory control of DA release by agents that modify EAA transmission.

This thesis examines acute effects of EAAs and modulators of EAA responses on primary cultures of DA-accumulating cells from the fetal rat brain. The dissociated mesencephalic cell cultures are developed and characterized as a model system for studying these effects. Our working hypothesis has been that DA-releasing cells in culture possess EAA receptors that can be activated to modulate DA release (see also section 2 "Aims and Objectives"). Cell culture techniques are used because of several inherent advantages that complement and extend

experimental possibilities achieved *in vivo* and in tissue slice studies. In particular they provide:

a. an opportunity for precise and accurate control over the composition of the extracellular environment to facilitate the study of cellular and ionic mechanisms underlying the DA release process and effects of EAAs on that release. For studying effects of EAAs, this is an important advantage of cell culture because the ubiquity of EAAs in the CNS and their multiple roles in cellular metabolism (see sections 1.2.1 and 1.2.2) lead to underestimates of their potency due to EAA uptake into other cell types. Diffusion through a neuropil may also result in the EAA-evoked release of intermediate transmitters which in turn modulate release from the DA neuron. These two possibilities are reduced in the dissociated cell culture model, where DA neurons are directly exposed to drugs added to the extracellular milieu (further discussion in section 1.5.1).

b. a reliable system for examining the pharmacology of functional responses (in this thesis, the release of radiolabelled DA) to EAA receptor stimulation. The ionic mechanisms of EAA receptor activation, role of second messenger systems, receptor-selectivity of EAA agonists and antagonists and the modulation of EAA actions by other transmitters may be studied. Use of cultures to explore EAA receptor pharmacology is possible because we have found that the cell cultures possess multiple EAA receptors with many of the pharmacological characteristics exhibited *in vivo* and in other *in vitro* systems.

c. a model for the study of dopaminergic transmission, in which the entire DA neuron (cell body and terminal fields) may be maintained *in vitro* for a prolonged period (several days to weeks) to permit (in future studies) examination of the cellular and molecular basis of long-term modulation of dopaminergic function (*e.g.* sensitization of DA neurons: see section 8 "General Discussion").

In this thesis, the cell culture model has been used to examine the role of EAA receptor subtypes in mediating effects of a range of naturally occurring EAA agonists. The action of phencyclidine (PCP: a psychotomimetic EAA receptor antagonist), and interactive effects of glycine (a potentiating allosteric modulator of EAA receptor function and an inhibitory neurotransmitter in brain areas containing DA cell

bodies) and Mg^{2+} (a voltage-dependent blocker of one EAA receptor subtype) are also explored (see section 2 "Aims and objectives").

The introduction of the thesis overviews well-studied characteristics of dopaminergic systems in the central nervous system (CNS). This is followed by discussion of the role EAAs play as excitatory transmitters in the brain. Evidence of important interactions between EAAs and dopaminergic transmission is then reviewed. Finally, the use of dissociated mesencephalic cell culture systems is discussed.

1.1 Dopamine as neurotransmitter in the CNS

The criteria that must be fulfilled for a substance to be considered a central neurotransmitter (Werman 1966) have been satisfied in the case of DA. To substantiate this notion, it has been demonstrated that:

a. specific neurons have mechanisms for the synthesis and storage of DA (Carlsson et al. 1962; Dahlström and Fuxe 1964; Fuxe 1965; Hartman and Hilaris 1980).

b. depolarization of these neurons results in Ca^{2+}-dependent release of DA (Farnebo and Hamberger 1971; Mulder and Snyder 1976; Patrick and Barchas 1976; Lane and Aprison 1977; Nieoullon et al. 1977*a*,*b*, 1978*a*,*b*).

c. specific mechanisms exist for terminating action of DA (in the case of DA, synaptic actions are principally terminated by reuptake [Iversen 1973; Horn 1979]).

d. exogenous DA mimics the action of the endogenous compound released upon neuronal stimulation and manipulations that affect actions of exogenous DA similarly modify responses to endogenously released DA (Bunney et al. 1973; Kitai et al. 1975,1976; Richardson et al. 1977; Siggins 1978; York 1979; Bunney 1979; Pinock 1983).

The application of these criteria to DA has been dealt with extensively in the literature (see also the historical review by Carlsson 1987) and will not be readdressed here. Since the experiments in this thesis deal with the regulation of DA turnover rather than postsynaptic effects of DA, only the first three of these criteria are discussed in detail in following sections (1.1.1 to 1.1.5).

Historical overview

The first important developments in establishing a role for DA in the CNS came from work with an early neuroleptic agent reserpine, a drug found to act through depletion of noradrenaline from central stores (Holzbauer and Vogt 1956). Carlsson and coworkers observed that administration of the NA precursor, L-dihydroxyphenylalanine (L-DOPA) reversed reserpine-induced sedation and hypokinesis (Carlsson et al. 1957), without enhancing noradrenaline levels, while brain levels of DA, the metabolic intermediate between L-DOPA and noradrenaline, were elevated (Carlsson et al. 1958). The reversal of extrapyramidal motor dysfunction by L-DOPA and the finding that DA was almost completely depleted in the brain of autopsied Parkinsonian patients (Ehringer and Hornykiewicz 1960) led to clinical trials of L-DOPA in treatment of Parkinson's disease in the early 1960s. At the same time, development of fluorescence microscopy (Falck et al. 1962) led to visualization of DA-containing neurons and mapping of DA pathways (Dahlström and Fuxe 1964). Electrophysiological (McLennan and York 1967; Connor 1970) and biochemical (Portig et al. 1968; Faull and Laverty 1969) studies confirmed that DA is the inhibitory transmitter of a major pathway arising mainly from substantia nigra (SN) to striatum, where it provides

as much as 21% of the axon terminals (Pickel et al. 1977). The biochemical and electrophysiological characteristics of pathways from midbrain DA neurons to various limbic and cortical structures have also been described (reviewed by Bannon et al. 1985). Calcium-dependent release of DA was shown to be induced by depolarizing stimuli *in vivo* (Nieoullon et al. 1977*a*,*b*, 1978*a*,*b*) and *in vitro* (Holz 1975; Mulder and Snyder 1976; Lane and Aprison 1977) and two DA receptor subtypes with differing distribution, pharmacological profile and adenylate cyclase linkage were identified (Kebabian and Calne 1979; see also Seeman 1980; Andersen et al. 1990). Putative physiological roles that have been described for nigrostriatal DA systems include modulation of motility, body posture and masticatory activity (reviewed by Costall and Naylor 1979). Mesocorticolimbic DA pathways have been implicated in the activation, regulation and organization of exploratory and locomotor behaviors (Fink and Smith 1980: Teitelbaum et al. 1982), drinking and eating (Ungerstedt 1971*b*; Ljundberg and Ungerstedt 1976), the rewarding properties of certain stimuli (see recent review by Wise and Rompré 1989) and in memory (Beninger 1983). From a clinical perspective, severe degeneration of the nigrostriatal DA pathway has been anatomically and neurochemically defined in Parkinson's disease (reviews by Hornykiewicz 1973, 1979). Also, the antipsychotic efficacy of DA receptor antagonists and post-mortem indications of altered dopaminergic transmission in brains from schizophrenic patients have led to the hypothesis that schizophrenia may be linked to pathological alteration of dopaminergic function (see review by Losonczy et al. 1987).

1.1.1 Anatomy of dopaminergic systems in the CNS

DA was first histochemically visualized in central neurons of the rat brain by Carlsson et al. (1962). The organization of dopaminergic systems (reviewed by Ungerstedt 1971a; Kim 1978; Lindvall and Björklund 1978) was subsequently revealed through fluorescence histochemistry, retrograde horseradish peroxidase tracing and immunohistochemical determination of DA and of the DA-synthesizing enzyme tyrosine hydroxylase (TH), often in combination with mechanical or chemical lesions with the selective neurotoxin for DA neurons, 6-hydroxydopamine (6-OHDA). The major ascending dopaminergic pathways were found to originate from cell bodies in the SN and ventral tegmental area (VTA) regions. In addition, cell bodies in a retrorubral field, between SN and VTA and once thought to constitute an extension of the SN, were subsequently found to have projections that contribute to the innervation of striatal, limbic and allocortical areas as well as to SN and VTA. It is now thought that these retrorubral neurons may be important mediators of direct and indirect interactions between the major DA systems, both at the level of the midbrain cell bodies and in their forebrain terminal fields (reviewed by Deutch et al. 1988).

Projections from midbrain DA neurons are characterized by highly ordered topography. According to traditional concepts and terminology, medial mesencephalic cell bodies of the SN project to the caudate-putamen (nigrostriatal pathway), while cell bodies in the more lateral VTA project to nucleus accumbens, amygdala and olfactory tubercle as well as to cingulate, prefrontal and entorhinal cortices (mesocorticolimbic system) (Ungerstedt 1971a). Dual contributions of SN and VTA neurons to nigrostriatal and mesocorticolimbic systems prompted

some authors to rename ascending midbrain DA projections the mesotelencephalic system and to rename subsystems the mesostriatal and mesocorticolimbic systems. (see Lindvall and Björklund 1978).

The other major DA pathways in the rat CNS are the periventricular, incertohypothalamic, tuberohypophyseal, periglomerular and retinal systems. These pathways are considerably smaller than mesostriatal and mesocorticolimbic systems in terms of length and distribution of axon fibres (see Lindvall and Björklund 1978).

1.1.2 Dopamine synthesis

Brain DA is synthesized from tyrosine. Tyrosine hydroxylase (TH), a mixed function oxidase located in the DA neuronal cytosol, catalyzes the rate-limiting step in catecholamine synthesis (Levitt et al. 1965) - conversion of the amino acid L-tyrosine to L-DOPA, through addition of an hydroxyl group in the meta position. TH requires a reduced pteridine cofactor, that is reoxidized by dihydropteridine reductase.

L-DOPA is converted to DA by L-aromatic amino acid decarboxylase (LAAAD) (Blaschko 1939). This enzyme is very active (100 - 1000 times more than TH) and the conversion proceeds at a fast rate, so that LAAAD is not normally rate-limiting in DA synthesis (Sourkes 1979).

1.1.3 Dopamine storage and release

Storage

DA is present in neurons both free in the cytoplasm and within membrane-bound synaptic vesicles in a concentrated form (Fuxe 1965; Hartman and Hilaris 1980). The storage of DA has been described by invoking an abstract concept of multiple "pools" of DA (Glowinski 1975). This notion implies that different molecules of DA do not behave identically in the neuron. In studies on disappearance of radiolabelled DA following brief exposure of striatal tissue to [^{3}H]tyrosine ("pulse-labelling") (Glowinski 1970; Besson et al. 1973; Gropetti et al. 1977) a biphasic rate of decline in the specific activity of released species was observed (indicating a two-pool kinetic model in which newly synthesized DA and a larger reserve of DA previously formed are differentially eliminated). [^{3}H]DA taken up into the neuron (as studied in the experiments described in this thesis) has been shown to exhibit characteristics similar to those of newly-synthesized DA (de Langen and Mulder 1979; de Langen et al. 1979; Cerrito et al. 1980). Newly-acquired DA (either newly-synthesized or newly-taken up) is preferentially utilized (metabolized or released) (Glowinski 1975). Based partly on these findings and on studies showing reversal of reserpine- or α-methyl-*p*-tyrosine- (α-mpt) induced behavioral effects by very small amounts of DA (Glowinski et al. 1966; Glowinski 1975; Shore and Dorris 1975; Gudelsky and Porter 1979), it has been proposed that newly-acquired DA may constitute the functional pool of transmitter (see Glowinski et al. 1972; Glowinski 1975; McMillen et al. 1980). The pool is likely located in storage vesicles adjacent to the neuronal membrane (Kuczenski 1973; Gershon et al. 1974). A reserve pool of DA, that

pool of DA, that supplies DA to the functional pool as the latter becomes depleted (McMillen et al. 1980), may be associated with older, degenerating vesicles in sites less favourable to extraneuronal release (Dahlstrom and Haggendal 1970).

Release

Transmitter release in response to a depolarizing stimulus, or "stimulus-secretion coupling", first described for noradrenaline release in the adrenal medulla (Douglas 1968), is generally Ca^{2+}-dependent and is one of the primary criteria that must be satisfied by putative neurotransmitters (Orrego 1979). In the case of DA and other catecholamines, a depolarizing stimulus causes DA vesicles to fuse with the presynaptic membrane and discharge quanta of neurotransmitter into the synaptic cleft through an exocytosis. The precise role of Ca^{2+} in the exocytotic process (Blaustein et al. 1981) is unknown, but is hypothesized to be related to mechanisms of membrane adhesiveness, contractility and permeability. Many of the effects of Ca^{2+} are catalyzed by the high-affinity Ca^{2+} receptor protein, calmodulin. The binding of Ca^{2+} to calmodulin has been linked to the phosphorylation of various synaptic proteins (particularly tubulin), leading to membrane fusion (DeLorenzo 1980, 1981), and is thought to be a necessary step for the contractile function of various microtubule and microfilament contractile proteins.

1.1.3.1 Release from terminal fields

Ca^{2+}-dependent DA release induced from central nerve endings by depolarizing agents such as elevated K^+, veratridine or electrical stimulation has been observed both *in vitro* (*e.g.* Farnebo and Hamberger 1971; Mulder and Snyder 1976; Patrick and Barchas 1976; Lane and Aprison 1977) and *in vivo* (Nieoullon et al. 1977*a*,*b*, 1978*a*,*b*). The Ca^{2+}-dependence of this release was further indicated by the findings that the Ca^{2+} ionophore A23187 also evoked DA release (Holz 1975), that *in vitro* depolarization was accompanied by Ca^{2+} uptake into striatal tissue (Blaustein 1975) and that depolarization-evoked DA release could be reduced by Ca^{2+} channel blockers such as high concentrations (10 mM) of Mg^{2+} (Farnebo 1971; Bustos et al. 1981).

That depolarization-induced transmitter release is largely mediated by an exocytotic process of vesicular fusion is supported by electron microscopic observations of synaptic vesicles in nerve endings (Fried and Blaustein 1976). In the case of DA, it is also supported by findings that:

a. both *in vivo* (Imperato and DiChiara 1984) and *in vitro* (Baldessarini 1975) stimulated DA release is reduced by pretreatment of animals with reserpine, an agent that prevents vesicular storage of DA.

b. depolarization-evoked DA release *in vitro* is inhibited by colchicine and vinblastine, agents that disrupt the function of microtubules and actomyosin-like proteins that are believed to function in exocytosis (Nicklas et al. 1973; Baldessarini 1975).

c. depolarization does not cause immediate increase in the extracellular concentrations of deaminated metabolites, suggesting that DA is not released into the cytoplasm (site of deamination) before being released into the synaptic cleft (Holz 1975).

1.1.3.2 Somatodendritic release

It was considered for many years that the release of DA occurred only at nerve terminals. However, DA can also be released from dendrites (see review by Chéramy et al. 1981). The mechanism for this release is apparently similar to that of terminal field release. DA release in the SN may be produced by stimulation of neurons that innervate SN or are involved in sensory motor processes (Nieoullon et al. 1977*b*, 1978*a*,*b*) or by stimulation of the SN itself (Aceves and Cuello 1981). Release is also induced by elevated K^+ (Geffen et al. 1976; Kerwin and Pycock 1979) or veratridine (Tagerud and Cuello 1979) and is reduced in the absence of external Ca^{2+} (Geffen et al. 1976). Furthermore, veratridine-evoked nigral [^{3}H]DA release may be reduced by application of tetrodotoxin (TTX)(Tagerud and Cuello 1979), indicating that DA dendrites possess fast voltage-sensitive Na^+ channels. Various neurotransmitters present at high levels in the SN have been shown to affect nigral release of DA (see section 1.1.5 "Regulation of dopamine release").

1.1.4 Dopamine uptake and catabolism

1.1.4.1 Dopamine uptake

Reuptake into the DA neuron is the major mechanism that terminates the action of DA released into the synapse. Transmitter taken back into the neuron is preferentially re-released (section 1.1.3), or is catabolized (section 1.1.4.2). Reuptake of DA permits use of a simple technique to monitor the DA-releasing effects of various transmitters and depolarizing stimuli in the experiments of this thesis. DA neurons

may be loaded with [^{3}H]DA and the releasing effects of pharmacological manipulations may be assessed by measuring radioactivity in the releasate. DA uptake is an active, temperature-dependent, saturable process (K_m = 0.13 μM and V_{max} = 25.3 pmol/100μg protein/2 min: Holz and Coyle 1974) that requires adenosine triphosphate (ATP), Na^+ and specific carrier molecules on the cell membrane (Coyle and Snyder 1969; Horn et al. 1971; Iversen 1973; Harris and Baldessarini 1973; Holz and Coyle 1974; Horn 1979).

Glial cells have also been shown capable of high affinity DA uptake, but do not release accumulated DA in response to depolarizing stimuli (Pelton et al. 1981; Barochovsky and Bradford 1987*a*). Other cell types may accumulate DA by lower affinity uptake processes. These cells include serotonergic neurons and brain capillary pericytes (Schoep and Azzaro 1982).

Experimentally, DA release is indicated by increased extracellular levels of DA and its metabolites. However, these levels more accurately reflect the net result of release, neuronal and extraneuronal uptake, catabolism, diffusion out of the tissue and binding to receptors and other sites. Thus, agents that block reuptake, by binding to and inhibiting the uptake carrier (*e.g.* cocaine, nomifensine, benztropine, phencyclidine), may cause an elevation of synaptic DA levels that mimics stimulated DA release. Also, inhibition (Bogdanski et al. 1970) and even reversal (Raiteri et al. 1979) of the high affinity presynaptic DA uptake process (*i.e.* a Ca^{2+}-independent release of DA) may be produced *in vitro*. This is accomplished by exposing the DA cell to low extracellular K^+ or by reducing extracellular Na^+ concentrations so as to decrease the inward-directed cellular transmembrane Na^+ ion gradient.

The *in vitro* DA release caused by reversal of the uptake carrier is Ca^{2+}-independent and is blocked by inhibitors of the carrier (Raiteri et al. 1979).

1.1.4.2 Metabolism

Catabolic inactivation of DA is accomplished by pathways involving monoamine oxidase (MAO) and/or catechol-*O*-methyl-transferase (COMT). MAO, which exists in two subtypes, and may be found both inside and outside the neuron (Demarest et al. 1980), deaminates DA, converting it (via the aldehyde intermediate) into 3,4-dihydroxphenylacetic acid (DOPAC). COMT is an enzyme localized exclusively in the extraneuronal compartment, that transfers a methyl group from S-adenosylmethionine to the meta hydroxy group of DA to form 3-methoxytyramine (3-MT), a minor DA metabolite. Extraneuronal DA is also metabolized into homovanillic acid (HVA), either through initial methylation, followed by deamination or by methylation followed by MAO-catalyzed deamination.

1.1.5 Regulation of dopamine release

Two mechanisms exist for the regulation of DA release (see reviews by Glowinski et al. 1979; Chesselet 1984). First, an elevated level of released DA may stimulate DA receptors on the presynaptic membrane to inhibit further transmitter release (autoreceptor inhibition). Second, transmitters other than DA may activate specific receptors on the DA neuron (at the level of the cell body or presynaptic terminals) to either stimulate or inhibit DA release (heteroreceptor regulation).

1.1.5.1. Autoreceptor inhibition

DA agonists and antagonists regulate electrically or K^+-evoked striatal release of DA *in vitro* by a presynaptic autoreceptor mechanism (Farnebo and Hamberger 1971; Westfall et al. 1976; Reimann et al. 1979; Cubeddu and Hoffmann 1982; Hoffmann and Cubeddu 1982; Cubeddu et al. 1983; Iuvone 1984; Parker and Cubeddu 1985) distinct from the mechanism mediating inhibitory effects of DA on DA biosynthesis (Bitran and Bustos 1982). The release-modulating autoreceptors have been pharmacologically characterized as D_2 type DA receptors (Helmreich et al. 1982; Stoof et al. 1982; Lehmann et al. 1983). They develop supersensitivity following chronic exposure to neuroleptics (Nowak et al. 1983) and desensitize rapidly when exposed to endogenous DA (Arbilla et al. 1985).

The presence of DA autoreceptors is considered a characteristic of nigrostriatal and mesolimbic DA systems that is not shared with the mesocortical pathway (reviewed by Bannon and Roth 1983; Roth et al. 1987). This difference may account for the higher rate of transmitter turnover and diminished responsiveness to DA agonists and antagonists (Bannon et al. 1981) as well as the lack of tolerance development following chronic neuroleptic drug administration (Bannon et al. 1982), within prefrontal cortex.

1.1.5.2 Heteroreceptor regulation

In vitro and *in vivo* studies have indicated that a number of transmitters other than DA itself may regulate DA release from striatum, nucleus accumbens, prefrontal cortex and/or olfactory tubercles (*e.g.* Marien et al. 1983; Rowell et al. 1987; Jaffé and Hernandez 1989; Kalivas et al. 1989*b*). These compounds are released from afferent

fibres or intrinsic neurons within these structures or within the ventral mesencephalic area (CNS area containing the SN and VTA). The best-characterized regulators of DA release include classical neurotransmitters (see Glowinski et al. 1979; Langer 1981; Chesselet 1984) [glutamate, glycine, GABA, norepinephrine, serotonin, acetylcholine (muscarinic and nicotinic)] and neuropeptides (reviewed by Chesselet 1984; Kalivas 1985) [tachykinins, enkephalins, cholecystokinin, somatostatin and neurotensin (reviewed by Quirion 1983)]. Certain neurotransmitters [particularly glutamate, GABA, glycine and substance P] that activate receptors near or on dendrites have also been found to regulate dendritic DA release (reviewed by Chéramy et al. 1981; Araneda and Bustos 1989). Evidence for important influences of glutamate on both terminal field and somatodendritic DA release is discussed in section 1.4 "Excitatory amino acid effects on dopaminergic transmission".

1.2 Excitatory amino acids (EAAs) as transmitters in the CNS

Glutamate and the related acidic amino acid, aspartate were first proposed as excitatory transmitter candidates in the CNS 30 years ago (Curtis et al. 1959; Curtis and Watkins 1960; Watkins 1962). A surge of interest in these and other EAAs has occurred more recently, largely in response to three major developments. First, glutamate, aspartate and possibly other EAAs have been found to be transmitters at a majority of excitatory synapses in the vertebrate CNS (reviewed by Cotman et al. 1987). Second, the development of pharmacological agents and manipulations to selectively modulate synaptic actions of EAAs has led

to the identification of EAA receptor subtypes with widespread distributions in the brain (reviewed by Young and Fagg 1990). Third, clinical observations and pharmacological studies with animal models have provided evidence linking EAA systems with a range of neurological diseases and disorders (Meldrum 1985; Choi 1988), possibly including schizophrenia (reviewed by Wachtel and Turski 1990). Use of EAA receptor antagonists has been proposed as adjuvant treatment of Parkinson's disease (Olney et al. 1987a; Klockgether and Turski 1989; Carlsson and Carlsson 1989a; Girault et al. 1990; Schmidt et al. 1990). Treatment with EAA agonists has been suggested in schizophrenia (Carlsson and Carlsson 1990). However, the latter proposal may not adequately take into account a large literature indicating stimulatory effects of EAAs on dopaminergic transmission (see sections 1.4.2 to 1.4.4, the experiments in this thesis and section 8 "General discussion").

EAAs as neurotoxins

In addition to their role as neurotransmitters, EAAs may activate EAA receptors to induce neurotoxic effects when elevated extracellular levels persist for longer than the brief period required for synaptic transmission. Lucas and Newhouse (1957) were the first to show that sustained exposure to physiologically relevant concentrations of glutamate may destroy retinal neurons. These findings were extended to actions of other EAAs on central neurons by the early work of Olney and coworkers, who were also the first to postulate that the neurotoxic actions of EAAs might be the cause of the neuronal cell loss in certain neurodegenerative disorders (Olney and Sharpe 1969; Olney et al. 1971;

Olney and De Gubareff 1978). *In vitro* experiments suggested that EAA neurotoxicity has two temporally distinct phases. The first, characterized by acute neuronal swelling and eventual osmotic lysis, depends on the presence of extracellular Na^+ and Cl^- and can be mimicked by other depolarizing agents (Rothman 1985; Olney et al. 1986; Choi 1987). The second, a delayed phase, is characterized by excessive Ca^{2+} influx, mobilization of intracellular Ca^{2+} stores, activation of second messenger systems, lipases and proteases, generation of free radicals and fatty acids, mitochondrial dysfunction and the depletion of energy stores (reviewed by Rothman and Olney 1987; Choi 1988). These disturbances in turn lead to destruction of the neuronal cytoskeleton and cellular membrane and ultimately to cell death.

Neurotoxic mechanisms may interfere in the measurement of functional responses to EAAs. For example, it is possible that any apparent EAA heteroreceptor-mediated DA release may be the result of an acute excitotoxic mechanism (whereby the release of transmitter results through lysis of DA cells). Such an explanation for EAA-evoked [^{3}H]DA release from mesencephalic cell cultures was examined in the experiments of section 3 "Glutamate stimulation of [^{3}H]dopamine release from dissociated cell cultures of rat ventral mesencephalon".

1.2.1 Dicarboxylic EAAs (glutamate, aspartate)

1.2.1.1 Distribution of pathways

Mapping of EAA pathways by immunohistochemical localization of EAAs or their synthesizing enzymes has been difficult because of the ubiquitous distribution and metabolic roles of EAAs in non-neuronal as

well as neuronal cells. Identification of these pathways has been possible through combined examination of several parameters - high-affinity uptake, Ca^{2+}-dependent release upon depolarization, synthetic enzyme levels and especially application of receptor mapping techniques - before and after lesioning of putative pathways (Watkins and Evans 1981; Fagg and Foster 1983; Fonnum 1984; Cotman et al. 1987).

Most glutamate and aspartate excitatory pathways originate in the neocortex and allocortex. Major glutamate projections are to hippocampus, caudate nucleus, nucleus accumbens, amygdala, superior colliculus, red nucleus, SN, and the pons. Minor glutamate efferents also project from the hippocampus to nucleus accumbens, lateral septum and hypothalamus (Cotman et al. 1987). A dipeptide of glutamate and aspartate, *N*-acetylaspartyl glutamate, has been proposed as an excitatory neurotransmitter in hippocampus (Bernstein et al. 1985) and in corticostriatal excitatory fibers (Koller et al. 1984).

1.2.1.2 Synthesis and storage

There are two distinct synthetic pathways for neurotransmitter glutamate. These pathways are characterized by the prototypic glutamate precursors, glucose and acetate. Glucose is metabolized, mainly within neuronal structures, into 2-oxoglutarate and aspartate (Balazs et al. 1970; Machiyama et al. 1970), with very little production of glutamine. Aspartate and 2-oxoglutarate are in turn converted to glutamate by aspartate aminotransferase, the relevant isoenzyme of which has been preferentially localized to presumed glutamatergic terminals (Altschuler et al. 1982) and has been shown in kinetic studies to be associated more with neuronal than glial structures (Berl and Clarke 1978). Some

additional 2-oxoglutarate derived via pyruvate carboxylase may be transported into neuronal pools from astroglial cells (Shank and Campbell 1982). Other glutamate precursors that are metabolized as glucose include glycerol, lactate, pyruvate, α-ketoglutarate and β-hydroxybutyrate (Clarke et al. 1975).

Acetate is converted into glutamine by glutamine synthetase, an enzyme that has been localized immunohistochemically in astroglial cells (Norenberg and Martinez-Hernandez 1979). Precusors that follow the pathway characterized by acetate are propionate, butyrate, citrate, leucine, GABA, aspartate, protein, and ammonia (Clarke et al. 1975). Glutamine from glial cells is converted to glutamate by phosphate-activated glutaminase, a mitochondrial enzyme preferentially localized in nerve terminals (Bradford and Ward 1976). Thus, astroglial cells provide glutamine for transmitter glutamate pools.

The relative importance of astroglial versus neuronal metabolism in the production of transmitter glutamate has not been satisfactorily resolved (Fonnum 1984). Owing to rapid diffusion of glutamate and ammonia, the end product inhibition of glutaminase was lost in experiments designed to address this question *in vitro* (Bradford et al. 1978; Hamberger et al. 1979*a*,*b*). This problem may have led to overestimation of the glial contribution to glutamate synthesis. However, it does appear that transmitter glutamate synthesis involves exchanges between astroglial and neuronal precursor pools and that synthesis is not a rate-limiting factor under normal conditions.

Estimates of glutamate and aspartate concentrations in the brain are 5 to 10 mM and 0.5 to 4 mM, respectively (Fonnum 1984; Robinson and Coyle 1987) and show little regional variation, presumably because of

many roles played by glutamate in cellular, and specifically neuronal function. For example, glutamate is a precursor for synthesis of important proteins and peptides including glutathione (Meister 1979) and for the inhibitory neurotransmitter, γ-aminobutyric acid (GABA) (Roberts and Frankel 1950) and also serves in the fixation of ammonia (Weil-Malherbe 1950). Glutamate is greatly enriched (10-fold relative to total tissue levels) in purified synaptic vesicles and the reduction in glutamate levels observed in lesion studies suggests that intraneuronal concentrations of glutamate may exceed 100 mM (see reviews by Fagg and Foster 1983; Robinson and Coyle 1987). Extraneuronal "free" concentrations of glutamate and aspartate in rat ventral tegmentum are 9 and 5 μm/g wet wt. (Gundlach and Beart 1982), while glutamate levels of 20 μM have been reported in human cerebrospinal fluid (Kim et al. 1981).

1.2.1.3 Depolarization-evoked release

Ca^{2+}-dependent release of glutamate or aspartate has been reported from brain slices, synaptosomes or neuronal cultures in response to high K^+ (Nadler et al. 1977, 1978; Reubi and Cuénod 1979; Rowlands and Roberts 1980; Pearce and Dutton 1981) or electrical field stimulation (De Belleroche and Bradford 1972; Potashner 1978) and *in vivo* by a push-pull cannula method (Girault et al. 1986). The Ca^{2+}-dependence of glutamate release was further indicated by observations that A23187 or Ca^{2+} evoked transmitter release (Levi et al. 1976). Glutamate and other transmitter amino acids are also released from glial cell preparations, although this release is Ca^{2+}-independent (Blaustein 1975; Weinreich and Hammerschlag 1975; Sellström and Hamberger 1977).

1.2.1.4 Reuptake

Uptake of EAAs into neurons and glia (Henn and Hamberger 1971; Hökfelt and Ljungdahl 1972; Hertz et al. 1979) is the main mechanism for the termination of transmitter action. Administration of the uptake inhibitors, L-glutamate dimethylester or D,L-*threo*-3-hydroxyaspartate prolongs the excitatory action of glutamate (Haldeman and McLennan 1973; Johnston et al. 1980). L-Glutamate and D- and L-aspartate are subject to uptake by a common high affinity transport process (Logan and Snyder 1971; Wofsey et al. 1971) that shows absolute dependence on Na^+ (Bennet et al. 1973) and also by a high affinity Cl^--dependent uptake process (Zaczek et al. 1987; Kessler et al. 1987). By exploiting its affinity for the uptake carrier(s), metabolically inactive D-aspartate has been used as a false transmitter to investigate the operational characteristics of EAA uptake and release (Minc-Golomb et al. 1987, 1989).

When taken into glia, glutamate is reconverted into glutamine which then diffuses out to neuron terminals and is hydrolysed to provide a source of transmitter glutamate - the "glutamine cycle" (Shank and Aprison 1981). The uptake by glial cells may change the effective concentration of EAA necessary to elicit a given response. This change is most noticeable *in situ* and in tissue slices (Mayer and Westbrook 1987). The problem is reduced in cell culture preparations (as in the experiments of this thesis), where the concentration of EAA bathing the neuronal surface may be more accurately controlled. Still, in the studies presented in this thesis, the effect of removing Na^+ and Cl^- from the extracellular milieu was assessed (sections 3 and 4) partly so as to evaluate whether uptake altered the responses to EAAs.

1.2.2 Sulfur-containing endogenous EAAs

Although there is much evidence pointing to glutamate and aspartate as the excitatory transmitters in many corticofugal projections, other endogenous EAAs may also be excitatory transmitter(s) in these pathways. Also, the identity of transmitters in less well-characterized excitatory fibers (*e.g.* thalamostriatal and thalamocortical projections) remains unclear. Recent attention has focussed on endogenous sulfur-containing amino acids L-cysteic acid [CA] and L-cysteine sulfinic acid [CSA] which are synthesized from methionine via a transsulfuration pathway through cysteine, and also on L-homocysteic acid [HCA], and L-homocysteine sulfinic acid [HCSA]) which are produced by an analogous pathway passing from methionine through homocysteine, (Do et al. 1988). All four of these EAAs:

a. have been detected in various parts of the brain, including neocortex, hippocampus, mesodiencephalon and striatum (Récasens et al. 1982; Do et al. 1986*a*,*b*,1988).

b. are released from these areas in a Ca^{2+}-dependent manner upon high K^+ depolarization (Baba et al. 1983; Do et al. 1986*a*,*b*, 1988).

c. are excitatory in spinal cord and central neurons (Curtis and Watkins 1960; Mewett et al. 1983; MacDonald and Wojtowicz 1982; Mayer and Westbrook 1984; Thomson 1986; Do et al. 1986*a*; Cuénod et al. 1986; Turski et al. 1987*b*).

d. evoke Ca^{2+}-dependent transmitter release from cortical neurons and cerebellar granule cells in culture (Dunlop et al. 1989).

e. are taken up by the same Na^+-dependent high affinity carrier as glutamate and aspartate (Cox et al. 1977; Iwata et al. 1982; Erecinska and Troeger 1986; Wilson and Patuszko 1986) and CA has also been shown to be a substrate for the Cl^--dependent glutamate uptake system (Koyama et al. 1989).

1.3 EAA receptors

Synaptic responses to EAAs are mediated by several receptor subtypes with distinct electrophysiological properties and distributions in the CNS. Until recently, three major EAA receptor subtypes were known. They were named after *N*-methyl-D-aspartate (NMDA), quisqualate and kainate, preferred agonists in a variety of electrophysiological and neurochemical studies (Mayer and Westbrook 1987). NMDA receptor binding was shown to open a membrane channel characterized by high conductance (main state about 50 pS) resulting in cation currents which excluded large organic ions (*e.g.* Tris and choline [Mayer and Westbrook 1987]), but allowed free passage of Na^+, Ca^{2+}. Quisqualate and kainate were found to produce fast responses mediated by membrane Na^+ channels (both have main conductance states of about 20 pS) that exclude Ca^{2+} ions (Ascher and Nowak 1988; Cull-Candy et al., 1988). A fourth receptor was proposed on the basis of potent (low micromolar) antagonist properties of 2-amino-4-phosphonobutyrate (APB) at a subpopulation of excitatory synapses (see Foster and Fagg 1984; Cotman and Iversen 1987; Monaghan et al. 1989). However, it has not yet been possible to identify a membrane binding site for APB. A Cl^--dependent [^{3}H]glutamate binding site which was thought to represent the APB receptor, was subsequently found to correspond to the Cl^--dependent EAA transport system, rather than to a synaptic receptor (Foster and Fagg 1984; Monaghan et al. 1989)

This receptor classification is now being revised to accomodate new receptor subtypes (see recent reviews by Watkins et al. 1990; Young and Fagg 1990). A metabotropic quisqualate-preferring receptor linked to inositol phosphate formation (Sladeczek et al. 1985; Sugiyama et al. 1987; Récasens et al. 1987; Weiss 1989) has been distinguished from the

ionotropic quisqualate receptor which is activated more selectively by α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) than by quisqualate itself. The latter has been appropriately renamed the AMPA receptor (Monaghan et al. 1989; Watkins et al. 1990).

It has been difficult to pharmacologically distinguish quisqualate/AMPA- from kainate-activated responses and considerable physiological evidence suggest that kainate and quisqualate receptors may act very similarly in many brain areas. These receptors are therefore often collectively referred to as "non-NMDA receptors". While patch-clamp study of cultured neurons have revealed differences in the average level of unitary conductance (higher with quisqualate/AMPA) and the probability of channel opening (higher with kainate), it has been proposed that these may correspond to subconductance states of a single EAA receptor supramolecular complex, encompassing both NMDA and non-NMDA receptor subtypes (Cull-Candy and Usowicz 1987; Jahr and Stevens 1987). This hypothesis has generated some controversy, but is now considered untenable for at least three reasons:

a. in certain cases, kainate may be 100 times more potent than AMPA in generating responses (*e.g.* in primary afferent C-fibres [Agrawal and Evans 1986]).

b. excitation produced by kainate and the kainate receptor-selective agonist, domoate does not desensitize. However, responses to quisqualate and AMPA show rapid desensitization and this desensitization, unlike that produced by NMDA, can be prevented by coapplication of the lectin concanavalin A (Mayer and Vyklicky 1989).

c. a functional kainate receptor has been cloned that is insensitive to both quisqualate and NMDA (Hollmann et al. 1989).

1.3.1 NMDA receptor

The NMDA receptor has been more thoroughly studied than have other EAA receptor subtypes. Several features of the modulation of NMDA receptor activity deserve particular attention (see recent review by Wood et al. 1990) and are described below. These are the blockade of NMDA receptor activity by channel blocking divalent cations (Mg^{2+}, Zn^{2+}) and drugs (phencyclidine [PCP], (+)-5-methyl-10,11-dihydroxy-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine [MK-801; dizocilpine]), and the NMDA potentiating effect of glycine. In addition, there is recent evidence that endogenous polyamines might modulate NMDA responses and suggestions that subtypes of NMDA receptors may exist (reviewed by Wood et al. 1990).

1.3.1.1 Effect of magnesium

NMDA receptor activation depends strongly on the voltage of the postsynaptic cell (MacDonald and Wojtowicz 1982; MacDonald et al. 1982). The voltage-dependence of channel opening has been linked to blockade of the open channel by Mg^{2+} ions. While NMDA receptor binding causes channel opening, at negative transmembrane potentials, the channel is immediately blocked by physiological concentrations of Mg^{2+} (0.8 to 1.2 mM). At more positive voltages, the Mg^{2+} ions are driven out of the channel, allowing free passage of Na^{+} and Ca^{2+} into the cell (Ault et al. 1980; Davies and Watkins 1980; Nowak et al. 1984; Mayer and Westbrook 1985). The ability of Mg^{2+} to produce voltage-dependent blockade of the NMDA receptor allows the receptor to act as an "input-sensitive amplifier of excitatory synaptic responses" (Young and Fagg

1990) (*i.e.* a small depolarizing input may alleviate the Mg^{2+} blockade so as to permit activation of an NMDA response).

1.3.1.2 Effect of zinc

Activity through the NMDA receptor channel is also blocked by micromolar concentrations of Zn^{2+}, at a site distinct from the one at which Mg^{2+} acts (Westbrook and Mayer 1987; Peters et al. 1987; Forsythe et al. 1988). Zinc antagonism of the NMDA receptor, unlike the blockade by Mg^{2+}, is not relieved by depolarization of the neuron (Westbrook and Mayer 1987).

1.3.1.3 Competitive antagonists

Pharmacological characterization of the NMDA receptor has been facilitated by the early availability of competitive EAA receptor antagonists with selectivity for the NMDA receptor. The first of these found to have some selectivity for NMDA responses were phosphonate-containing glutamate analogs including D-2-amino-5-phosphonopentanoate (APV; Davies and Watkins 1982; Evans et al. 1982) and D-2-amino-7-phosphonoheptanoate (APH; Perkins et al. 1982) . A more recently described NMDA antagonist of this class, 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP; Davies et al. 1986; Lehmann et al. 1987) is the most selective competitive NMDA receptor antagonist now widely available.

1.3.1.4 Uncompetitive antagonists

NMDA receptors are blocked by phencyclidine (PCP) and related dissociative anaesthetics (see recent review by Lodge and Johnson 1990)

at a site within the NMDA receptor ionophore that is distinct from the binding sites for Mg^{2+} and Zn^{2+} (MacDonald and Nowak 1990). This antagonism is generally referred to as "noncompetitive" (Kemp et al. 1987; Lodge and Johnson 1990), but has also been called "uncompetitive" (Pennefather and Quastel 1982), since the degree of block *in vitro* is directly related to the presence of the agonist (Honey et al. 1985; MacDonald et al. 1987). A similar use-dependent blockade of the receptor is produced by the dibenzocycloheptenеimine, MK-801 (Huettner and Bean 1988), the most potent NMDA antagonist within this class of compounds (Wong et al. 1986).

The PCP binding site associated with the NMDA receptor is also known as the PCP receptor (Quirion et al. 1987) and is only one of the many molecular targets for PCP within the CNS (see reviews by Quirion 1986; Contreras et al. 1987). PCP is also a potent ligand at the sigma binding site (Quirion et al. 1987) and is an antagonist at K^+, Na^+, Ca^{2+} and Ca^{2+}-dependent K^+ channels as well as at the neuronal high affinity DA uptake site (reviewed by Lazdunski et al. 1983).

It has been recently proposed that a unique "NMDA-uncoupled" PCP receptor might exist in rodent cortex (Wood and Rao 1989; Rao et al. 1988, 1989, 1990). The evidence for such a site is that PCP, ketamine and MK-801 stimulated cortical DA release (section 1.4.6.1 "Phencyclidine and dopamine"), while competitive NMDA receptor antagonists and the sigma ligand, rimcazole were inactive (Wood and Rao 1989). However, it has not yet been determined whether this site might correspond to one of the other known molecular targets for PCP in the CNS (*e.g.* a cation channel or the dopamine uptake site).

1.3.1.5 Glycine and glycine antagonists

Low micromolar concentrations of glycine activate a strychnine-insensitive glycine binding site allosterically associated with the NMDA receptor to potentiate responses to NMDA (Johnson and Ascher 1987; Reynolds et al. 1987; Bonhaus et al. 1989). It is now thought that glycine acts as a coagonist with NMDA, as it has been shown in isolated cell preparations that the modulatory site must be occupied for NMDA currents to be elicited (Forsythe et al. 1988; Reynolds and Miller 1988; review by Thomson 1989). A recent study of NMDA receptors expressed in *Xenopus* oocytes has indicated that the role of glycine is to decrease NMDA receptor desensitization in addition to enabling channel opening by the NMDA agonist (Lerma et al. 1990).

Several noncompetive NMDA receptor antagonists have been found to act through the strychnine-insensitive glycine site on the NMDA receptor complex. Kynurenate, a broad-spectrum EAA antagonist that blocks responses at quisqualate/AMPA and kainate receptors (Ganong et al. 1983; Jahr and Jessel 1985), has been found to at least partly antagonize NMDA responses through competitive antagonism of the glycine binding site (Birch et al. 1988; Bertolino et al. 1989). However, a derivative of this compound, 7-chlorokynurenate, may be a more potent antagonist at the glycine site and a much weaker antagonist of the quisqualate/AMPA and kainate receptors than kynurenate (Frey et al. 1987; Kemp et al. 1988). 7-chlorokynurenate and 3-amino-1-hydroxy-2-pyrrolidone (HA-966), a less potent NMDA antagonist that also has this action (Kemp et al 1988; Donald et al. 1988), are the most selective antagonists currently available for the strychnine-insensitive glycine site. Finally, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 6-cyano-7-nitroquinoxaline-2,3-

dione (CNQX), initially heralded as selective non-NMDA receptor antagonists (Honoré et al. 1988), while remaining the most potent non-NMDA receptor antagonists currently available, have also been found to be potent antagonists of this glycine site (Birch et al. 1988; Foster 1988).

1.3.2 Quisqualate ionotropic and metabotropic receptors

Quisqualate receptors have been recently subdivided into two distinct receptor subtypes. An ionotropic receptor activated by quisqualate and more selectively, by AMPA has been renamed the AMPA receptor (Monaghan et al. 1989). AMPA is without effect on membrane binding sites radiolabelled by NMDA and kainate receptor ligands. The AMPA receptor is thought to mediate a fast component of EPSPs in many central excitatory pathways and has therefore been called a "general purpose" EAA receptor (Young and Fagg 1990). No specific antagonists for this receptor have been identified. However, certain analogues of kynurenic acid, and the quinoxalinediones, DNQX and CNQX, have some selectivity for [^{3}H]AMPA binding sites and block AMPA- and quisqualate-evoked excitation (Honoré et al. 1988). Activity at the AMPA receptor is potentiated by Zn^{2+} (Peters et al. 1987), further distinquishing this receptor from the NMDA receptor.

A "metabotropic" quisqualate receptor, linked to a delayed second messenger response involving generation of 1,4,5-trisphosphate (IP_3), mobilization of intracellular Ca^{2+}, and activation of protein kinase C, has been recently identified (Sladeczek et al. 1985; Récasens et al. 1987; Sugiyama et al. 1987; Doble and Perrier 1989). The metabotropic

receptor is activated by glutamate, quisqualate, ibotenate and trans-1-aminocyclopentane-1,3-dicarboxylate (ACPD), but not by NMDA, AMPA or kainate (Watkins et al. 1990). Antagonists at the AMPA receptor (Weiss 1989) or NMDA receptor (Sladeczek et al. 1985) are ineffective at this site. However, activation of the metabotropic receptor may be blocked by pertussis toxin (Wroblewski et al. 1987) or L-serine-*O*-phosphate (Doble and Perrier 1989). Thus, the action of glutamate at the metabotropic receptor is distinct from a previously reported inhibition of carbachol-stimulated IP_3 turnover, which has the pharmacological profile of an NMDA receptor (Baudry et al. 1986).

1.3.3 Kainate receptor

The neurophysiological properties of the kainate receptor are poorly understood, largely due to the difficulty in pharmacologically distinguishing this receptor from the AMPA receptor. Like the AMPA and metabotropic quisqualate receptors, the kainate receptor is insensitive to NMDA receptor antagonists, APV, APH, CPP, PCP and MK-801. No potent and specific antagonists of the kainate receptor have yet been identified. However, the EAA receptor antagonists, 6-D-glutamylaminomethylsulphonate (GAMS) (Croucher et al. 1984; Jones et al. 1984) has shown some selectivity for kainate responses relative to those mediated by quisqualate/AMPA receptors in cat spinal cord (Davies and Watkins 1985) and cultured rat cortical neurons (Drejer et al. 1987). In several studies, the broad spectrum EAA antagonists, kynurenate and *cis*-2,3-piperidine dicarboxylic acid (PDA; Watkins and Evans 1981) have also been found to preferentially inhibit kainate responses (Coleman et

al. 1986; Gallo et al. 1987*a*,*b*, 1989). Responses at the kainate receptor are further distinguished from quisqualate/AMPA and NMDA responses by insensitivity to Zn^{2+} (Peters et al. 1987).

The kainate receptor is also distinguished from other EAA receptor subtypes in that no endogenous ligand has yet been identified for this site. However, domoic acid, a neurotoxin isolated from the sea algae, *Chondria armata*, may be a more specific agonist for kainate receptors than kainate itself.

1.3.4 Receptor mechanisms for endogenous EAAs

Glutamate, aspartate, CA, CSA, HCA and HCSA, are considered "mixed agonists" (Mayer and Westbrook 1984, 1987; Watkins et al. 1990). Unlike the prototypic selective agonists quisqualate, kainate and NMDA, these endogenous EAAs evoke responses that are only partially susceptible to blockade by selective NMDA receptor antagonists (Watkins and Evans 1981). Thus, an understanding of excitatory transmission under physiological conditions in systems where multiple EAA receptors and agonists are present demands that the relative importance of NMDA and non-NMDA receptor activation be clarified for responses to glutamate, aspartate and a widening list of putative neurotransmitters. One of the aims of the experiments reported in this thesis (section 5), was to characterize EAA receptor-involvement in responses to naturally occurring EAAs in the mesencephalic cell culture model.

Glutamate responses were initially thought to be mediated predominantly by quisqualate receptors (Davies et al. 1982; McLennan 1983). More recently, current-voltage relationships and partial

antagonism of glutamate responses by APV in voltage clamp experiments on cultured spinal cord neurons (Mayer and Westbrook 1984), as well as the high affinity of glutamate for each of NMDA, quisqualate/AMPA and kainate receptors in binding studies (Murphy et al. 1987; Pullan et al. 1987; Olverman et al. 1988) have made it difficult to identify a preferred receptor subtype for this ligand. It has been suggested that in some systems where multiple EAA receptors are present, low concentrations of glutamate may interact preferentially with NMDA receptors, while high concentrations act at non-NMDA receptors (Mayer and Westbrook 1987).

L-Aspartate acts at both APV-sensitive and APV-insensitive sites in slices or cerebral cortex (Harrison and Simmonds 1985; Surtees and Collins 1985). However, in spinal cord neurons aspartate produced NMDA-like depolarizations, suggesting that it may have higher affinity for NMDA receptors than for non-NMDA sites (Mayer and Westbrook 1984).

Until recently, the EAA receptor mechanisms underlying actions of CA, CSA and HCSA have not, received much attention, but are thought to involve both NMDA and non-NMDA components (Pullan et al. 1987). By comparison, HCA has been extensively studied and there is a controversial proposal that HCA may be the endogenous ligand for the NMDA receptor in certain systems (Knöpfel et al. 1987; Lehman et al. 1988). It is generally agreed that exogenous HCA produces depolarizing shifts similar to those produced by aspartate or NMDA (MacDonald and Wojtowicz 1982; Herrling et al. 1983; Mayer and Westbrook 1984; Do et al. 1986*a*). In systems where NMDA, quisqualate and kainate receptors produce different responses, electrophysiological (Do et al. 1986*a*) and excitotoxic (Olney et al. 1987*b*) actions of HCA were found to be

selectively mediated by NMDA receptors. In neocortical neurons responses to HCA were actually more affected by NMDA receptor antagonists than were glutamate-induced depolarizations (Knöpfel et al. 1987). However, in other systems, HCA has been found to produce actions like quisqualate (but not kainate) that do not resemble NMDA responses (Davies et al 1985; Gulat-Marnay et al. 1987). Also, in binding studies, HCA interacts with quisqualate/AMPA-type receptors with the same potency as at the NMDA receptor (Murphy and Williams 1987; Murphy et al. 1987; Olverman et al. 1988).

1.4 EAA effects on dopaminergic transmission

Anatomical, electrophysiological and neurochemical studies have pointed to major regulatory effects of EAAs on dopaminergic transmission (sections 1.4.1 and 1.4.2). The physiological relevance of this regulation is supported by behavioral studies (section 1.4.3). Synaptic mechanisms underlying regulation of terminal field and somatodendritic DA release by EAAs (sections 1.4.4 and 1.4.5) have been investigated *in vitro* in tissue slice and synaptosomal preparations, and *in vivo* by push-pull canula, microdialysis and voltametric techniques. These studies have yielded some conflicting results, as outlined in the following sections (1.4.3, 1.4.4 and 1.4.5). In particular, questions remain concerning:

a. characterization of EAA receptor subtypes mediating the interaction with DA.

b. synaptic localization of these receptors.

c. identification of endogenous transmitter candidates that act at these receptors.

d. effects of endogenous and exogenous modulators of EAA receptor pharmacology (*e.g.* Mg^{2+}, Zn^{2+}, glycine, and EAA receptor antagonists, including PCP) on DA transmission.

A major aim of the experiments reported in this thesis was to clarify these issues (see section 2).

1.4.1 Distribution of EAA pathways in dopaminergic brain regions

1.4.1.1 Projections to striatum, nucleus accumbens and olfactory tubercles

Corticostriatal projections are the main input to the neostriatum (Graybiel and Ragsdale 1979) and are excitatory (Spencer 1976). The following evidence indicates that EAAs are the neurotransmitters of these excitatory inputs. Cortical lesions reduce striatal glutamate tissue levels (Kim et al. 1977; Fonnum et al. 1981*a*,*b*; Sandberg et al. 1985), high affinity uptake (Divac et al. 1977; McGeer et al. 1977; Carter 198[illegible], 1982; Young et al. 1981; Walaas 1981; Fonnum et al. 1981*a*,*b*) synthesis and release (Reubi and Cuénod 1979; Rowlands and Roberts 1980; Druce et al. 1982) and Ca^{2+}-dependent K^{+}-stimulated glutamate release *in vivo* (Girault et al. 1986). Parallel findings have been reported for aspartate (Druce et al. 1982; Sandberg et al. 1985; Girault et al. 1986). Electrical stimulation of the frontal (Godukhin et al. 1980), visual and motor cortices (Nieoullon et al. 1978*a*) has been shown to evoke the release of radiolabelled L-glutamate from the ipsilateral striatum *in vivo*. The excitation of striatal neurons elicited by cortical stimulation, or by iontophoretic application of glutamate were largely suppressed by the EAA receptor antagonists glutamate diethyl ester (GDEE) (Spencer 1976).

The nucleus accumbens also receives cortical input from both the allocortex and neocortex, which may be glutamatergic (Walaas and Fonnum 1979; Carter 1980). Lesions of the frontal cortex result in reductions in the high affinity uptake of glutamate in nucleus accumbens (Walaas and Fonnum 1979; Carter 1980; Walaas 1981) and olfactory tubercles (Fonnum et al. 1981a).

1.4.1.2 Projections to substantia nigra and VTA

The SN and VTA receive excitatory afferents from the cortex (Carter 1982; Nieoullon and Dusticier 1983). Lesions of cortical areas result in reductions in nigral glutamate content (Kornhuber et al. 1984) and uptake (Kerkerian et al. 1983; Nieoullon and Dusticier 1983), and of aspartate levels and uptake in VTA (Christie et al. 1985). Electrical stimulation of the visual and motor cortices induces release of radiolabelled L-glutamate from SN *in vivo* (Nieoullon et al. 1978a). There is also electrophysiological and pharmacological evidence for a minor glutamate pathway projecting from the pedunculopontine nucleus to SN pars compacta (Scarnatti et al. 1984, 1986, 1987). In addition, both SN and VTA regions contain low to moderate density of glutamate-immunoreactive neuronal perikarya (Ottersen and Storm-Mathisen 1984).

1.4.2 Electrophysiological effects of EAAs on dopamine neurons

Iontophoretic administration of glutamate increases the firing frequency of DA neurons in both the SN and VTA (Scarnati and Pacitti 1982; Grace and Bunney 1984*a*,*b*, 1986). EAA receptor antagonists may also modulate DA neuron firing. For example, 2-amino-6-

trifluoromethoxybenzothiazole (PK 26124), a novel anticonvulsant found to block neuronal excitation mediated by EAAs, decreased the firing of spontaneously active neurons in both the nigrostriatal and mesocorticolimbic systems of the rat *in vivo*, resulting in decreased release of DA from both terminal fields and cell body regions (Coston et al. 1986).

1.4.3 Behavioral effects of EAAs on dopamine systems

Studies of stereotypy (gnawing, licking, and sniffing) and/or locomotor behaviors after local or systemic application of EAA agonists or antagonists provide behavioral/physiological evidence for modulatory effects of EAAs on dopaminergic function (see sections 1.4.3.1 to 1.4.3.3). However, DA systems may not be involved in all of these behavioral effects of EAA agonists and antagonists. For example, MK-801 caused pronounced, neuroleptic-resistant locomotor stimulation in rats (Raffa et al. 1989) and MK-801, PCP and ketamine produced similar effects in mice depleted of monoaminergic stores by pretreatment with reserpine or α-mpt (Carlsson and Carlsson 1989*a*,*b*, 1990).

1.4.3.1 Administration into striatum

Microinjection of AMPA into striata of rats forced to swim in a water basin, abolished an escape response that was found sensitive to agonists and antagonists of DA transmission (Cools and Peeters 1987). The effect of AMPA was facilitated by low concentrations of apomorphine, was completely inhibited by kynurenate or GDEE and was potently affected, but only partially blocked by APH. In addition, haloperidol-

sensitive enhancement of stereotyped sniffing (but not sterotyped oral movements) was induced by local injection of APV (Schmidt 1986). These data may be interpreted as indicating a role for both NMDA and non-NMDA receptors (possibly of the quisqualate/AMPA subtype) in mediating striatal dopaminergic transmission. The report by Cools and Peeters (1987) suggests that activation of NMDA and non-NMDA receptors leads to DA-mimetic behavioral effects. Conversely, in the study by Schmidt (1986) the NMDA receptor-mediated effect of glutamate was opposite to that of DA. Together, these results are consistent with a synaptic localization of EAA receptors on both DA terminals and inhibitory interneurons in the striatum (see section 1.4.5.1).

1.4.3.2 Administration into nucleus accumbens

Bilateral microinjections of NMDA, quisqualate, or kainate into the nucleus accumbens significantly increased locomotion in rats (Donzanti and Uretsky 1983, 1984). These effects were attenuated by pretreatment with reserpine, or the DA receptor antagonist haloperidol, suggesting the mediation of accumbal DA activity (Donzanti and Uretsky 1983). Similar observations with NMDA, quisqualate, kainate and folates containing a glutamate moiety were reported by Stephens et al. (1986). Blockade of the hypermotility by NMDA and non-NMDA antagonists indicated that multiple EAA receptor subtypes were involved in these locomotor effects (Donzanti and Uretsky 1984; Stephens et al. 1986). However, the specificity of EAA effects is called into question by the finding that NMDA was equally potent in inducing locomotor behavior when injections were made into the lateral ventricle (O'Neill et al. 1986). Further, the involvement of dopaminergic systems may be challenged because the

pattern of locomotor behavior induced by NMDA administration into either nucleus accumbens or cerebral ventricle (episodic wild running) fundamentally differs from the behavior induced by d-amphetamine (generalized locomotor activation and exploratory activity) (O'Neill et al. 1986).

1.4.3.3 Administration into substantia nigra or VTA

Bilateral acute application of either NMDA or kainate to the SN pars compacta or VTA, enhanced locomotor activity and sniffing in the rat (Pycock and Dawbarn 1980). A greater sniffing response was seen when injections were made into SN pars compacta, while the locomotor response was more marked after intra-VTA injection. This selectivity is consistent with the general notion that nigrostriatal and mesolimbic DA pathways mediate predominantly stereotyped and locomotor responses respectively (Costall et al. 1977). Both responses were blocked by prior systemic administration of the DA receptor blocker, fluphenazine, suggesting that they were at least partly mediated by increases in dopaminergic transmission.

In some studies, injections of kainate or NMDA into SN produced no significant behavioral alterations (Jackson and Kelly 1984; Turski et al. 1987*a*), or inhibition of locomotor activity (Wirtshafter and McWilliams 1987). In at least one of these studies, the site of injection was clearly in SN pars reticulata, caudal to the SN pars compacta (Turski et al. 1987*a*). Administration of NMDA or kainate into this structure has been shown to induce sedation and catalepsy (Pycock and Dawbarn 1980). These effects may be related to EAA activation of inhibitory nigral interneurons (perhaps GABAergic) that regulate

nigrostriatal DA transmission. This hypothesis is supported by the differential effects of 6-OHDA and elecrolytic lesions of SN. Thus, while unilateral injection of 6-OHDA produces a turning of the body toward the side of the lesion (Ungerstedt and Arbuthnott 1970), a non-selective electrolytic lesion of the SN causes turning that is contralateral to the side of the lesion (Costall et al. 1976; Schwartz et al. 1976). Olianas et al. (1978) demonstrated that unilateral microinjection of kainate into the SN pars reticulata region induced an acute episode of ipsilateral and contralateral circling, followed by a chronic contralateral circling behavior. The contralateral component of the acute turning response was abolished by previous 6-OHDA lesioning of DA neurons in the SN. However, the ipsilateral turning and chronic contralateral turning were unaffected by 6-OHDA lesions and thus appeared to be independent of altered nigrostriatal DA neurotransmission. The ipsilateral turning may be explained by an initial stimulatory effect of kainate on a neuron inhibitory to the DA cell, while the chronic contralateral turning might reflect the destruction of this inhibitory input.

One recent study provides the clearest demonstration of correlations between the behavioral effects of EAA injection into the VTA and DA turnover in mesocorticolimbic systems (Kalivas et al. 1989*b*). In this study, a stimulation of locomotor activity was produced by intra-VTA injection of glutamate or kainate, but not NMDA or quisqualate. The behavioral activation induced by glutamate was blocked by pretreatment with haloperidol and was accompanied by an increase in DA turnover in the nucleus accumbens, as measured by *in vivo* dialysis. Intra-VTA glutamate, but not kainate, also caused an increase in DA

turnover in the prefrontal cortex. This increase was blocked when the NMDA antagonist, CPP was coadministered with glutamate. The physiological relevance of NMDA receptor activation was supported by a blockade of footshock stress-induced activation of prefrontal cortical DA turnover by intra-VTA injection of CPP. Kalivas and coworkers (1989*b*) concluded that NMDA and kainate receptors preferentially mediate EAA activation of VTA DA neurons projecting to the prefrontal cortex and nucleus acumbens, respectively.

1.4.4 Role of EAA receptors in the regulation of dopamine release

Multiple EAA receptor subtypes may be involved in the regulation of DA release by EAA agonists from both dopaminergic terminal field and dendritic regions. The relative importance of non-NMDA and NMDA receptors remains unclear, but may vary between brain regions, as indicated in the reports summarized above (section 1.4.3).

1.4.4.1 Release from terminal fields

Giorguieff and coworkers were the first to show that glutamate evokes the release of [^{3}H]DA from tissue slices of rat striatum *in vitro* (Giorguieff et al. 1977). These observations were confirmed by other groups (Roberts and Sharif 1978; Roberts and Anderson 1979; Marien et al. 1983; Snell and Johnson 1986) and were extended to nucleus accumbens (Roberts and Anderson 1979; Marien et al. 1983; Jones et al. 1987), as well as olfactory tubercle (Marien et al. 1983). In each region, [^{3}H]DA release was strongly inhibited by millimolar concentrations of Mg^{2+}, suggesting involvement of the NMDA receptor (Marien et al. 1983).

Glutamate was similarly found to stimulate Ca^{2+}-dependent release of endogenous DA from striatal slices (Jhamandas and Marien 1987; Clow and Jhamandas 1989). This release was also largely blocked by Mg^{2+}, or by the NMDA receptor antagonist, APH.

Some discrepancies have emerged between studies concerning effects of high concentrations of glutamate on striatal DA release. Glutamate concentrations ranging from 0.1 to 10 mM have been reported to evoke release of endogenous DA (Jhamandas and Marien 1987; Clow and Jhamandas 1989) and concentrations of 10 μM to 10 mM have been reported to evoke the release of [^{3}H]DA (Roberts and Anderson 1979; Marien et al. 1983) from striatal tissue slices. However in an *in vivo* experiment that assessed glutamate-evoked release of [^{3}H]DA using a push-pull cannula technique, Chéramy et al. (1986) observed that micro- and even nanomolar concentrations of glutamate enhanced DA release and that higher concentrations of glutamate (100 μM) actually inhibited the release. These findings have not been confirmed by other investigators in either *in vivo* or *in vitro* studies. For example, very high concentrations of glutamate (10 mM) induced DA release, while lower concentrations were without effect in an *in vivo* study that involved microdialysis delivery of glutamate coupled with sensitive voltammetric analysis of local DA release (Moghaddam et al. 1990). It remains possible that differences between studies in the apparent dose-response relationship for glutamate-evoked DA release might be explained by glutamate uptake into striatal tissue and/or by glutamate-evoked release of other agents that affect DA release from the striatum. This issue would appear particularly well-suited to investigation in the mesencephalic cell culture system, as discussed in section 1.5.1.

Aside from glutamate, other EAAs including kainate, cysteate, aspartate, NMDA, homocysteate, and proline, as well as the inhibitory amino acids, glycine, and GABA have been reported to evoke [^{3}H]DA release from the striatal slice (Roberts and Anderson 1979). The effects of NMDA have been best characterized (Snell and Johnson 1986; Carter et al. 1988; Clow and Jhamandas 1989; Woodward and Gonzales 1990). NMDA stimulated release of exogenous (Snell and Johnson 1986) and endogenous DA (Woodward and Gonzales 1990) from striatal slices at threshold concentrations of 10 to 25 μM NMDA and from striatum *in vivo* (in the presence of 1.2 mM Mg^{2+}) at concentrations of 1 to 10 mM NMDA (Carter et al. 1988) . NMDA-evoked DA release from striatal slices was completely blocked by Mg^{2+}, PCP, APV and APH (Snell and Johnson 1986; Clow and Jhamandas 1989; Woodward and Gonzales 1990). Snell and Johnson (1986) showed that quisqualate and kainate can also evoke release of [^{3}H]DA from striatum and that the pharmacological profile of this release was distinct from the release evoked by NMDA (APH- and Mg^{2+}-insensitive). These observations were confirmed for endogenous DA by Clow and Jhamandas (1989). In the trans-striatal dialysis study, NMDA and kainate, but not quisqualate, evoked DA release. Kainate-evoked release was distinguished from the NMDA response by diminished sensitivity to APV and PCP (Carter et al. 1988). In addition, Butcher et al. (1986) reported that both glutamate and APB facilitated potassium evoked [^{3}H]DA release from striatal slices. Together, these observations provide evidence for multiple EAA receptor subtypes in the striatum. It remains unclear which of the non-NMDA receptor subtypes might be present.

There is good evidence for stimulatory effects of EAAs in mesocorticolimbic DA systems. As reported in section 1.4.3.3, Kalivas et al. (1989*b*) demonstrated that intra-VTA microinjection of glutamate, NMDA or kainate resulted in enhanced DA turnover in nucleus accumbens and/or prefrontal cortex. Activation of the NMDA receptor (demonstrated by sensitivity to CPP injecion) preferentially modulated DA turnover in prefrontal cortex, while DA transmission in the nucleus accumbens was predominantly mediated by a non-NMDA receptor subtype (Kalivas et al. 1989*b*). However, when administered directly to the nucleus accumbens rather than into the VTA, effects of EAAs on DA release may be mediated by both NMDA and non-NMDA receptors. This notion is supported by a study with accumbal tissue slices in which NMDA, kainate and quisqualate (Kalivas et al. [1989*b*] found that intra-VTA quisqualate was inactive in increasing DA turnover in VTA, nucleus accumbens and prefrontal cortex) were found to stimulate release of [^{3}H]DA (Jones et al. 1987). The release evoked by NMDA was reduced by PCP, largely blocked by APV and completely inhibited by Mg^{2+}. Kainate was less effective at inducing [^{3}H]DA release. Also, the kainate response was not inhibited by PCP and was only marginally attenuated by Mg^{2+} or APV. Quisqualate was the least effective agonist inducing release of [^{3}H]DA and this release was largely unaffected by PCP.

1.4.4.2 Somatodendritic release

Within SN, the releasing effects of EAAs have been less well characterized. Roberts and Anderson (1979) were the first to show that a stimulatory effect of glutamate on dendritic release of [^{3}H]DA from tissue slices of rat SN. As a percentage of total [^{3}H]DA uptake into

the slice, the release evoked from this area by 100 μM glutamate was less than 50% of that released in striatum or nucleus accumbens. Marien et al. (1983) extended these observations by demonstrating that glutamate-stimulated nigral release of [^{3}H]DA was significantly reduced by Mg^{2+}. Similarly, NMDA-evoked [^{3}H]DA release from the SN was found by Araneda and Bustos (1989) to be entirely blocked by Mg^{2+}. The role of non-NMDA receptors in the releasing effects of glutamate and other EAAs from SN has not been established.

1.4.5 Synaptic localization of EAA receptors

The synaptic localization of EAA receptors in dopaminergic regions of the brain has not yet been clarified. Most of the studies have focussed on rat striatum or nucleus accumbens, where various groups have reported contradictory findings. However, there may be some agreement with respect to localization of receptor sites in the SN. In other regions (*i.e.* olfactory tubercle, VTA), less information is available. As reviewed below, studies addressing receptor localization have relied on selective brain lesions and/or on comparing EAA-stimulated DA release in the presence and absence of TTX.

<u>Lesion studies</u>

Three general types of lesions have been used:

a. Selective lesions of dopaminergic pathways are made by unilateral injection of 6-OHDA into the cell body area of the relevant dopaminergic pathway. The interpretation of a loss of receptors after this lesion is that receptors were located on terminals and/or dendrites of the dopaminergic neuron itself.

b. Lesions of intrinsic innervation that destroy interneurons at the site of injection, while sparing both fibers of passage and terminals of axons projecting to the region (Schwarcz and Coyle 1977; Hattori and McGeer 1977) may be produced (in the long-term) after local injection of an EAA (usually kainate, ibotenate or quinolinate). Although these lesions should cause a selective reduction of receptors on interneurons, there are cautionary reports that kainate lesions might in some cases also have effects on terminals (Meibach et al. 1978; Butcher and Rogers 1978).

c. Since projections from cerebral cortex account for most excitatory innervation of dopaminergic cell body and terminal field areas, decortication may be performed to selectively remove receptors located presynaptically with respect to the excitatory cortical innervation.

Studies with tetrodotoxin (TTX)

TTX has been shown not to block the Na^+ channels opened by EAAs (Zieglgansberger and Puil 1972; Luini et al. 1981). Experiments on *in vitro* DA release confirmed that TTX (0.1 to 1 μM) inhibits depolarization-evoked release of DA in striatum (Starke et al. 1978) or SN (Tagerud and Cuello 1979). However, TTX does not reduce the spontaneous release of DA, but actually increases it (Nieoullon et al. 1977*a*). Thus, blocking the propagation of regenerative Na^+ action potentials with TTX provides an indication that any residual EAA-stimulated release of a transmitter is mediated by a direct effect of the EAA on the transmitter-releasing neuron close to the site of transmitter release.

1.4.5.1 Striatum

There is good evidence for the presence of multiple EAA receptor subtypes in the striatum (see review of receptor distribution by Cotman et al. 1987). However, the synaptic localization of these receptors

remains unclear. EAA receptors are probably not located presynaptically on terminals of excitatory afferents since:

a. Ablation of the frontal cortex produced no difference in glutamate-stimulated [^{3}H]DA release from lesioned compared to unlesioned striatum, when tested one month after lesioning (Roberts and Anderson 1979).

b. Unilateral decortication resulted in no significant changes in the density of NMDA, AMPA or kainate receptor binding within the denervated striatum relative to its contralateral control (Greenamyre and Young 1989).

In a single study, DL-[^{3}H]APB binding in the striatum was reduced after lesion of the nigrostriatal DA pathway or intrinsic innervation, but not after decortication (Butcher et al. 1986). Thus, APB receptors may be distributed on nigrostriatal DA terminals as well as on striatal interneurons.

There is some controversy concerning localization of the major EAA receptor subtypes with respect to dopaminergic terminals and striatal interneurons. Some evidence suggests that EAA receptors are localized on nigrostriatal DA terminals:

a. Roberts et al. (1982) observed a 40% reduction in glutamate binding in rat striatum after nigral lesioning with 6-OHDA, clearly indicating a presynaptic location of EAA receptors on nigrostriatal DA terminals.

b. In striatal tissue slices three groups have found glutamate-evoked [^{3}H]DA release to be TTX-insensitive, suggesting a presynaptic action on DA terminals (Gioruieff et al. 1977; Roberts and Anderson 1979; Marien et al. 1983).

c. These findings were confirmed for endogenous DA by Jhamandas and Marien (1987) and were ascribed to actions at the NMDA receptor on the basis of sensitivity to Mg^{2+} and APV (Marien et al. 1983; Jhamandas and Marien 1987). NMDA evoked release of [^{3}H]DA that was partially TTX-sensitive (Snell and Johnson 1986) and release of endogenous DA that was TTX-insensitive (Clow and Jhamandas 1989). Release of endogenous DA evoked by quisqualate and kainate was partially inhibited by TTX (Clow and Jhamandas 1989).

A convincing case may also be made that EAA receptors are overwhelmingly distributed on striatal interneurons. For example:

a. De Belleroche and Bradford (1980) found that glutamate did not influence release of [^{14}C]DA from striatal synaptosomes, indicating that the receptors are not on nigrostriatal dopaminergic terminals.

b. NMDA, AMPA and kainate receptor densities were decreased by 92%, 80% and 81%, respectively three months after destruction of striatal intrinsic innervation by intrastriatal injection of quinolinic acid (Greenamyre and Young 1989).

c. A trans-striatal microdialysis study revealed *in vivo* NMDA-evoked DA release to be largely TTX-sensitive, while the release evoked by kainate was only partially blocked by TTX (Carter et al. 1988). More recently, it has been reported that NMDA-evoked release of DA from striatal tissue slices was completely blocked by TTX (Woodward and Gonzales 1990).

d. Binding of a PCP analog ([^{3}H]1-[1-(2-thienyl)-cyclohexyl]piperidine; [^{3}H]TCP) to the NMDA/PCP receptor complex in rat striatum was unaffected after 6-OHDA lesions of the nigrostriatal DA pathway (Gundlach et al. 1986).

These findings are consistent with the proposal that NMDA receptors are localized on striatal interneurons and suggest that they are not distributed presynaptically on dopaminergic terminals in this region. It remains possible that a proportion of the remaining AMPA or kainate receptors might be located either on EAA terminals, or on terminals of the nigrostriatal DA projection. Alternatively, the EAA receptors may be distributed on axons or terminals of dopaminergic neurons in such a way that their effect still requires the activation of voltage-dependent Na^{+} channels in order to produce transmitter release.

In summary, it remains unclear whether EAA receptors (and particularly NMDA receptors) are located on DA terminals in the striatum. In the experiments reported in this thesis, the issue of TTX-sensitivity of EAA-evoked DA release was examined. We show for NMDA-

evoked DA release that conflicting results may be due to the residual presence of Mg^{2+} in the experimental preparation (see section 4).

1.4.5.2 Nucleus accumbens

Quirion and coworkers reported the existence of binding sites for PCP (PCP/NMDA receptor complex) in the nucleus accumbens. Lesions of the dopaminergic innervation to the nucleus accumbens resulted in the loss of 62% of the binding sites, suggesting that a large proportion of these sites are located on dopaminergic terminals in the nucleus accumbens (French et al. 1985). This hypothesis was reinforced by the observation that glutamate-evoked release of [^{3}H]DA from tissue slices of nucleus accumbens (as well as olfactory tubercle) was insensitive to 0.3 μM TTX (Marien et al. 1983). The EAA receptor subtype mediating this effect was not characterized. However, Jones et al. (1987) found that NMDA-evoked [^{3}H]DA release was completely blocked by TTX and kainate-evoked [^{3}H]DA release was partially TTX-sensitive. Thus, a discrepancy has emerged with results from the lesion study indicating the presence of NMDA receptors on DA terminals and the release study in the presence of TTX suggesting a localization on other elements in this structure. As in striatum (section 1.4.5.1), the controversy centers on the NMDA receptor, which is known to be particularly susceptible to the blocking actions of endogenous cations (sections 1.3.1.1 and 1.3.1.2). The experiments in this thesis provide a demonstration of how the TTX-sensitivity of NMDA-evoked [^{3}H]DA release may be manipulated by extracellular Mg^{2+} (section 4).

1.4.5.3 Substantia nigra

Marien et al. (1983) reported that glutamate-induced release from nigral slices was partially sensitive to TTX. However, Araneda and Bustos (1989) found that NMDA-evoked endogenous DA release was completely blocked by TTX. These latter results suggest that NMDA receptors in SN are located on interneurons in this region. It is possible that non-NMDA receptors may be responsible for the TTX-insensitive component of glutamate-evoked [^{3}H]DA release and that these receptors may be located on the dopaminergic neurons. As in other brain regions (sections 1.4.5.1 and 1.4.5.2), the possibility that NMDA receptors are responsible for directly stimulating DA release (either somatodendritic or terminal field DA release) has not yet been convincingly demonstrated. The question of whether NMDA receptors may mediate TTX-insensitive transmitter release from the DA neuron has been addressed in section 4 of this thesis.

1.4.6 Modulators of EAA responses affect dopamine transmission

1.4.6.1 Phencyclidine (PCP) and dopamine

Phencyclidine (PCP) is a potent inhibitor of NMDA-evoked DA release from tissue slices (Snell and Johnson 1986) and intact brain (Carter et al. 1988) through its action at a site within the NMDA receptor channel (see section 1.3.1.4), referred to as the PCP receptor (Quirion et al. 1987). Paradoxically, PCP and its analogues also have stimulatory effects on brain DA systems. The important psychotomimetic effects of PCP-related compounds have been attributed to their activation of DA turnover (see reviews by Domino and Luby 1981; Johnson

1983; but see Carlsson and Carlsson 1990). As reviewed below, both activation of a novel PCP receptor and inhibition of DA uptake have received attention as potential explanations for PCP-enhanced DA turnover. The evidence for the former comes from *in vivo* studies, while reports of *in vitro* experiments favor interference with the DA uptake process. The actual cellular mechanism is unknown. It also remains unclear whether PCP interacts directly with DA neurons or indirectly stimulates DA transmission through activation of a polysynaptic pathway.

PCP has been found to evoke DA release *in vivo* from mesocorticolimbic, but not mesostriatal DA terminal fields (Gratton et al. 1987; Deutch et al. 1987; Carboni et al. 1989; Wood and Rao 1989; Rao et al. 1989, 1990) and to increase the firing rates of neurons in the VTA (French 1989; French and Ceci 1989). When tested, the enhancement of DA turnover induced by PCP was found also with MK-801, but not with competitive NMDA receptor antagonists or the sigma ligand, rimcazole (Wood and Rao 1989; Rao et al. 1989, 1990). The unique pharmacological characteristics of this interaction led to the suggestion of a new "NMDA-uncoupled form of the PCP receptor" (Wood and Rao 1989).

While PCP enhanced activity of DA neurons in the VTA *in vivo* (French 1989; French and Ceci 1989), it suppressed their activity *in vitro*, in slices of VTA and SN (Trulson and Arasteh 1987). Transmitter release studies with striatal tissue slices and synaptosomes revealed PCP-induced increases in DA or [^{3}H]DA release (Ary and Komiskey 1982; Vickroy and Johnson 1982; Bowyer et al. 1984; Snell et al. 1984; Snell and Johnson 1986). In other work, 10 μM PCP, amphetamine or the DA uptake inhibitor nomifensine, had no effect on spontaneous DA release

from striatal slices (Buxton et al. 1989). However, when the slices were depolarized in the presence of these agents, each drug caused a measurable increase in extracellular DA concentrations. The effects of PCP and the potent DA uptake inhibitor nomifensine were attenuated by the acetylating agent, metaphit, while the amphetamine effect was unaffected by this treatment. These data provide evidence that the apparent DA release caused by PCP in striatal slices might be mediated by blockade of DA reuptake.

The effect of PCP on [^{3}H]DA release from mesencephalic cell cultures was studied in this thesis. Since some stimulatory action of PCP-related compounds was observed, inhibition of DA reuptake and the presence of a novel PCP receptor were evaluated as possible underlying mechanisms (see section 6).

1.4.6.2 Effects of glycine on NMDA receptors and dopamine systems

Glycine interacts with at least two distinct receptors: a strychnine-insensitive site allosterically associated with the NMDA receptor (see section 1.3.1.5) and an inhibitory glycine receptor that is selectively blocked by strychnine (Curtis et al. 1971; Tebecis and DiMaria 1972). Both strychnine-insensitive and strychnine-sensitive actions of glycine may be implicated in the regulation of dopaminergic transmission.

Strychnine-insensitive effects on NMDA receptor-mediated DA release

Although glycine-potentiated NMDA receptor function has been widely reported, exogenous glycine failed to enhance NMDA-evoked release of [^{3}H]DA (Ransom and Deschenes 1989) or endogenous DA (Woodward and

Gonzales 1990) from striatal slices. Glycine modulation of NMDA-stimulated [^{3}H]DA release could be demonstrated nonetheless, by using kynurenate, an agent that blocks NMDA responses through competitive inhibition of glycine binding at the strychnine-insensitive glycine site (Birch et al. 1988; Bertolino et al. 1989; Danysz et al. 1989). Kynurenate inhibited NMDA-evoked release of [^{3}H]DA from the slice and the inhibition was reversed by coincubation with 30 to 100 μM glycine (Ransom and Deschenes 1989). This observation suggests that glycine may be required to elicit NMDA-stimulated DA release from striatum and that endogenous glycine levels in the striatal slice may be sufficient to maximally saturate this glycine binding site, in the absence of kynurenate.

Strychnine-sensitive effects of glycine on DA release

Inhibitory glycine receptors have been demonstrated by receptor autoradiographic methods, in brain regions containing dopaminergic cell bodies and nerve terminals (Young and Snyder 1973; Zarbin et al. 1981; De Montis et al. 1982; Frostholm and Rotter 1985) and have been implicated in inhibition of DA neuron firing in the SN (Crossman et al. 1974; Dray and Gonye 1975; Mercuri et al. 1988, 1990, but see Feltz 1971). The inhibitory effect was not blocked by TTX (Mercuri et al. 1988, 1989), suggesting that glycine-induced hyperpolarization and/or depolarization was the result of direct action on the neurons from which recordings were taken. Also, [^{3}H]strychnine binding studies revealed a 28% reduction in the B_{max} of glycine receptors in the ipsilateral rat SN following 6-OHDA lesions of the nigrostriatal dopaminergic pathway (De Montis et al. 1982). Together, these results suggest that a significant

proportion of strychnine-sensitive glycine receptors are present on the DA neurons themselves.

In the cat SN, *in vivo* application of glycine resulted in a reduction in [^{3}H]DA release from the ipsilateral caudate nucleus (Chéramy et al. 1978), while glycine (100 μM) markedly stimulated *in vitro* release of [^{3}H]DA from slices of rat SN (Kerwin and Pycock 1979; Araneda and Bustos 1989). These data suggest that when administered in the SN, glycine enhances nigral somatodendritic DA release. This in turn may inhibit dopaminergic activity in striatum via autoreceptor feedback at the level of dopaminergic cell bodies. There is less evidence for this mechanism in other DA systems. In slices of rat ventral tegmentum, strychnine-sensitive glycine receptors did not affect spontaneous [^{3}H]DA release: However, glycine did potentiate [^{3}H]DA release evoked by proveratrine A stimulation (Gundlach and Beart 1982).

In dopaminergic terminal regions, variable effects of glycine on DA release have been observed. Glycine stimulated strychnine-sensitive release of [^{3}H]DA (Roberts and Anderson 1979; Giorguieff-Chesselet et al. 1979), but not endogenous DA (Woodward and Gonzales 1990) from rat striatal slices. When infused into nucleus accumbens, low doses (12.5 or 25 μg/24 hr) moderated DA-induced hyperactivity, while a higher dose (50 μg/24 hr) stimulated locomotor activity (Barnes et al. 1986).

1.5 Mesencephalic cell culture systems

Primary dissociated cell cultures of fetal murine ventral mesecephalon, the region containing cell bodies of origin for mesostriatal as well as mesocorticolimbic DA pathways of the adult CNS,

have been used to examine various aspects of DA neuron development, function and degeneration. Like their counterparts *in situ*, the cultured cells express TH (Prochiantz et al. 1979; Barochovsky and Bradford 1987*a*; Silva et al. 1988), become capable of DA synthesis (Prochiantz et al. 1979; Barochovsky and Bradford 1987*a*), depolarization-induced DA release (Daguet et al. 1980; Berger et al. 1982; Barochovsky and Bradford 1985, 1987*a*,*b*; Ahnert-Hilger et al. 1986; Peacock et al. 1988) and binding (Heyer et al. 1986), exhibit DA histofluorescence (Prochiantz et al. 1979; Hemmendinger et al. 1981; Berger et al. 1982; Heyer 1984) and respond to cholinergic agents (Barochovsky and Bradford 1987*b*; Peacock et al. 1988). Histochemically visualized DA neurons from mesencephalic cell cultures exhibit a distinctive prolonged action potential duration (Silva et al. 1988), as they do *in situ* or in tissue slices (Bunney et al. 1973; Pinnock 1983).

Mesencephalic cell cultures have been used to examine the promotion of DA neuron differentiation in response to striatal target neurons (Prochiantz et al. 1979; di Porzio et al. 1980; Hemmendinger et al. 1981) and the neuronotrophic effects of a striatal-derived factor (Tomozawa and Appel 1986; Dal Toso 1988) and of basic fibroblast growth factor (Ferrari et al. 1989). However, they have been most useful in elucidating the cytotoxic mechanism of action for the selective DA neurotoxin, 1-methyl-4-phenylpyridinium (MPTP) (Sanchez-Ramos et al. 1986, 1988; Mytilineou and Friedman 1988; Schinelli et al. 1988; Michel et al. 1989, 1990).

1.5.1 Application of cell culture methodology to study of dopaminergic transmission

Use of primary mesencephalic cell cultures has important advantages over tissue slice and *in vivo* approaches for examining certain aspects of EAA heteroreceptor regulation of DA transmission.

Ready access to target cells minimizes problems of diffusion through a neuropil and affords precise control over the extracellular environment bathing target cells. In working with EAAs, this control is particularly welcome since EAAs are taken up by and released from many cell types. The uptake, release and interconversion between transmitter and metabolic pools may confound interpretation of tissue slice and *in vivo* experiments with EAAs. Typically, it can lead to an underestimation of EAA agonist potency (Mayer and Westbrook 1987). Cell culture techniques have also afforded better control over concentrations of ions and endogenous modulators of EAA receptor function. For example, characterization of the coagonist role of glycine at the NMDA receptor and of the receptor blocking actions of Mg^{2+} and Zn^{2+} were only made possible through application of cell culture techniques, since the low concentrations of these agents endogenously present *in situ* are active in modulating EAA receptor function.

The entire intact neuron is present in cultures and can be maintained long-term. This makes it possible to study chronic as well as acute effects of pharmacological manipulations on the function of DA cells. In this regard, cell culture presents distinct advantages over both *in vivo* and tissue slice approaches for studying effects of ions and drugs.

The presence of the entire neuron also makes it possible to detect actions of EAAs on DA release that are initiated at receptors on the cell body, dendrites, or axon terminals. Information concerning the synaptic localization of EAA receptors modulating DA release is provided by assessing the role of action potentials in the EAA-stimulated release, as indicated by sensitivity to inhibition by TTX. In tissue slice studies, EAA-evoked DA release has been variously described as sensitive, insensitive, or partially sensitive to inhibition by TTX (section 1.4.5 "Synaptic localization of EAA receptors"). It is possible that EAAs may evoke DA release through a TTX-resistant indirect mechanism (*i.e.* without the propagation of a Na^+ action potential) if they cause release of an intermediate transmitter which then acts upon a DA neuron near the site of DA release. Use of the culture model may clarify whether EAA receptors are indeed located on DA neurons. In the dissociated cell cultures, normal neural connectivity is disrupted. Thus, the mesencephalic cell culture model may allow assessment of direct effects on DA neurons, while reducing the opportunity for indirect, polysynaptic responses. In the presence of TTX, any indirect effects of EAAs on DA release are even further disfavoured, so that only EAA receptors localized presynaptically on the DA-releasing neurons will induce DA release.

Certain caveats apply to the interpretation of results from cell culture experiments and there are some questions for which the model is ill-suited. For example, it is not possible to distinguish between somatodendritic and terminal field transmitter release since the entire DA neuron is present in culture and DA neurons from nigrostriatal as well as mesocorticolimbic pathways are simultaneously present (although

techniques have recently been developed that may permit the selective culturing of neurons from VTA or SN [see Masuko et al. 1989; Rayport et al. 1989]).

The maturational state of EAA receptors and of the DA cell must also be considered. Prenatal tissue is cultured, because adult tissue in which neural processes are developed does not survive dissociation and culture procedures well. Responses in cell cultures may therefore resemble *in situ* results from immature rather than adult brain. In cultured cortical neurons, the development of EAA agonist-evoked transmitter release has been found to parallel the ontogeny of EAA receptor subtypes *in situ* (Drejer et al. 1987). Cultured neurons from hippocampus, septum and neocortex develop responsiveness to EAAs after different numbers of days in culture (Köller et al. 1990). Thus, it is possible that failure of EAAs to evoke transmitter release from cultured DA neurons may only indicate that longer periods of maintenance are required. The developmental profiles of EAA receptors in mesencephalic cultures were investigated in this thesis (Appendix A). In general, the development of DA neuronal characteristics in cell culture has been shown to reflect ontogeny *in situ* (Ahnert-Hilger et al. 1986). Functional ionic channels and depolarization-evoked release of [^{3}H]DA similar to the response of adult tissue slices has been reported as early as three days in culture, while an optimal response was obtained between 5 and 8 days in culture (Ahnert-Hilger et al. 1986; Barochovsky and Bradford 1987*a*). However, survival of neuronal elements and the expression of transmitter phenotypes (Black et al. 1984) are sensitive to the modulatory influence of cellular plating density, depolarization conditions, and the synthesis or release into the milieu of neurotrophic

agents. These factors in addition to the loss and/or reorganization of afferent and efferent connections inherent in a dissociated cell culture model may lead to functional characteristics of mesencephalic neurons that differ from those encountered *in situ*.

1.5.2 Cell culture methodology

Techniques for growing primary dissociated mesencephalic cell cultures were pioneered by Prochiantz and coworkers (1979). When fetal rat tissue is used, the ventral mesencephalic area (Fig. 1) is dissected on gestation day 15 (of a 21 day gestation period) - (according to a dissection protocol described in detail by Björklund et al. 1983; Commissiong and Toffano 1986; König et al. 1989). By this time, DA neurons in SN, VTA and the retrorubral field have completed final mitosis (Sprecht et al. 1981). Tissue may be dissociated mechanically, without use of trypsinization. The cell suspensions are diluted in medium and maintained in the presence of 5-10 % fetal calf serum. Heterogenous cell cultures are obtained. They contain neuronal, fibroblast and glial elements. Over days in culture, non-neuronal cell types may proliferate, eventually replacing neuronal cells. The inclusion of elevated K^+ (20-25 mM) in the growing medium has been found to enhance the survival of neurons (Kostenko et al. 1982) and development of DA neuronal characteristics (Barochovsky and Bradford 1987*a*).

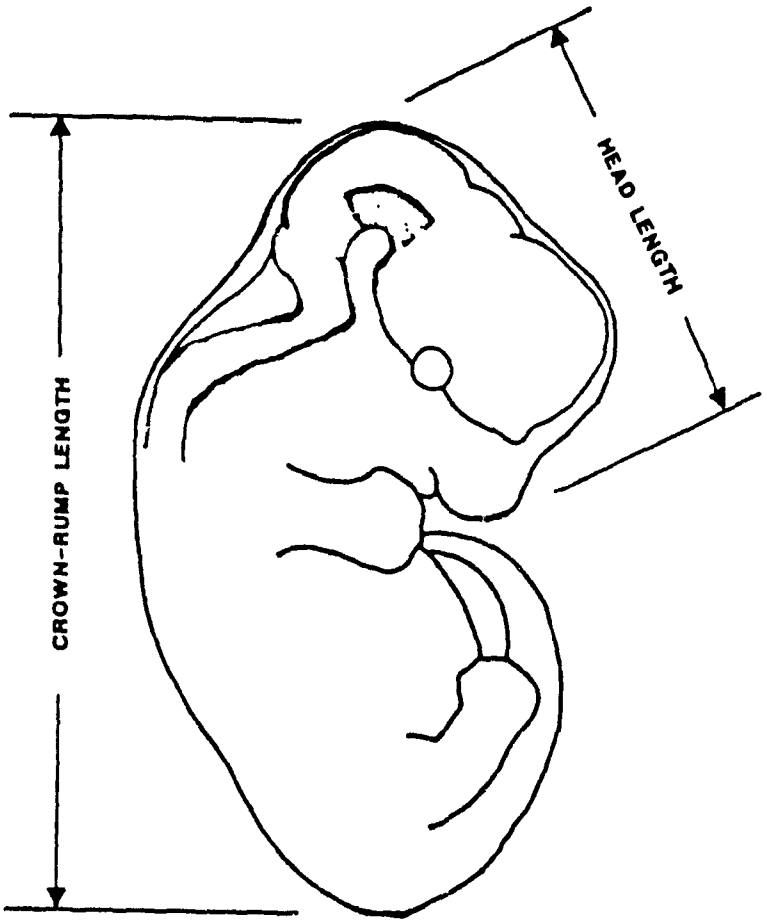

Fig. 1 Ventral mesencephalon (stippled area) of the rat fetus on gestation day 15 (adapted from König et al. 1989).

2. AIMS AND OBJECTIVES

The overall aim of this thesis was to determine the effect of EAAs on the release of DA from mesencephalic cells in dissociated cell culture. Mesencephalic cell cultures have been previously used to investigate DA uptake and release evoked by potassium and veratridine, the maturation of DA systems and the neurotoxic effects of MPTP. We hypothesize that the DA-releasing cells also possess EAA heteroreceptors that can be activated to modulate DA release. If this is true, then the cultures may provide a system for studying cellular and ionic aspects of EAA-DA interactions and also a functional assay for investigating EAA receptor pharmacology. In addition, mesencephalic cell cultures might facilitate the study of acute and long-term modulation of DA release by other transmitters, second messengers and drug treatments. The objectives and hypotheses of specific parts of this thesis are listed below. The following constitutes connecting text between the published papers and manuscript comprising sections 3 to 7 of this thesis (as required by university thesis preparation guidelines[a]).

2.1 Effect of glutamate on [^{3}H]DA release

In the first set of experiments (section 3: *J. Neurochem.* 52, 1300-1310 [1989]), mesencephalic cells were exposed to glutamate to

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[a] "...It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as an integral part of the thesis. .In such instances, connecting texts are mandatory and supplementary explanatory material is almost always necessary . "

reprinted from the Guidelines Concerning Thesis Preparation, Faculty of Graduate Studies and Research, McGill University, March 1989.

determine whether these cells possessed functional EAA receptors and whether glutamate would stimulate EAA receptors to induce Ca^{2+}-dependent release of DA from the cells. Since EAAs may be neurotoxic under certain conditions, the possibility that any [^{3}H]DA release induced by exposure to glutamate might be caused by an acute excitotoxic mechanism was addressed. A further aim was to study some of the basic ionic mechanisms underlying glutamate-stimulated [^{3}H]DA release - the involvement of action potential generation through voltage-sensitive Na^+ or Ca^{2+} channels and the role of Na^+ ion flux (either through receptor- or voltage-linked channels).

2.2 The role of EAA receptor subtypes in mediating EAA-evoked [^{3}H]DA release

Given that [^{3}H]DA release was stimulated by glutamate, the second series of experiments (section 4: *Synapse* **5**, 271-280 [1990]) was designed to determine which of the known EAA receptor subtypes might be involved in mediating EAA-evoked [^{3}H]DA release. Prototypic EAA receptor agonists and antagonists with affinity for NMDA and non-NMDA receptors were tested and the ionic dependence(s) of responses to agonists were examined. These experiments tested two related hypotheses:

a. that the pharmacological and ionic profile of responses to EAAs (*e.g.* sensitivity to EAA agonists and antagonists, Mg^{2+} and Zn^{2+}) matches the profile of EAA responses mediated by various EAA receptor subtypes in other cultured cell systems and *in vivo* (*e.g.* action potentials, excitotoxic responses and stimulated release of transmitters).

b. that EAA-evoked [^{3}H]DA release from mesencephalic cell cultures pharmacologically resembles EAA-evoked DA release evoked by these agents in tissue slices and *in vivo*.

As a part of (b) above, these experiments assessed the TTX-sensitivity of EAA-evoked [^{3}H]DA release, to determine whether EAAs might interact directly with DA neurons (as discussed in section 1.4.5).

2.3 Characterization of effects of naturally occurring EAAs on [^{3}H]DA release

Given that multiple EAA receptor subtypes were found in the mesencephalic cell cultures, the third series of experiments (section 5: *J. Neurochem.* 55, 268-275 [1990]) examined the role of these receptors in mediating any effects of EAAs endogenous to the ventral mesencephalon and to the terminal field regions of DA projections on [^{3}H]DA release. The role of EAA receptor subtypes in mediating effects of L-glutamate, L-aspartate, L-HCA, L-HCSA, L-CA, and L-CSA were examined over a relevant range of concentrations. By examining effects of these EAA agonists in the presence of the selective and potent NMDA receptor antagonist, MK-801, it was hypothesized that the component of release due to NMDA receptor activation might be distinguished from that mediated by non-NMDA receptors.

2.4 Effects of phencyclidine on spontaneous [^{3}H]DA release

In the fourth part of the thesis (section 6: *Can. J. Physiol. Pharmacol.* 68, 1200-1207 [1990]), the cellular target for the DA-releasing effect of PCP-like compounds was examined. The mesencephalic

culture model was used to assess several possible explanations for these effects. These included the hypothesis that PCP produces DA release by interaction with a site on dopaminergic neurons that has the pharmacological characteristics of an "NMDA-uncoupled PCP receptor" (Rao et al. 1989, 1990; Wood and Rao 1989; Wood et al. 1990) and an alternate hypothesis that PCP increases extrasynaptic DA concentrations by blocking DA reuptake (*e.g.* Buxton et al. 1989).

2.5 Effects of glycine on spontaneous and EAA-evoked [^{3}H]DA release

The fifth set of experiments (section 7: *Molec. Pharmacol.* [submitted]) was done to examine the effects of glycine on spontaneous and EAA-evoked [^{3}H]DA release. Both strychnine-sensitive and strychnine-insensitive glycine modulation of dopaminergic transmission has been reported. Glycine may have a strychnine-insensitive coagonist effect at the NMDA receptor. By itself, glycine has also been reported to modulate dopaminergic transmission by a strychnine-sensitive glycine mechanism. In these studies, the relationship between these effects was investigated at the level of the DA-releasing cells themselves.

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Glutamate Stimulation of [^{3}H]Dopamine Release from Dissociated Cell Cultures of Rat Ventral Mesencephalon

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Abstract: In dissociated cell cultures of fetal rat ventral mesencephalon preloaded with [^{3}H]dopamine, glutamate (10^{-5}–10^{-2} *M*) stimulated the release of [^{3}H]dopamine. Glutamate stimulation of [^{3}H]dopamine release was Ca^{2+} dependent and was blocked by the glutamate antagonist, *cis*-2,3-pipendine dicarboxylic acid. Glutamate stimulation of [^{3}H]dopamine release was not due to glutamate neurotoxicity because (1) glutamate did not cause release of a cytosolic marker, lactate dehydrogenase, and (2) preincubation of cultures with glutamate did not impair subsequent ability of the cells to take up or release [^{3}H]dopamine. Thus, these dissociated cell cultures appear to provide a good model system to characterize glutamate stimulation of dopamine release. Release of [^{3}H]dopamine from these cultures was stimulated by veratridine, an activator of voltage-sensitive Na^+ channels, and this stimulation was blocked by tetrodotoxin However, glutamate-stimulated [^{3}H]dopamine release was not blocked by tetrodotoxin or Zn^{2+}. Substitution of NaCl in the extracellular medium by sucrose, LiCl, or Na_2SO_4 had no effect on glutamate stimulation of [^{3}H]dopamine release; however, release was inhibited when NaCl was replaced by choline chloride or *N*-methyl-D-glucamine HCl. Glutamate-stimulated [^{3}H]-dopamine release was well maintained (60–82% of control) in the presence of Co^{2+}, which blocks Ca^{2+} action potentials, and was unaffected by the local anesthetic, lidocaine. These results are discussed in terms of the receptor and ionic mechanisms involved in the stimulation of dopamine release by excitatory amino acids. **Key Words:** Dopamine—Excitatory amino acids—Glutamate—Mesencephalon—Tissue culture. **Mount H. et al.** Glutamate stimulation of [^{3}H]dopamine release from dissociated cell cultures of rat ventral mesencephalon. *J Neurochem* 52, 1300–1310 (1989).

Glutamate is one of the major excitatory neurotransmitters in the CNS (for review see Fonnum, 1984). There is good evidence for an interaction of glutamate with brain dopaminergic transmission, particularly with the nigrostriatal dopaminergic pathway and also with the mesolimbic [ventral tegmental area (VTA)–nucleus accumbens] pathway. In the substantia nigra, the striatum, and the nucleus accumbens, glutamate has been demonstrated to be present (Kim et al., 1977; Walaas and Fonnum, 1979; Fonnum et al., 1981*a*; Korf and Venema, 1983; Kornhuber et al., 1984), to be taken up by a high-affinity mechanism (Walaas and Fonnum, 1979; Fonnum et al., 1981*a,b*; Carter, 1982; Kerkerian et al., 1983), and/or released in response to stimulation (Reubi and Cuenod, 1979; Godukhin et al., 1980; Rowlands and Roberts, 1980; Girault et al., 1986), and moderate densities of glutamate receptor sites have been identified in these regions (Greenamyre et al., 1984; Halpain et al., 1984; Rainbow et al., 1984; Monaghan and Cotman, 1985). Glutamate or glutamate agonists have been demonstrated to stimulate release of dopamine (DA) (or of [^{3}H]DA following administration of [^{3}H]DA or a radiolabelled precursor) from slices of the substantia nigra (Roberts and Anderson, 1979; Marien et al., 1983), striatum (Giorgieff et al., 1977; Roberts and Sharif, 1978; Roberts and Anderson, 1979; Marien et al., 1983; Snell and Johnson, 1986; Jhamandas and Marien, 1987), and nucleus accumbens (Roberts and Anderson, 1979; Marien et al., 1983; Jones et al., 1987) and from the striatum in vivo (Chéramy et al., 1986). Electrophysiological studies have also shown that local application of glutamate to the substantia nigra caused excitation of identified DA neurons (Grace and Bunney, 1984*a,b*). In addition,

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Abbreviations used DA, dopamine; DMEM, Dulbecco's modified Eagle's medium, DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid, KRH, Krebs–Ringer–HEPES buffer; LDH, lactate dehydrogenase, NA, noradrenaline; *cis*-2,3-PDA, (±)-*cis*-2,3-pipendine dicarboxylic acid; TTX, tetrodotoxin; VTA, ventral tegmental area.

local application of glutamate or glutamate agonists to the substantia nigra (Pycock and Dawbarn, 1980; Arnt, 1981*a*), VTA (Pycock and Dawbarn, 1980), or nucleus accumbens (Arnt, 1981*b*; Donzanti and Uretsky, 1983) produced alterations in DA-mediated motor behavior.

Although the preceding studies with brain slices or local application of drugs to brain in situ provided good evidence for glutamate stimulation of dopaminergic transmission, a cell culture system containing dissociated DA neurons may have some inherent advantages for examination of the mechanisms involved in glutamate stimulation of DA release at the cellular level. For example, in studies of the ionic dependence of DA release, the cell culture system allows one to strictly control the composition of the extracellular environment, circumventing problems of diffusional barriers to ions and drugs. Also, cultures contain an intact neuron (i.e., cell bodies together with their processes or neurites) rather than only cell bodies or nerve terminals, and this is advantageous, for example, in studies utilizing pharmacological blockers to test the involvement of action potentials in glutamate stimulation of DA release.

The fetal rat ventral mesencephalon contains the cell bodies of origin of the major brain DA pathways (nigrostriatal, mesolimbic, and mesocortical). The present study indicates that cultures of dissociated cells from this area can be used to characterize glutamate stimulation of DA release. The study also examines the involvement of Na^+ or Ca^{2+} action potentials and of Na^+ in this release process.

A preliminary report of some of these results has been presented at a meeting (Mount et al., 1987).

MATERIALS AND METHODS

Materials

3,4-[7-^{3}H(N)]Dihydroxyphenylethylamine ([^{3}H]DA, 18.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). L-Glutamate (Na salt) was obtained from BDH Chemicals (Montreal, Quebec, Canada), desipramine, pargyline, tetrodotoxin (TTX), and veratridine were from Sigma Chemical (St. Louis, MO, U.S.A.), (±)-*cis*-2,3-piperidine dicarboxylic acid (*cis*-2,3-PDA) was from Cambridge Research Biochemicals (Harston, U.K.) and benztropine was from Aldrich Chemical (Milwaukee, WI, U.S.A.). Fluoxetine was a generous gift from Eli Lilly (Indianapolis, IN, U.S.A.).

Cell culture

Pregnant Sprague–Dawley rats were anesthetized with sodium methohexital on gestation day 15. A piece (approximate dimensions 3.5 mm × 3.5 mm × 1.0 mm) of the rostral portion of the ventral mesencephalon was dissected from each fetus as described by Commissiong and Toffano (1986); this portion of the ventral mesencephalon contains >90% of the DA content of the mesencephalon (Commissiong and Toffano, 1986, and confirmed by us). Tissue from 8–12 fetuses per dam was pooled in 1 ml of Dulbecco's modified Eagle's medium (DMEM; GIBCO Canada, Burlington, Ontario, Canada) containing glucose (1.0 g/L), pyruvate (110 mg/L), glutamine (548 mg/L), and sodium bicarbonate (110 mg/L). The tissue was distributed into three test tubes each containing 1 ml of DMEM and dissociated mechanically by successive gentle triturations through a Pasteur pipet (11 times) and a 25-gauge needle (two times). The dissociated cells (Fig. 1A) were pooled and diluted to a concentration of 10^6 cells/ml in DMEM containing 10% vol/vol heat-inactivated fetal calf serum (GIBCO Canada) and nystatin (24 U/ml). Cells were grown on 24-well Multiwell plates previously coated with poly-D-lysine (20 μg/ml). Cultures were maintained for as long as 8 days at 37°C in moist air containing 10% CO_2. During this time, the initially rounded cells flatten out and many extend processes to form a dense network (Fig. 1B).

Release experiments

[^{3}H]DA release experiments were performed on cells, after 4–6 days in culture (except where specifically noted), as follows. Each culture was washed once with 0.5 ml of Krebs–Ringer–HEPES buffer (KRH). The cells were then loaded with [^{3}H]DA by incubating them for 20 min at 37°C with KRH containing [^{3}H]DA (5×10^{-8} *M*) and desipramine (5×10^{-5} *M*). After the loading, cells were rinsed four times with KRH over a 20-min period at 23°C to remove unbound radioactivity. To measure release of [^{3}H]DA, cultures were incubated for 5 min at 23°C with 0.5 ml of KRH (basal release period) followed by 5 min with 0.5 ml of KRH containing a secretagogue (stimulated release period), and radioactivity in the release media was measured by liquid scintillation spectrometry (Beckman LS 7500). At the end of the experiment, residual intracellular radioactivity was extracted from the cells by incubating them for 30 min with 0.5 ml of acidic ethanol (95% ethanol/5% 0.1 *M* HCl), and this was similarly counted. In most calculations (except where specifically mentioned), [^{3}H]DA release is expressed as a percentage of the total intracellular content of [^{3}H]DA present in each culture at the time of the release incubation, to correct for variations in the amount of [^{3}H]DA taken up by different cultures.

The composition (m*M*) of the standard KRH buffer (pH 7.4) was: NaCl, 125; KCl, 4.8; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.2; glucose, 5.6; HEPES, 25; pargyline, 0.1; and ascorbate, 1.0. In some experiments this buffer was altered as follows. High K^+ buffer contained 60 m*M* KCl and 65 m*M* NaCl; for the Ca^{2+}-free buffer, Ca^{2+} was omitted; for the Cl^--substituted buffer, 125 m*M* NaCl was substituted by 62.5 m*M* Na_2SO_4; in Na^+-free buffers, 125 m*M* NaCl was substituted by either 225 m*M* sucrose, 125 m*M* LiCl, 125 m*M* choline chloride, or 125 m*M* *N*-methyl-D-glucamine HCl.

HPLC determination of [^{3}H]DA and metabolites

Each cell culture was loaded with [^{3}H]DA and subsequently incubated for 5 min with KRH buffer (to assess basal release) followed by 5 min with 10^{-4} *M* glutamate, as described above except that 0.25 ml of release medium was used instead of 0.5 ml. The release media were collected, and the identities of the radiolabelled compounds in the media were analyzed as follows. Release media from four cultures were pooled, acidified to pH 3–3.5 with 1 *M* HCl, and lyophilized under reduced pressure. The residue was resuspended in 0.9 ml of methanol, sonicated for 30 min, and centrifuged. Aliquots (0.7 ml) of the supernatant from triplicate samples were combined, concentrated under nitrogen, and then lyophilized. The residue was resuspended in 0.3 ml of 0.1 *M* sodium acetate buffer containing catecholamine standards [DA, noradrenaline (NA), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) each at a concentration of 10 pg/μl]. An aliquot (0.02 ml) of the sample was injected onto

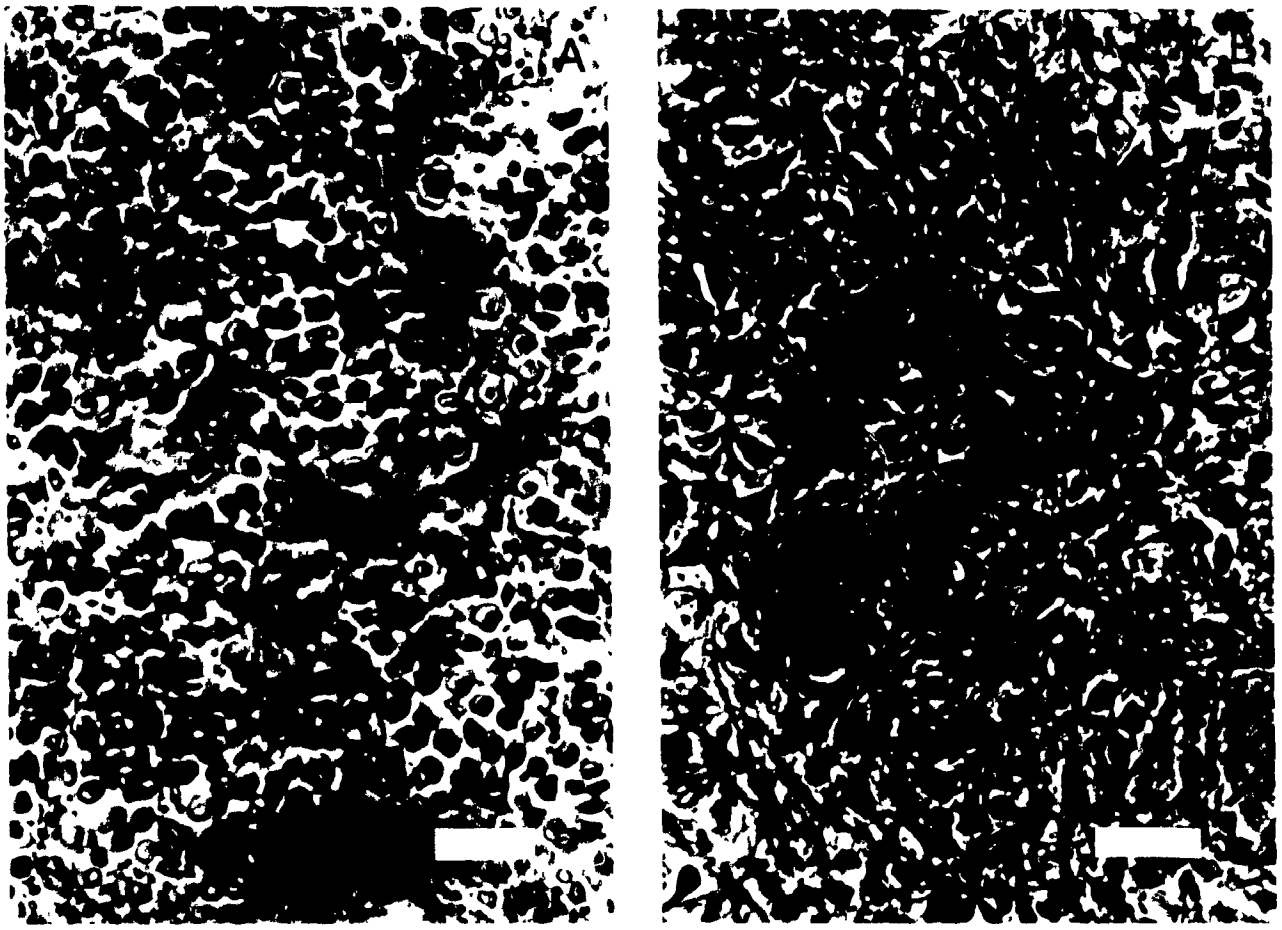

FIG. 1. Phase-contrast micrographs of cells from the ventral mesencephalon A Dissociated cells at the time of plating B: Dissociated cells after 5 days in culture Bar = 50 μm

the HPLC column, and radioactivity was determined in fractions eluted from the column at 30-s intervals The HPLC conditions used for separation and detection of DA and its metabolites were those described by Renner and Luine (1984), except that a Novapak C18 column (3.9 × 15 cm, Waters Associates) was used, and the mobile phase contained 250 mg/L sodium octyl sulfate and no acetonitrile. Average recoveries of DA, NA, HVA, and DOPAC standards added to the initial release medium and extracted following the above procedure were 82%, 83%, 111%, and 103%, respectively.

Lactate dehydrogenase (LDH) activity

LDH activity was assayed according to the method of Wróblewski and LaDue (1955) Control cultures incubated with standard KRH buffer for 30 min released a measurable amount of LDH activity into the medium, this amount of activity was approximately the lower limit of sensitivity of the LDH assay.

Statistics

The significance of differences between groups was analyzed using a one-way analysis of variance with post-hoc *t* tests.

RESULTS

Glutamate-stimulated [^{3}H]DA release

When incubated with [^{3}H]DA, dissociated cell cultures of the rat ventral mesencephalon took up [^{3}H]DA from the extracellular medium (see Fig. 3, control uptake) Glutamate, in concentrations from 10^{-5} to 10^{-3} *M*, stimulated the release of [^{3}H]DA from cell cultures previously loaded with [^{3}H]DA (Fig 2). Maximal stimulation of [^{3}H]DA release was achieved with 10^{-4} *M* glutamate; at this concentration, glutamate stimulated release of 6.6% of the total amount of [^{3}H]DA taken up by the cells. Expressed as percentage increase over basal [^{3}H]DA release, the stimulation of [^{3}H]DA release produced by glutamate (10^{-6} *M*, 10^{-5} *M*, 10^{-4} *M*, and 10^{-3} *M*) was 0 ± 6 7 (18), 48.8 ± 7 2 (34), 123 0 ± 11 9 (34), and 115.8 ± 10.2 (34), respectively [data expressed as (glutamate-stimulated [^{3}H]DA release − basal [^{3}H]DA release)/basal [^{3}H]DA release × 100; mean ± SEM from the number of cultures shown in parentheses]. In the absence of extracellular Ca^{2+}, glutamate did not stimulate release of [^{3}H]DA (Fig. 2), indicating that glutamate-stimulated [^{3}H]DA release is a Ca^{2+}-dependent process. As a function of the age of the culture, glutamate-stimulated [^{3}H]DA release was maximal on days 4–6 in culture; at an earlier time in culture (day 2), glutamate was unable to stimulate release of [^{3}H]DA, and the glutamate response was submaximal by day 8 in culture (data not shown). The inability of the cultures to release [^{3}H]DA

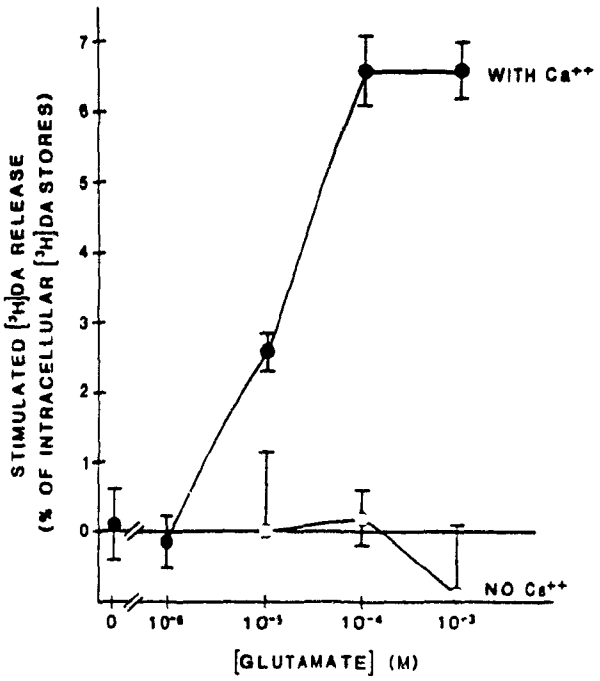

FIG. 2. Glutamate stimulation of [^{3}H]DA release in the presence and absence of extracellular Ca^{2+}. Dissociated cells from the ventral mesencephalon, maintained in culture for 4–6 days, were loaded with [^{3}H]DA as described in Materials and Methods. Following this, the cultures were incubated for 5 min with buffer alone (to determine basal [^{3}H]DA release), followed by a 5-min incubation with the indicated concentration of glutamate, in the presence ("With Ca^{2+}") or absence ("No Ca^{2+}") of 2.2 m*M* Ca^{2+}. Basal [^{3}H]DA release has been subtracted from glutamate-stimulated [^{3}H]DA release as described in Materials and Methods, and glutamate-stimulated [^{3}H]DA release is expressed as a percentage of the total amount of [^{3}H]DA taken up by the cells. Results are the means ± SEM from 18–43 cultures in the presence of Ca^{2+} and from seven or eight cultures in the absence of Ca^{2+}. There was no significant difference in basal [^{3}H]DA release in the presence and absence of Ca^{2+}. In the presence of Ca^{2+}, glutamate produced a significant stimulation of [^{3}H]DA release ($F = 65.5$, $df = 4$, $p < 0.0001$), values significantly different from 0 glutamate are at 10^{-5} *M* ($p < 0.0005$), 10^{-4} *M* ($p < 0.0005$), and 10^{-3} *M* ($p < 0.0005$) glutamate. Stimulation of [^{3}H]DA release by glutamate (10^{-5}, 10^{-4}, and 10^{-3} *M*) was significantly ($p < 0.005$) decreased in the absence of Ca^{2+}.

in response to glutamate on day 2 was accompanied by a deficiency in the ability of the cells to take up [^{3}H]DA from the medium; for example, in a single-cell preparation, cells cultured for 2, 6, and 8 days took up 2,053 ± 256 dpm, 53,138 ± 6,442 dpm, and 40,400 ± 3,400 dpm (means ± SEM from 22–24 cultures) of [^{3}H]DA, respectively.

A depolarizing concentration (6×10^{-2} *M*) of K^+ also stimulated the release of [^{3}H]DA from [^{3}H]DA-loaded cultures. The amount of [^{3}H]DA released by K^+ (25.3 ± 1.1% of intracellular [^{3}H]DA stores: mean ± SEM from 36 cultures) was much greater than the maximal amount of [^{3}H]DA released by glutamate. In the absence of extracellular Ca^{2+}, K^+-stimulated [^{3}H]DA release was reduced to 8.5 ± 0.4% of intracellular [^{3}H]DA stores (mean ± SEM from eight cultures). K^+-stimulated [^{3}H]DA release was maximal and well maintained from days 5–8 in culture; K^+ was unable to stimulate [^{3}H]DA release on day 2 in culture, and the response was submaximal on day 4 (data not shown).

Characterization of [^{3}H]DA taken up and released

In cell cultures from the ventral mesencephalon, dopaminergic neurons represent only a fraction of the total cell population (Berger et al., 1982). In order to determine which cell type is able to take up [^{3}H]DA, monoamine uptake inhibitors, which are relatively specific for particular amines, were tested for their effects on [^{3}H]DA uptake (Fig. 3). The DA uptake inhibitor benztropine (5×10^{-6} *M*) reduced [^{3}H]DA uptake to 12% of the control value, whereas the serotonin uptake inhibitor fluoxetine (10^{-6} *M*) had no effect and the NA uptake inhibitor desipramine (5×10^{-6} *M*) had only a slight effect on [^{3}H]DA uptake.

To test whether the [^{3}H]DA taken up by the cells remained and was released as unmetabolized [^{3}H]DA, cell cultures were loaded with [^{3}H]DA and incubated according to the protocol used for standard release experiments, and the identities of the radioactive compounds released into the medium were determined by HPLC. The HPLC system used provided good separation between dopamine and each of its major metabolites, such as NA, HVA, and DOPAC (Fig. 4a). The only radioactive catecholamine detected in release media from cells exposed to 10^{-4} *M* glutamate for 5

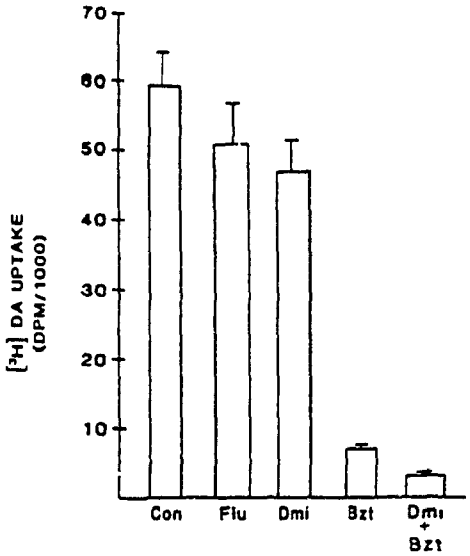

FIG. 3. Effects of monoamine uptake inhibitors on [^{3}H]DA uptake. Mesencephalic cell cultures were loaded with [^{3}H]DA as described in Materials and Methods in the presence of buffer alone (Con) or in the presence of buffer containing 10^{-6} *M* fluoxetine (Flu), 5×10^{-6} *M* desipramine (Dmi), 5×10^{-6} *M* benztropine (Bzt), or 5×10^{-6} *M* desipramine plus 5×10^{-6} *M* benztropine (Dmi + Bzt). Results are the means + SEM from 12–16 cultures. Drug treatment produced a significant effect ($F = 46.9$, $df = 4$, $p < 0.0001$), values significantly different from control are Dmi ($p < 0.05$), Bzt ($p < 0.0005$), and Dmi + Bzt ($p < 0.0005$).

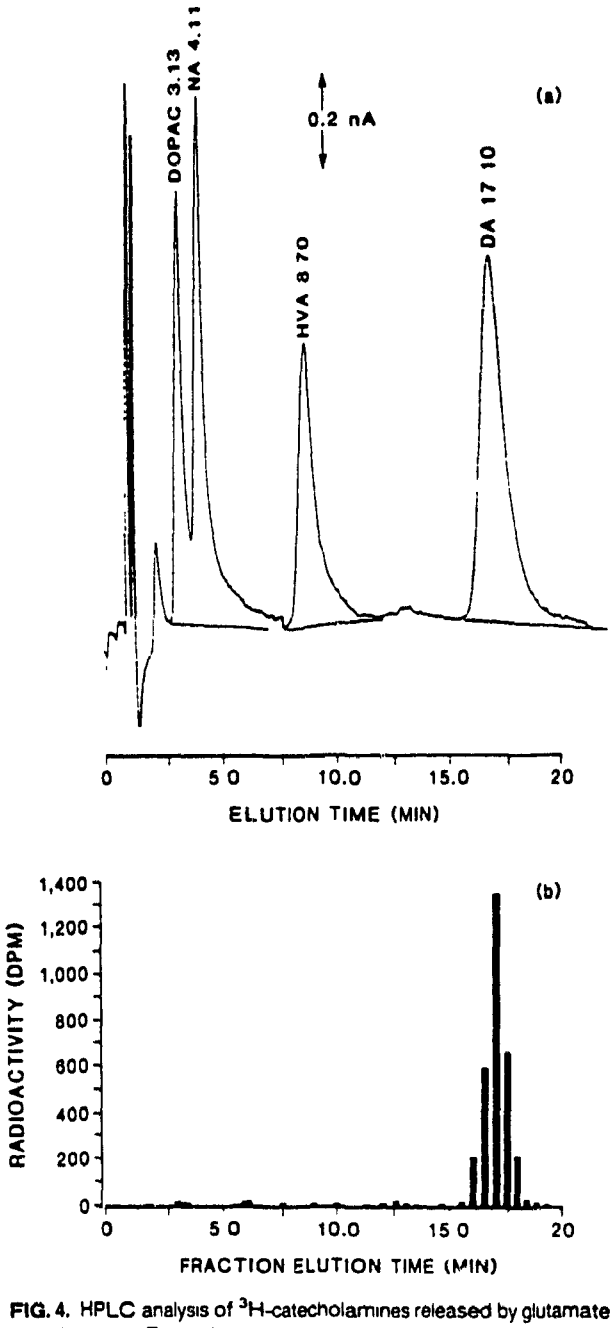

FIG. 4. HPLC analysis of ^{3}H-catecholamines released by glutamate stimulation. **a:** Trace showing chromatographic separation of catecholamine standards 1 ng each of DOPAC, NA, HVA, and DA **b:** Elution pattern of the ^{3}H released by stimulation of mesencephalic cells with glutamate Mesencephalic cell cultures were loaded with [^{3}H]DA Release of ^{3}H-catecholamines was then stimulated by incubating the cells with 10^{-4} *M* glutamate for 5 min, and ^{3}H-catecholamines in the release medium were extracted and separated by HPLC.

min was [^{3}H]DA (Fig. 4). [^{3}H]DA was also the only radioactive catecholamine detected in release media from a 5-min basal release incubation (data not shown). In addition, in cell cultures extracted just prior to the time at which release was normally tested, >90% of the cell content of ^{3}H comigrated with authentic DA (data not shown)

On the mechanism of glutamate-stimulated [^{3}H]DA release

cis-2,3-PDA is a nonselective glutamate receptor antagonist capable of blocking responses at three glutamate receptor subtypes (*N*-methyl-D-aspartate-, kainate-, and quisqualate-preferring; for review see Fagg et al., 1986). Glutamate stimulation of [^{3}H]DA release from mesencephalic cultures was almost completely abolished by 5×10^{-3} *M* *cis*-2,3-PDA (Table 1), indicating that this stimulation is a glutamate receptor-mediated response As expected, *cis*-2,3-PDA (up to 5×10^{-3} *M*) had no effect on K^+-induced [^{3}H]DA release (data not shown)

Glutamate and glutamate analogues can be toxic to some neurons under certain conditions. The following experiments were performed to determine whether glutamate-stimulated release of [^{3}H]DA from mesencephalic cultures is the result of stimulus–secretion coupling (i.e., physiological release) or the result of neurotoxic damage to the cells. To examine the integrity of the general cell population in the culture, the release of LDH, a cytosolic marker, was measured following a half-hour incubation with glutamate (10^{-3} *M*) or K^+ (6×10^{-2} *M*). (It should be noted that in experiments measuring [^{3}H]DA release, cultures were routinely incubated with glutamate or K^+ for a period of 5 min.) Whereas exposure of the cell cultures to a hypotonic medium caused the release of measurable amounts of LDH activity into the medium, neither glutamate nor K^+ induced release of LDH from the cultures (Fig. 5) In order to examine specifically the functional integrity of the dopaminergic cell population following exposure to glutamate, we preexposed cultures to glutamate (10^{-4} *M*) for 10 min and then examined the ability of the cells to take up [^{3}H]DA (Table 2, part a) and to release it in response to stimulation by glutamate (Table 2, part b) or K^+ (Table 2, part c).

TABLE 1. *Effects of* cis-*2,3-PDA on glutamate-stimulated release of [^{3}H]DA*

	[^{3}H]DA release (percent intracellular [^{3}H]DA stores)
Glutamate	7.6 ± 0.7
Glutamate + *cis*-2,3-PDA (5×10^{-4} *M*)	6.9 ± 0.3
Glutamate + *cis*-2,3-PDA (10^{-3} *M*)	5.4 ± 1.1
Glutamate + *cis*-2,3-PDA (2.5×10^{-3} *M*)	4.1 ± 0.8[a]
Glutamate + *cis*-2,3-PDA (5×10^{-3} *M*)	1.0 ± 0.2[b]

Mesencephalic cell cultures, preloaded with [^{3}H]DA, were incubated for 5 min with the indicated concentration of *cis*-2,3-PDA, followed by a 5-min incubation with the same concentration of *cis*-2,3-PDA plus 10^{-4} *M* glutamate [^{3}H]DA release shown is that measured during the second 5-min incubation period *cis*-2,3-PDA alone had no effect on basal [^{3}H]DA release Basal [^{3}H]DA release has been subtracted from glutamate-stimulated [^{3}H]DA release. Results are the means ± SEM from three or four cultures

cis-2,3-PDA produced a significant ($F = 11.6$, $df = 4$, $p < 0.005$) inhibition of glutamate-stimulated [^{3}H]DA release, [a]$p < 0.025$, [b]$p < 0.0005$

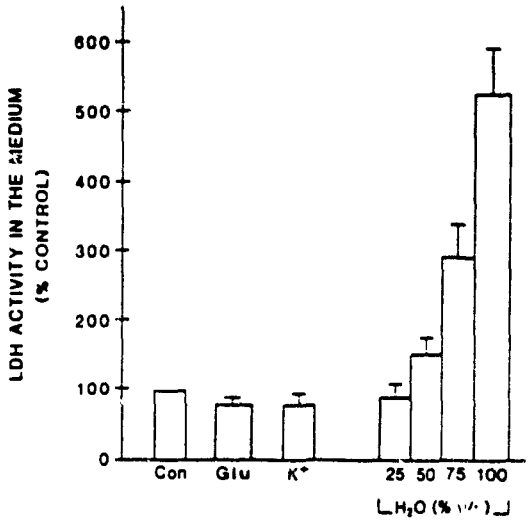

FIG. 5. Effects of glutamate, K^+, and hypotonicity on the release of LDH into the medium. Mesencephalic cell cultures were incubated for 30 min with buffer containing 10^{-3} *M* glutamate (Glu), 6×10^{-2} *M* K^+, or a mixture of buffer and water as indicated. Following this, LDH activity in the medium was measured. Results are the means ± SEM from three to six cultures. Treatment of cultures produced a significant effect on LDH activity in the medium ($F = 24.9$, $df = 6$, $p < 0.0001$). A significant increase in LDH in the medium was produced only by incubating cultures with 75% H_2O ($p < 0.01$) and 100% H_2O ($p < 0.005$).

Exposure of the cells to glutamate did not impair their subsequent ability to accumulate [^{3}H]DA or to release it in response to stimulation.

The objective of the next series of experiments was to determine whether voltage-sensitive Na^+ channels, activated by a glutamate-induced depolarization of the neuron, might be involved in the stimulation of [^{3}H]DA release by glutamate. Two classes of voltage-sensitive Na^+ channels have been identified according to their sensitivities to pharmacologic blockade. One class of Na^+ channels is potently inhibited by TTX, whereas TTX-resistant Na^+ channels are potently inhibited by sea anemone toxins, Cd^{2+}, or Zn^{2+} (Frelin et al., 1986). Neither TTX (10^{-8}–10^{-5} *M*, Fig. 6a) nor Zn^{2+} (5×10^{-6}–5×10^{-4} *M*, Fig. 6b) had any significant effect on glutamate-stimulated [^{3}H]DA release from mesencephalic cultures. TTX (10^{-8}–10^{-7} *M*) produced a small (~15%) decrease in basal [^{3}H]DA release, whereas Zn^{2+} had no effect on basal release (data not shown).

Figure 7 shows the results of experiments designed to determine whether extracellular Na^+ is necessary for glutamate stimulation of [^{3}H]DA release. Glutamate stimulation of [^{3}H]DA release was well maintained when NaCl in the medium was substituted by an isoosmotic concentration of sucrose; Cl^- substitution alone (by SO_4^{2-}) also had no effect on glutamate stimulation of [^{3}H]DA release. [It should be noted that it was important to preincubate the cells in the Na^+-free medium in these experiments because introduction of the cells to a Na^+-free medium stimulates release of [^{3}H]DA. However, the level of this release falls to baseline after 20 min in the Na^+-free medium. Stimulation of catecholamine release following introduction of other neurosecretory cell types to Na^+-free medium has been previously observed (Lastowecka and Trifaró, 1974).]

Further experiments (also shown in Fig. 7) tested the effects of replacing Na^+ in the medium with several other cations. Li^+ could be substituted for Na^+ in maintaining glutamate-stimulated [^{3}H]DA release. However, when 125 m*M* Na^+ in the medium was replaced by choline, glutamate-stimulated [^{3}H]DA release was completely abolished. Addition of lower concentrations (10 μM or 10 m*M*) of choline to standard NaCl-containing medium had no effect on glutamate-stimulated [^{3}H]DA release (data not shown). Complete replacement of NaCl in the medium by *N*-methyl-D-glucamine HCl produced a partial inhibition of glutamate-stimulated [^{3}H]DA release. As expected, [^{3}H]DA release stimulated by direct K^+ depolarization was well maintained when choline was substituted for Na^+, or SO_4^{2-} was substituted for Cl^- (data not shown).

Although neither TTX nor Zn^{2+} had any effect on glutamate-stimulated [^{3}H]DA release, veratridine (10^{-5}

TABLE 2. *Effects of preincubation with glutamate on subsequent [^{3}H]DA uptake (a) and [^{3}H]DA release in response to stimulation with glutamate (b) and K^+ (c)*

a [^{3}H]DA uptake	dpm
Preincubation with	
Buffer	26,801 ± 1,735 (32)
Glutamate	33,791 ± 4,018 (16)
b Glutamate-stimulated [^{3}H]DA release	**Percent intracellular [^{3}H]DA stores**
Preincubation with	
Buffer	10.7 ± 0.9 (11)
Glutamate	11.3 ± 1.1 (12)
c K^+-stimulated [^{3}H]DA release	**Percent intracellular [^{3}H]DA stores**
Preincubation with	
Buffer	25.9 ± 1.0 (12)
Glutamate	29.0 ± 1.2 (12)

Mesencephalic cell cultures were preincubated for 10 min with buffer or buffer containing 10^{-4} *M* glutamate. Following this the cultures were washed and loaded with [^{3}H]DA as described in Materials and Methods, in the absence of glutamate. Cultures were then incubated for 5 min with buffer (to determine basal [^{3}H]DA release), followed by a 5-min incubation with 10^{-4} *M* glutamate (b) or 6×10^{-2} *M* K^+ (c). For (a), [^{3}H]DA uptake was calculated as the sum of [^{3}H]DA released into the medium during the release incubations plus the residual [^{3}H]DA left in the cells following the release incubations. For (b) and (c), basal [^{3}H]DA release has been subtracted from stimulated [^{3}H]DA release. Results are the means ± SEM from the number of cultures shown in parentheses.

Preincubation with glutamate produced significant increases in subsequent [^{3}H]DA uptake ($p < 0.05$) and K^+-stimulated [^{3}H]DA release ($p < 0.05$) and had no effect on glutamate-stimulated [^{3}H]DA release.

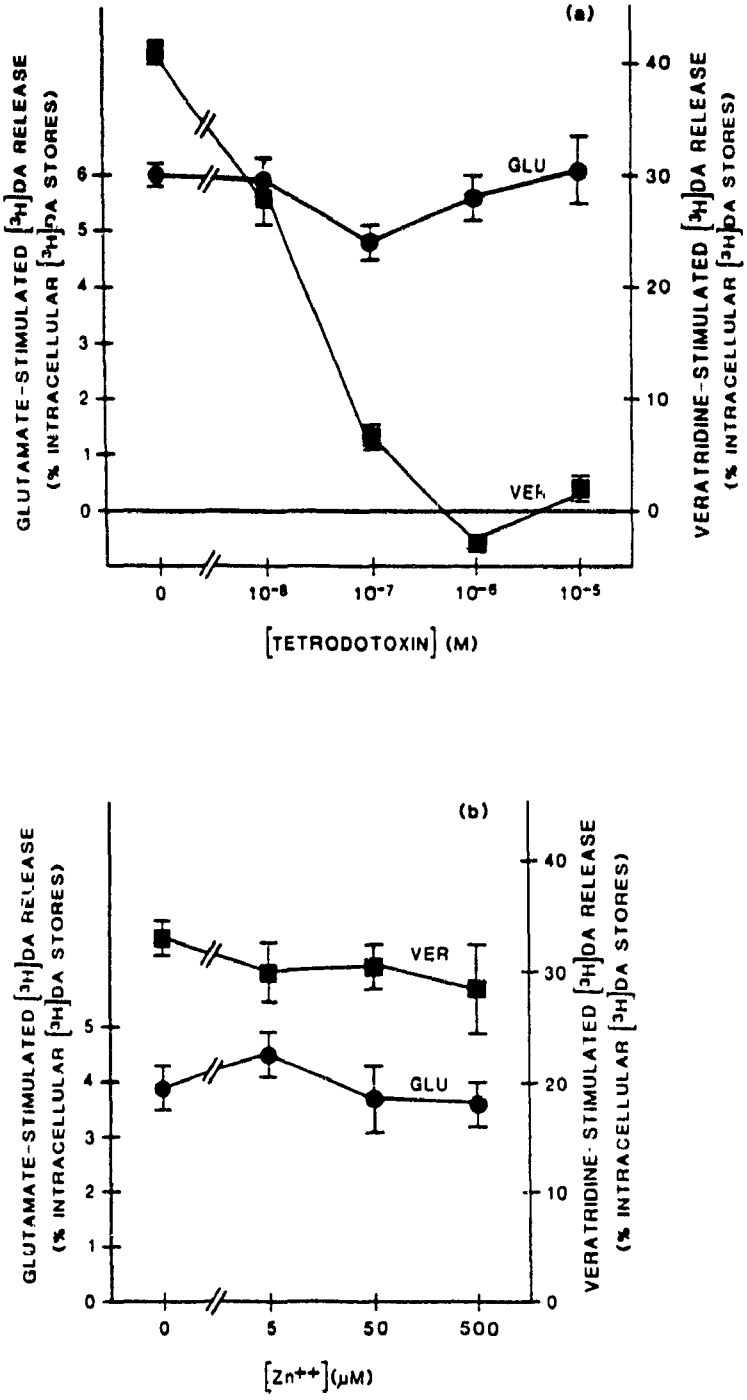

FIG. 6. Effects of TTX (a) and Zn^{2+} (b) on stimulation of [^{3}H]DA release by veratridine or glutamate. Mesencephalic cell cultures, preloaded with [^{3}H]DA, were incubated for 15 min with the indicated concentrations of TTX or for 5 min with the indicated concentrations of $ZnSO_4$. This was followed by a 5-min incubation with the same concentration of TTX or Zn^{2+} plus 10^{-5} *M* veratridine or 10^{-4} *M* glutamate. Basal [^{3}H]DA release has been subtracted from veratridine- or glutamate-stimulated [^{3}H]DA release. Results are the means ± SEM from 4–12 cultures, except for the value for veratridine-stimulated [^{3}H]DA release in the presence of 10^{-6} *M* TTX that is the mean from two cultures. TTX produced a significant ($F = 82.4$, $df = 4$, $p < 0.0001$) inhibition of veratridine-stimulated [^{3}H]DA release; significant inhibition was produced by 10^{-8} *M* ($p < 0.01$), 10^{-7} *M* ($p < 0.0005$), 10^{-6} *M* ($p < 0.0005$), and 10^{-5} *M* ($p < 0.0005$) TTX. TTX had no significant effect ($F = 1.6$, $df = 4$, $p > 0.10$) on glutamate-stimulated [^{3}H]DA release. Zn^{2+} had no significant effect on either veratridine- ($F = 0.5$, $df = 3$, $p > 0.25$) or glutamate-stimulated ($F = 0.8$, $df = 3$, $p > 0.25$) [^{3}H]DA release.

M), an agent known to activate voltage-sensitive Na^+ channels, was able to stimulate [^{3}H]DA release from mesencephalic cultures (Fig. 6a and b); the magnitude of release evoked by veratridine was much greater than the maximal release evoked by glutamate (compare Fig. 6a and b with Fig. 2). In addition, veratridine-stimulated [^{3}H]DA release was blocked by TTX (Fig. 6a). Zn^{2+} (5×10^{-6}–5×10^{-4} *M*) had no effect on veratridine-stimulated [^{3}H]DA release (Fig. 6b).

In some neurons, TTX-resistant action potentials can also be generated by inward Ca^{2+} currents, and these Ca^{2+} action potentials can be blocked by omitting Ca^{2+} or by adding 2×10^{-3} *M* Co^{2+} or 10^{-2} *M* Mg^{2+} to the extracellular medium (Williams et al., 1984; Williams and Marshall, 1987). To test whether Ca^{2+} action potentials are necessary for glutamate stimulation of [^{3}H]DA release from mesencephalic cells, glutamate stimulation of [^{3}H]DA release was tested in the presence of 2×10^{-3} *M* Co^{2+}; glutamate-stimulated [^{3}H]DA release was maintained at 60–82% of control values in the presence of Co^{2+} (Table 3). In addition, lidocaine (2×10^{-4} *M*), which has been shown to completely abolish action potentials in dissociated cerebral cortex cell cultures (Romijn et al., 1981), had no effect

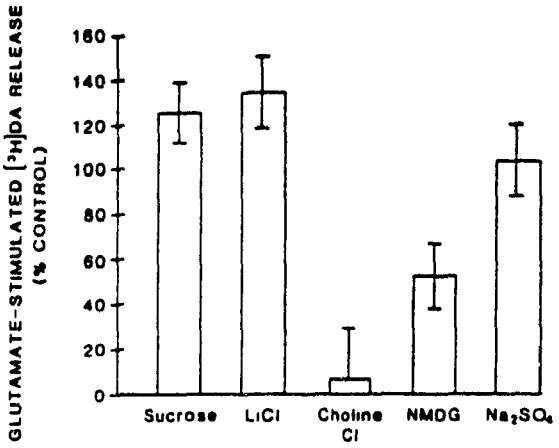

FIG. 7. Effects of extracellular NaCl substitutions on the stimulation of [^{3}H]DA release by glutamate. Mesencephalic cell cultures, preloaded with [^{3}H]DA, were incubated for 20 min with either standard buffer containing the usual 125 mM NaCl (control) or standard buffer, in which 125 mM NaCl was replaced with 225 mM sucrose, 125 mM LiCl, 125 mM choline chloride, 125 mM *N*-methyl-D-glucamine (NMDG), or 62.5 mM Na_2SO_4. Following this, the cultures were incubated for 5 min in a buffer of the same composition to determine basal [^{3}H]DA release, followed by a 5-min incubation with the same buffer containing 10^{-4} *M* glutamate (except for experiments with choline chloride, in which 10^{-3} *M* glutamate was used). Results are the means ± SEM from 4–14 cultures. Basal [^{3}H]DA release has been subtracted from stimulated [^{3}H]DA release. In control cultures, release of [^{3}H]DA in response to stimulation by 10^{-4} *M* and 10^{-3} *M* glutamate was 3.7 ± 0.5% (mean ± SEM from 40 cultures) and 4.3 ± 1.0% (mean ± SEM from four cultures) of intracellular [^{3}H]DA stores, respectively. Values significantly different from control are choline chloride ($p < 0.025$) and NMDG ($p < 0.025$), other values are not significantly different ($p > 0.05$) from control. Statistical analysis was performed on raw data before normalization to percent control.

on glutamate-stimulated [^{3}H]DA release from mesencephalic cultures (Table 3).

DISCUSSION

The present study shows that cultures of dissociated cells from fetal rat ventral mesencephalon take up [^{3}H]DA into dopaminergic neurons, as [^{3}H]DA uptake was strongly inhibited by a low concentration of benztropine, a relatively specific inhibitor of DA uptake into dopaminergic neurons, but not by desipramine or fluoxetine, inhibitors of NA and serotonin uptake, respectively. Previous studies by Prochiantz et al. (1979) and Daguet et al. (1980) have shown that dissociated cell cultures from fetal mouse mesencephalon also take up [^{3}H]DA into dopaminergic neurons by a benztropine-sensitive mechanism and release it in response to stimulation by K^+ or veratridine.

In the present study, glutamate stimulated a Ca^{2+}-dependent release of [^{3}H]DA from mesencephalic cell cultures, and this release was inhibited by a glutamate antagonist, *cis*-2,3-PDA, indicating that stimulation of release was mediated by glutamate receptors. Glutamate and some of its analogues are known to be toxic to some populations of neurons under certain conditions. However, the following observations indicate that glutamate stimulation of [^{3}H]DA release from mesencephalic cultures is not due to neurotoxic damage. (1) Exposure of the cells to a concentration of glutamate that produces maximal stimulation of [^{3}H]DA release did not cause release of LDH, a cytosolic marker, into the medium. (2) Extracellular Na^+, Cl^-, and Ca^{2+} have all been implicated as necessary agents in the development of glutamate-induced neurotoxicity (Choi, 1985; Rothman, 1985; Garthwaite et al., 1986; Olney et al., 1986). In the present study, glutamate stimulated [^{3}H]DA release in the absence of both Na^+ and Cl^-, indicating that neither Na^+- nor Cl^--dependent neurotoxicity is responsible for the release. (Because stimulus–secretion coupling is almost certainly Ca^{2+} dependent, it is not possible to distinguish between neurotoxicity and physiological release on the basis of Ca^{2+} dependence.) (3) Preexposure of the cultures to glutamate had no effect on the subsequent ability of the dopaminergic neurons to take up and retain [^{3}H]DA or to release it in response to stimulation by glutamate or K^+. This indicates that these neurons were not leaking [^{3}H]DA. Taken together, these results indicate that glutamate stimulation of [^{3}H]DA release under conditions of the present study is the result of stimulus–secretion coupling rather than neurotoxicity.

Veratridine stimulated release of [^{3}H]DA from mesencephalic cultures in a TTX-sensitive manner. This indicates that TTX-sensitive, voltage-sensitive Na^+ channels are present on at least some of the DA neurons in these cultures. However, glutamate stimulation of [^{3}H]DA release was not inhibited by either TTX or Zn^{2+}. This suggests that depolarization-induced activation of voltage-sensitive Na^+ channels is not necessary for glutamate-evoked release. In addition, Co^{2+}, which blocks Ca^{2+} action potentials, and lidocaine,

TABLE 3. *Effects of Co^{2+} and lidocaine on glutamate-stimulated release of [^{3}H]DA*

	Percent of control glutamate-stimulated [^{3}H]DA release
Glutamate (10^{-4} *M*) + Co^{2+}	60.3 ± 7.9*
Glutamate (10^{-3} *M*) + Co^{2+}	82.4 ± 8.9*
Glutamate (10^{-4} *M*) + lidocaine	100.6 ± 8.3
Glutamate (10^{-3} *M*) + lidocaine	94.0 ± 4.2

Mesencephalic cell cultures, preloaded with [^{3}H]DA, were incubated for 5 min with either standard buffer alone or standard buffer containing Co^{2+} (2×10^{-3} *M*) or lidocaine (2×10^{-4} *M*) (for the measurement of basal [^{3}H]DA release), followed by a 5-min incubation with the same concentration of Co^{2+} or lidocaine and the indicated concentration of glutamate (for the measurement of glutamate-stimulated [^{3}H]DA release). Results are the means ± SEM from eight or nine cultures.

Co^{2+} produced a significant inhibition of glutamate-stimulated [^{3}H]DA release ($F = 7.1$, $df = 3$, $p < 0.025$); *$p < 0.05$. Lidocaine had no significant effect on glutamate-stimulated [^{3}H]DA release ($p > 0.25$). Statistical analysis was performed on raw data before normalization to percent control.

which completely blocks all action potentials in cultured cerebral cortex, had only minimal or no effect on glutamate-stimulated [^{3}H]DA release. These observations indicate that there is no requirement for action potentials to be generated, in either the DA neurons themselves or in interneurons, in order for glutamate to stimulate [^{3}H]DA release. This is consistent with previous studies showing that glutamate stimulation of [^{3}H]DA release from sliced tissue or from in situ brain areas containing either DA nerve terminals (e.g., striatum: Roberts and Shanf, 1978; Marien et al., 1983; Chéramy et al., 1986; Snell and Johnson, 1986; Jhamandas and Marien, 1987) or cell bodies with their dendritic fields (e.g., substantia nigra: Chéramy et al., 1981; Marien et al., 1983) was either completely or at least partially TTX insensitive. However, neither of these preparations contains the entire DA neuron through which an action potential might be propagated. We have found that action potentials are not required for glutamate stimulation of DA release from intact DA neurons in culture. However, it should be noted that one cannot exclude the possibility that processes on cultured DA neurons might possess some different properties from their counterparts in vivo.

The ability of glutamate to stimulate DA release from dissociated DA neurons in culture, without generation of action potentials in interneurons, is consistent with the notion that glutamate *directly* stimulates DA neurons (as opposed to an indirect stimulation of dopaminergic neurons through a polysynaptic pathway). However, it is also possible that glutamate directly stimulates the neurites of an interneuron to cause release of a secondary transmitter; the secondary transmitter would then directly stimulate the neurites of the DA neuron to release DA, and this would occur without action potential generation in either the interneuron or the DA neuron. This, latter, mechanism seems unlikely because direct stimulation of neurites (either dendrites or nerve terminals) is not a commonly reported mode of action for transmitters other than glutamate. Of several neurotransmitters we have tested so far (substance P and substance K, neurotensin, μ, κ, and δ opioids, and nicotinic cholinergic agonists), none produced as great a release of [^{3}H]DA as did glutamate (unpublished observations).

Glutamate stimulation of [^{3}H]DA release from mesencephalic cultures was unaffected by replacement of NaCl in the medium by sucrose or Na_2SO_4. This indicates that the glutamate response does not require changes in permeability to either Na^+ or Cl^- and is consistent with the notion that activation of voltage-sensitive Na^+ channels is not necessary for this response. The Na^+ independence of the response also suggests that glutamate-induced depolarizations may not be due to increased Na^+ permeability through glutamate receptor-linked Na^+ channels but may involve another mechanism such as deactivation of K^+ efflux, as has previously been suggested by Shapovalov et al. (1978) and Engberg et al. (1979). An alternative interpretation is that glutamate-induced depolarization does involve an increased Na^+ permeability when physiological concentrations of Na^+ are present but that in the absence of Na^+, other ions (such as Ca^{2+}) can permeate a relatively nonspecific glutamate receptor-linked ion channel. In fact, several studies (Bührle and Sonnhof, 1983; Zanotto and Heinemann, 1983; MacDermott et al., 1986) indicate that glutamate does increase membrane permeability to both Na^+ and Ca^{2+} through glutamate receptor-linked channels.

Electrophysiological studies with spinal cord neurons have demonstrated that depolarizations evoked by glutamate or its analogues are greatly attenuated when Na^+ is substituted by choline or Tris (Hösli et al., 1973; MacDonald, 1984; Mayer and Westbrook, 1985; Nistri et al., 1985). However, Mayer and Westbrook (1987) suggested that large organic cations such as choline or Tris may block glutamate receptor-linked ion channels, thereby confounding the interpretation of Na^+ substitution experiments. Consistent with this report, we found that glutamate stimulation of [^{3}H]DA release was completely inhibited by replacement of Na^+ by choline and partially inhibited by replacement with *N*-methyl-D-glucamine; these inhibitory effects were not seen when Na^+ was replaced with Li^+ or an inert substance such as sucrose.

In conclusion, the present study indicates that cultures of dissociated cells from fetal rat ventral mesencephalon are an excellent model system in which to characterize glutamate stimulation of DA release from intact DA neurons and to study the pharmacology and molecular and ionic mechanisms involved in this process. The cultures may also be particularly useful in studying the effects of more long-term changes in the extracellular environment on the glutamate response; for example, compared with in vivo experiments, in some cases it may be easier and more economical to chronically treat cultures with drugs and factors than to treat the animal, in vivo.

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SYNAPSE 5 271–280 (1990)

Subtypes of Excitatory Amino Acid Receptors Involved in the Stimulation of [^{3}H]Dopamine Release From Cell Cultures of Rat Ventral Mesencephalon

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KEY WORDS Tissue culture, Quisqualic acid, Kainic acid, N-methyl-D-aspartic acid

ABSTRACT N-methyl-D-aspartic acid (NMDA), quisqualic acid (QUIS), and kainic acid (KAIN), respective agonists for three excitatory amino acid (EAA) receptor subtypes, stimulated [^{3}H]dopamine ([^{3}H]DA) release from dissociated cell cultures of fetal rat ventral mesencephalon Release evoked by all three agonists was Ca^{2+}-dependent and inhibited by broad-spectrum antagonists (D,L-*cis*-2,3-piperidine dicarboxylic acid [PDA] and kynurenic acid [KYN]) However, both of these antagonists were more potent against KAIN than against QUIS and only KAIN-evoked release was blocked by γ-D-glutamyl-aminomethyl sulfonic acid (GAMS, IC_{50} 700 μM) NMDA-stimulated [^{3}H]DA release was selectively inhibited by competitive (3-[2-carboxypiperazine-4-yl]propyl-1-phosphonic acid [CPP] and D,L-2-amino-5-phosphonovaleric acid [APV]) and non-competitive (phencyclidine and MK-801) NMDA receptor antagonists In 1 2 mM Mg^{2+}, NMDA-stimulated [^{3}H]DA release was Na^+-dependent and inhibited by tetrodotoxin (TTX, 2 μM) or by the local anaesthetic, lidocaine (200 μM) However, in 0 Mg^{2+}, NMDA-evoked release was not inhibited by TTX or lidocaine Thus, TTX-sensitivity of the NMDA response in 1 2 mM Mg^{2+} apparently occurs because Na^+-action potentials are required to alleviate a Mg^{2+} blockade. Neither QUIS- nor KAIN-evoked release was affected by Mg^{2+} or TTX When extracellular NaCl was replaced by sucrose or Na_2SO_4, the QUIS response was increased. KAIN-evoked release was unaffected by the sucrose substitution and was attenuated in the Na_2SO_4-containing buffer

It is concluded that NMDA and QUIS/KAIN release [^{3}H]DA via separate receptor subtypes

INTRODUCTION

Major dopaminergic pathways in the brain are known to project from the substantia nigra, pars compacta to the striatum (nigrostriatal pathway), and from cell bodies mainly in the ventral tegmental area (VTA) to medial prefrontal, cingulate, and entorhinal cortices (mesocortical) and to nucleus accumbens, olfactory tubercules, and amygdala (mesolimbic) (reviewed by Lindvall and Bjorklund, 1978) It has also been established that excitatory amino acids (EAAs) are important transmitters in excitatory corticocortical neurotransmission (see review by Cotman et al., 1987) and in corticofugal inputs to substantia nigra (Carter, 1980, 1982; Kerkerian et al., 1983, Kornhuber et al., 1984; Nieoullon and Dusticier, 1983), striatum (Carter, 1980, 1982; Fonnum et al., 1981a b; Girault et al., 1986, Kerkerian et al., 1983, Kim et al., 1977, Kornhuber and Kornhuber, 1986, Reubi and Cuenod, 1979; Roberts et al., 1982; Rowlands and Roberts, 1980, Sandberg et al., 1985, Spencer, 1976), VTA (Christie et al., 1985), and nucleus accumbens (Carter, 1980; Walaas, 1981). This localization of EAA inputs suggested that EAAs might interact with dopaminergic transmission at the level of dopamine (DA) cell bodies and/or terminal fields An ability of EAAs to stimulate dopaminergic neurotransmission has been confirmed in behavioral (Arnt 1981a,b, Cools and Peeters, 1987, Donzanti and Uretsky, 1983, Olianas et al., 1978, Pycock and Dawbarn, 1980) electrophysiological (Grace and Bunney, 1984a,b; Scarnati and Pacitti, 1982), and biochemical (Carter et al., 1988, Cheramy et al., 1986, Clow and Jhamandas, 1989; Giorgueff et al., 1977, Jhamandas and Marien, 1987, Jones et al., 1987, Marien et al., 1983, Roberts and Anderson, 1979; Roberts and Sharif, 1978) studies

EAAs have been shown to bind at and act upon at least three distinct receptor subtypes, with some selectivity, respectively, for N-methyl-D-aspartic acid (NMDA), quisqualic acid (QUIS), and kainic acid (KAIN) (see reviews by Mayer and Westbrook, 1987;

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McLennan, 1982, Watkins and Evans, 1981) NMDA receptors have clearly been implicated in the release of DA in brain slices of substantia nigra (Marien et al., 1983), striatum (Clow and Jhamandas, 1989, Jhamandas and Marien, 1987, Roberts and Anderson, 1979, Snell and Johnson, 1986), nucleus accumbens (Marien et al., 1983) and olfactory tubercule (Marien et al., 1983) and in vivo, in trans-striatal dialysis experiments (Carter et al., 1988) In order to investigate the neuronal circuitry involved in this NMDA response, most of the above studies have examined the sensitivity of NMDA receptor-stimulated DA release to inhibition by tetrodotoxin (TTX), a blocker of voltage-sensitive Na^+ channels The rationale behind these experiments has been that direct effects of NMDA agonists on the DA neuron terminals (or dendrites) should be TTX-insensitive (i e, not requiring regenerative action potentials), while TTX-sensitivity might indicate activation by the NMDA agonist of a polysynaptic pathway involving an interneuron In the striatum alone, results from such experiments have been contradictory, with NMDA receptor-stimulated DA release reported to be completely TTX-sensitive (Carter et al, 1988) completely TTX-insensitive (Clow and Jhamandas, 1989), or partially TTX-sensitive (Jhamandas and Marien, 1987, Snell and Johnson, 1986)

KAIN has also been shown to evoke the release of DA from slices of striatum (Clow and Jhamandas, 1989, Roberts and Anderson, 1979, Roberts and Sharif, 1978, Snell and Johnson, 1986) and nucleus accumbens (Jones et al, 1987), as well as from striatum in vivo (Carter et al, 1988) Where tested, KAIN-evoked DA release has been reported consistently to be partially TTX-sensitive (Carter et al, 1988, Jones et al, 1987) Although a role for striatal QUIS receptor activation in the modulation of DA-mediated behaviors has been reported (Cools and Peeters 1987) the effect of QUIS on DA release remains less clear QUIS stimulated DA release from slices of striatum (Clow and Jhamandas, 1989, Snell and Johnson, 1986) and nucleus accumbens (Jones et al, 1987), but had no effect on release in trans-striatal dialysis experiments (Carter et al, 1988)

Dissociated cell culture systems may offer certain advantages over tissue slice and dialysis techniques for the elucidation of receptor mechanisms specifically at the level of the target neuron A stricter control over the composition of the extracellular environment can be achieved, facilitating the manipulation of neuronal mechanisms by drugs or ionic alterations We have recently used cultures of dissociated cells from fetal rat ventral mesencephalon, the area of brain containing cell bodies of origin for nigrostriatal, mesocortical and mesolimbic dopaminergic pathways, to study the effects of the endogenous EAA, glutamate on release of [^{3}H]DA from dopaminergic neurons (Mount et al, 1989) It was found that glutamate-stimulated [^{3}H]DA release was Ca^{2+}-dependent and insensitive to inhibition of voltage-regulated Na^+-channels by TTX or Zn^{2+} Glutamate can activate all three EAA receptor subtypes (NMDA, QUIS, KAIN), Thus, the first objective of the present experiments was to characterize which EAA receptor subtypes can mediate [^{3}H]DA release from dissociated cell cultures of fetal rat mesencephalon The second objective was to examine the involvement of various ions (Ca^{2+}, Mg^{2+}, Na^+) and of regenerative action potentials in the EAA-stimulated release of [^{3}H]DA

A preliminary report of some of these results has been presented at a meeting (Boksa et al., 1989)

MATERIALS AND METHODS

Materials

3,4-[7,8-^{3}H(N)]dihydroxyphenylethylamine ([^{3}H]DA, 18.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U S.A.). D,L-*cis*-2,3-piperidine dicarboxylic acid (PDA) came from Cambridge Research Biochemicals Ltd (Harston, UK) and 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) came from Tocris Neuramin (Buckhurst Hill, UK) N-methyl-D-aspartic acid (NMDA), quisqualic acid (QUIS), kainic acid (KAIN), kynurenic acid (KYN), γ-D-glutamylaminomethyl-sulfonic acid (GAMS), D,L-2-amino-5-phosphonovaleric acid (APV), desipramine hydrochloride, and pargyline hydrochloride were obtained from Sigma Chemical Co (St Louis, MO) Phencyclidine (PCP) and MK-801 were generous gifts from NIDA (Baltimore, MD) and Merck, Sharp & Dohme (Harlow, UK), respectively Other chemicals were reagent grade and came from regular commercial sources

Cell culture methods

Mesencephalic cell cultures were prepared as previously described (Mount et al, 1989) Briefly, anterior ventral mesencephalic tissue was dissected from the brains of Sprague-Dawley rat embryos on gestation day 15 The pieces of tissue were mechanically dissociated and diluted to a density of approximately 10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM, Gibco Canada Burlington Ont, Canada) containing nystatin (24 U/ml), KCl (20 mM), and 10% v/v heat-inactivated fetal calf serum (Gibco Canada) Cells were grown on 24-well Multiwell plates that had been previously coated with poly-D-lysine (20 μg/ml) Cultures were maintained at 37° C in an incubator supplied with humid air containing 10% CO_2

Release experiments

Release experiments were performed on the fifth or sixth day in culture and involved the following general protocol Cells were rinsed with Krebs-Ringer-HEPES buffer (KRH) (500 μl) and then loaded with [^{3}H]DA (50 nM) in a 20 min incubation at 37°C, in the presence of desipramine (50 μM) (to inhibit [^{3}H]DA uptake into noradrenergic neurons) In previous work, uptake of [^{3}H]DA into mesencephalic cell cultures was shown to be overwhelmingly into dopaminergic neurons, as the uptake was inhibited almost completely by the DA uptake inhibitor, benztropine, but only slightly attenuated by the noradrenaline uptake inhibitor, desipramine, and unaltered by the serotonin uptake inhibitor, fluoxetine (Mount et al, 1989) After [^{3}H]DA loading, each well was rinsed four times with KRH over a 25 min period at 23°C By this time spontaneous [^{3}H]DA release reached a steady rate of 4–6% total [^{3}H]DA content per 5 min collection period KRH buffer (500 μl/well) from a 5 min incubation (spontaneous release period), followed immediately by a 5 min incubation in the presence of an EAA agonist (stimulated release period), were collected, and [^{3}H]DA release was quantitated by the amount of radioactivity in each aliquot of buffer collected For ion-substitution experiments, modified KRH was used for all rinses and incubations following [^{3}H]DA loading When antagonists

were tested, cells were exposed to these during the spontaneous release incubation as well as during exposure to the agonist. Following release incubations, radioactivity remaining in the cells was extracted by 30 min incubation with acidified ethanol (500 μl, 95% ethanol/5% 0.1 M HCl) Radioactive species released into the extracellular environment under spontaneous and glutamate-stimulated conditions, as well as total cellular content of [^{3}H]labelled compounds in the cultures, have previously been characterized by HPLC (Mount et al., 1989) The only catecholamine detected in release incubation media from cells exposed to 100 μM glutamate for 5 min was [^{3}H]DA Also, more than 90% of the radioactivity detected in cells at the time of release eluted with dopamine standards.

For data presentation, spontaneous [^{3}H]DA release was subtracted from release stimulated by the EAA agonist. The net release evoked by the agonist was expressed as a % of the total [^{3}H]DA uptake into cells

The composition (mM) of the KRH buffer (pH 7 4) was NaCl, 125, KCl, 4.8; HEPES, 25, NaOH, 5, $MgSO_4$, 1.2; KH_2PO_4, 1.2; D-glucose, 5.6; $CaCl_2$, 2.2; pargyline, 0.1; ascorbate, 1.0 NaCl-free KRH was prepared by substituting 125 mM NaCl by 225 mM sucrose: for Cl^--substituted buffer, 125 mM NaCl was substituted by 62.5 mM Na_2SO_4. When Ca^{2+} or Mg^{2+} was omitted from the KRH, no substitutions were made.

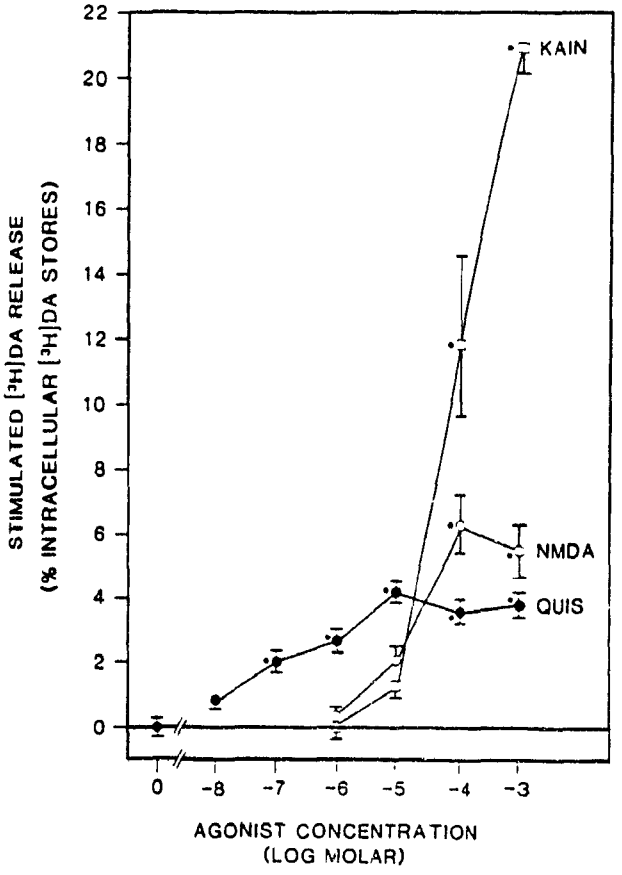


Fig 1 Dose-response curves for the stimulation of [^{3}H]DA release from mesencephalic cell cultures by N-methyl-D-aspartic acid (NMDA), quisqualic acid (QUIS), and kainic acid (KAIN) Spontaneous [^{3}H]DA release has been subtracted from [^{3}H]DA released in the presence of agonists (NMDA, QUIS and KAIN in the indicated concentrations) The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells, as described in Materials and Methods Results are the means ± SEM from 5–8 27–35, and 6–11 cultures, for NMDA, QUIS, and KAIN, respectively Significant [^{3}H]DA release was evoked by NMDA ($F=27\,8$, $df=4\,33$, $P< 001$), QUIS ($F=13\,7$, $df=6.195$, $P< 001$) and KAIN ($F=117\,7$, $df=4\,34$, $P< 001$) *$P< 05$, compared to control buffer

Statistics

Release data were subjected to unpaired t-tests, or to one-way analysis of variance, followed by a Newman-Keuls test for multiple comparisons, as appropriate The accepted level of statistical significance was $\alpha \leq 05$.

RESULTS

Effects of EAA agonists on [^{3}H]DA release

Initial experiments tested whether [^{3}H]DA release from mesencephalic cell cultures could be stimulated by NMDA, QUIS and KAIN, agonists that activate three subtypes of EAA receptors Each of these agonists stimulated [^{3}H]DA release from the cultures (Fig 1) QUIS was the most potent, while efficacy at high agonist concentrations was KAIN >> NMDA > QUIS. This order of agonist efficacy was conserved when the release was measured over periods ranging from 1 to 11 min in duration (data not shown)

Figure 2 indicates that when Ca^{2+} was omitted from the extracellular environment, NMDA- and QUIS-evoked [^{3}H]DA release were completely abolished, while KAIN-evoked release was attenuated by more than 90% Omission of Ca^{2+} from the extracellular environment had no significant effect on spontaneous [^{3}H]DA release from cell cultures (data not shown).

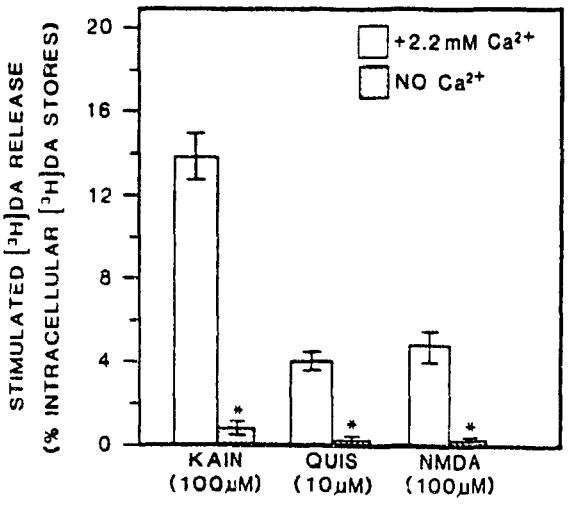


Fig 2 Ca^{2+}-dependence of [^{3}H]DA release stimulated by N-methyl-D-aspartic acid (NMDA), quisqualic acid (QUIS) and kainic acid (KAIN) Cells were loaded with [^{3}H]DA in control KRH buffer (2 2 mM $CaCl_2$) Subsequent rinses, spontaneous release, and release in the presence of agonists (NMDA QUIS and KAIN in the indicated concentrations) were collected either in control KRH or in $CaCl_2$-free KRH (0 mM $CaCl_2$ added) Results are the means ± SEM from 8–9 cultures Spontaneous [^{3}H]DA release has been subtracted from agonist-stimulated [^{3}H]DA release Release stimulated by each agonist in the absence of $CaCl_2$, was significantly (*$P< 001$) attenuated in comparison to the corresponding release in the presence of $CaCl_2$ Only KAIN stimulated any [^{3}H]DA release in $CaCl_2$-free KRH ($P< 001$) Exclusion of $CaCl_2$ from the KRH had no effect on spontaneous [^{3}H]DA release (data not shown)

Effects of EAA antagonists on EAA-stimulated [^{3}H]DA release

Kynurenic acid (KYN; Fig. 3) and D,L-*cis*-2,3-piperidine dicarboxylic acid (PDA; data not shown), two broad-spectrum EAA antagonists that block actions of EAAs at all three EAA receptor subtypes (Ganong et al., 1983; Jahr and Jessel, 1985, Watkins and Evans, 1981), inhibited [^{3}H]DA release stimulated by QUIS, KAIN, and NMDA, without affecting spontaneous [^{3}H]DA release. Both KYN and PDA were more potent against NMDA- and KAIN-evoked [^{3}H]DA release than against the QUIS-response (IC_{50}s for PDA inhibition of NMDA, KAIN, and QUIS responses were 460, 1,100, and 3,000 μM, respectively). GAMS (Croucher et al., 1984, Jones et al., 1984; Davies and Watkins, 1985) inhibited KAIN-stimulated [^{3}H]DA release at concentrations that were ineffective against either QUIS- or NMDA-stimulated [^{3}H]DA release (Fig. 3).

The competitive NMDA receptor antagonist 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP), (Davies et al., 1986, Lehmann et al., 1987) completely inhibited NMDA-stimulated [^{3}H]DA release at concentrations that had no effect on the KAIN or QUIS responses (Fig. 4). Another competitive NMDA receptor antagonist, D,L-2-amino-5-phosphonovaleric acid (APV) (Evans et al., 1982; Davies and Watkins, 1982), inhibited responses to NMDA much more potently than release stimulated by QUIS or KAIN (Fig. 4). Two noncompetitive NMDA antagonists, phencyclidine (PCP) (Anis et al., 1983) and MK-801 (Wong et al., 1986), potently inhibited the release stimulated by NMDA, but not the release stimulated by QUIS or KAIN (Fig. 5). Spontaneous release of [^{3}H]DA was increased by 100 μM PCP or MK-801 (by 2% of intracellular [^{3}H]DA stores), but was unaffected by CPP or APV (data not shown).

Effect of Mg^{2+} on [^{3}H]DA release stimulated by NMDA

A well-established characteristic of responses at the NMDA receptor is voltage-dependent blockade of receptor activation by low millimolar concentrations of Mg^{2+} (Ault et al., 1980, Davies and Watkins, 1980; Mayer and Westbrook, 1985, Nowak et al., 1984). Since release experiments were performed in Mg^{2+} (1.2 mM)-containing release buffer, it was reasoned that removal of Mg^{2+} from the buffer would increase NMDA-stimu-

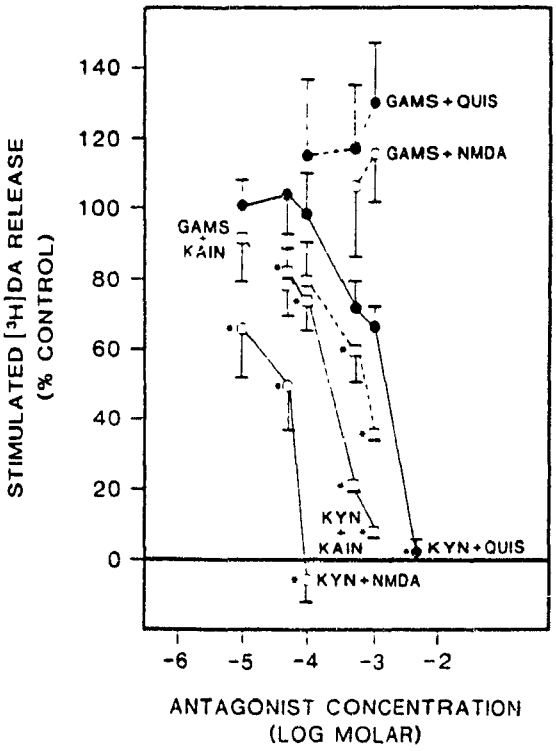


Fig. 3. Effects of the broad-spectrum EAA antagonists kynurenic acid (KYN) and γ-D-glutamylaminomethyl sulfonic acid (GAMS) on EAA-evoked [^{3}H]DA release. Cells were exposed to KYN or GAMS during the spontaneous incubation and in the presence of the EAA agonist (NMDA, 100 μM; QUIS, 10 μM; KAIN, 100 μM). Spontaneous [^{3}H]DA release has been subtracted from agonist-stimulated [^{3}H]DA release. Results are the means ± SEM from 6–14 cultures for KYN and 3–5 cultures for GAMS, transformed to % of release evoked by NMDA, QUIS, or KAIN in the absence of antagonist. KYN significantly antagonized the release stimulated by NMDA ($F = 16.6$, $df = 3,23$, $P < .001$), QUIS ($F = 10.1$, $df = 6,70$, $p < .001$) and KAIN ($F = 49.2$, $df = 4,57$, $p < .001$). GAMS significantly antagonized the [^{3}H]DA release evoked by KAIN ($F = 7.0$, $df = 4,19$, $P < .005$), but failed to alter the release evoked by NMDA ($F = .16$, $df = 2,9$, $P = .854$), or QUIS ($F = .63$, $df = 3,14$, $P = .61$). *$P < .05$, compared to release in the absence of antagonists. Statistical analyses were performed on raw data before transformation to % control.

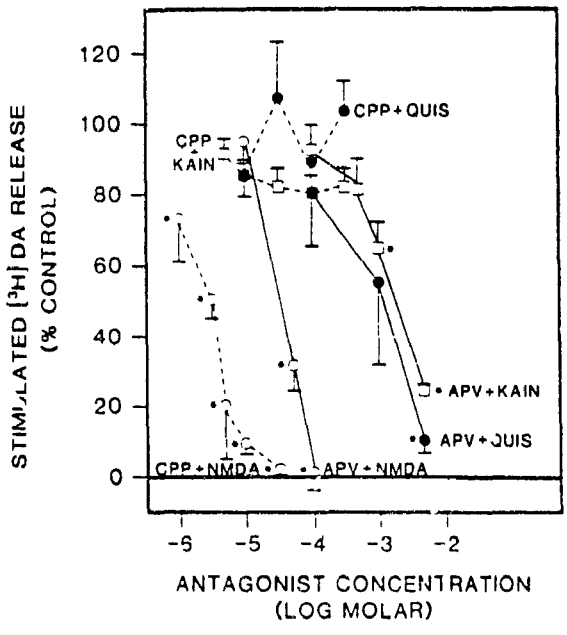


Fig. 4. Effects of the selective competitive NMDA receptor antagonists D,L-2-amino-5-phosphonovaleric acid (APV) and 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) on EAA-evoked [^{3}H]DA release. Cells were exposed to antagonists during the spontaneous incubation and in the presence of the EAA agonists (NMDA, 100 μM; QUIS, 10 μM; KAIN, 100 μM). Spontaneous [^{3}H]DA release has been subtracted from EAA-stimulated [^{3}H]DA release. Results are the means ± SEM from 5–21 cultures for APV and 4–12 cultures for CPP, transformed into % of release evoked by NMDA, QUIS, or KAIN in the absence of antagonist. APV significantly antagonized the release stimulated by NMDA ($F = 47.1$, $df = 3,71$, $P < .001$), QUIS ($F = 3.4$, $df = 3,28$, $P < .05$) and KAIN ($F = 16.2$, $df = 4,40$, $P < .001$). CPP significantly antagonized [^{3}H]DA release evoked by NMDA ($F = 26.6$, $df = 5,48$, $P < .001$) but failed to alter the release evoked by QUIS ($F = .68$, $df = 4,24$, $P = .61$), or KAIN ($F = 2.0$, $df = 5,57$, $P = .086$). *$P < .05$, compared to release in the absence of antagonists. Statistical analyses were performed on raw data before transformation to % control.

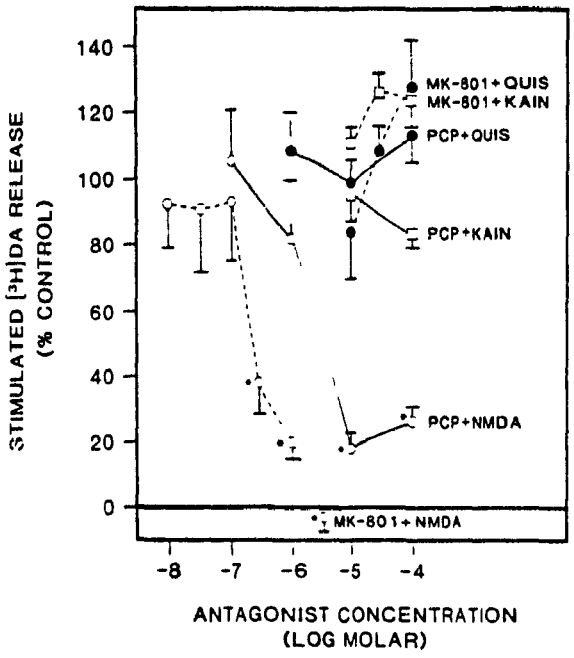
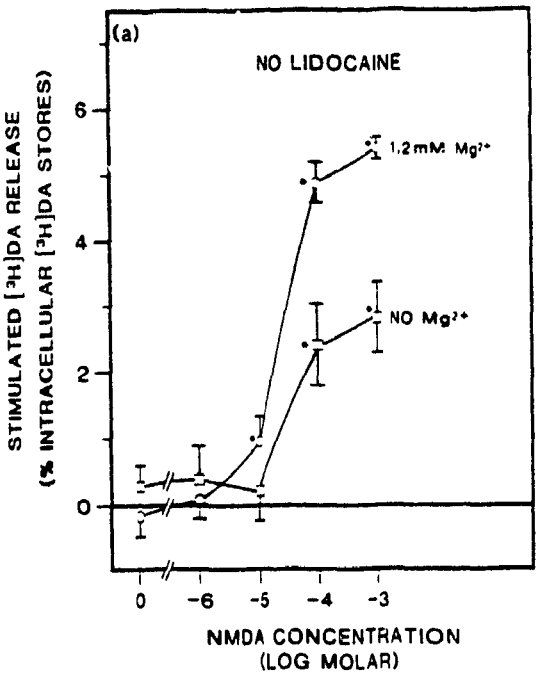


Fig 5. Effects of the noncompetitive NMDA receptor antagonists, phencyclidine (PCP) and MK-801 on EAA-evoked [^{3}H]DA release Cells were exposed to antagonists during the spontaneous incubation and in the presence of the EAA agonists (NMDA, 100 μM, QUIS, 10 μM· KAIN, 100 μM) Spontaneous [^{3}H]DA release has been subtracted from EAA-stimulated [^{3}H]DA release Results are the means ± SEM from 5–19 cultures for PCP and 5–11 cultures for MK-801, transformed into % of release evoked by NMDA QUIS, or KAIN in the absence of antagonist PCP significantly antagonized the release stimulated by NMDA ($F=14.2$, $df=4,24$ $P<.001$) but not release stimulated by QUIS ($F=.7$, $df=3,39$, $P=.51$) or KAIN ($F=2.3$, $df=2,46$ $P=.11$) Similarly, MK-801 significantly antagonized [^{3}H]-DA release evoked by NMDA ($F=13.7$, $df=6,46$, $P<.001$), but failed to alter release evoked by QUIS ($F=2.2$ $df=3,23$, $P=.12$), or KAIN ($F=2.9$, $df=3,20$, $P=.06$) *$P<.05$, compared to release in the absence of antagonists Statistical analyses were performed on raw data before transformation to % control



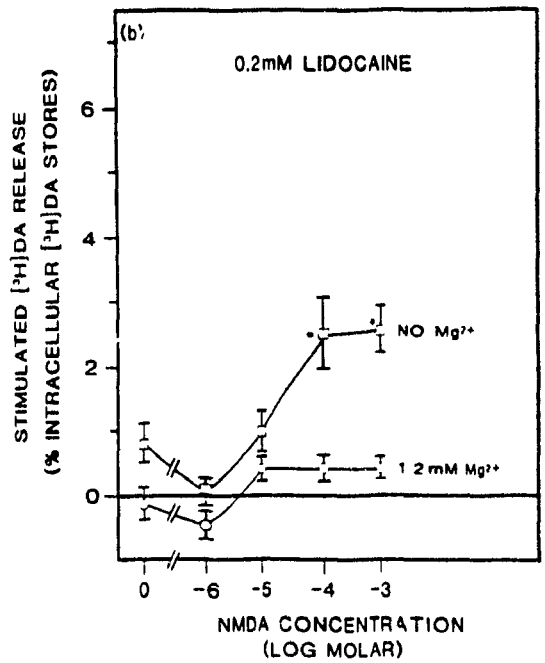


Fig 6 Effects of removing Mg^{2+} from the control KRH buffer on NMDA-stimulated [^{3}H]DA release (a) in the absence of lidocaine and (b) in the presence of lidocaine Spontaneous [^{3}H]DA release has been subtracted from agonist-stimulated [^{3}H]DA release Results are the means ± SEM from 4–6 cultures a: In the absence of lidocaine, NMDA evoked significant [^{3}H]DA release both in the presence of Mg^{2+} ($F=81.4$, $df=4,20$, $P<.001$) and in Mg^{2+}-free KRH ($F=7.0$, $df=4,18$, $P<.005$) However, release evoked by NMDA (100 μM or 1 mM) was significantly greater in the presence of Mg^{2+} than in its absence ($P<.001$) Exclusion of Mg^{2+} from the KRH had no effect on spontaneous [^{3}H]DA release b: In the presence of lidocaine, NMDA stimulated release of [^{3}H]DA in Mg^{2+}-free KRH ($F=9.53$, $df=4,25$, $P<.001$) However, in the presence of lidocaine and Mg^{2+} ($F=4.7$, $df=4,25$, $P<.01$), NMDA produced no significant stimulation of [^{3}H]DA release *$P<.05$, compared to 0 M NMDA.

lated [^{3}H]DA release, if the effect was mediated through activation of the NMDA receptor. Surprisingly, removal of Mg^{2+} did not increase the NMDA-evoked [^{3}H]DA release, but decreased it (Fig 6a). However, when ongoing electrical activity in the cultures was dampened by the local anaesthetic, lidocaine (0.2 mM) (Fig. 6b), NMDA responses were greater in the absence of Mg^{2+} than in its presence.

The role of regenerative Na^+-action potentials in the [^{3}H]DA release stimulated by EAAs

To examine further the role of propagated activity in the [^{3}H]DA release stimulated by NMDA and other EAAs, effects of the voltage-sensitive Na^+ channel blocker, TTX, were investigated As in results obtained in the previous section with lidocaine (see Fig. 6), removal of Mg^{2+} from the standard Mg^{2+} (1.2 mM)-containing KRH buffer decreased NMDA-stimulated [^{3}H]DA release in the absence of TTX (Fig 7a, compare columns 1 and 3). However, when TTX was present to dampen ongoing action potentials, NMDA-evoked release was greater in the absence of Mg^{2+} than in its presence (Fig. 7a, compare columns 2 and 4). In addition, Fig. 7a shows that in the presence of Mg^{2+}, NMDA-stimulated [^{3}H]DA release was completely inhibited by

TTX (Fig. 7a, compare columns 1 and 2), while in the absence of Mg^{2+}, the NMDA response was insensitive to inhibition by TTX (Fig. 7a, compare columns 3 and 4). Figure 7b shows that [^{3}H]DA release stimulated by either KAIN or QUIS was unaffected by TTX (in the presence of 1.2 mM Mg^{2+}).

Role of extracellular Na^+ in EAA-stimulated [^{3}H]DA release

As shown in Figure 8, NMDA-, KAIN-, and QUIS-evoked [^{3}H]DA release were differentially affected by alteration of the extracellular Na^+ concentration. NMDA-stimulated release was completely abolished when NaCl in the release buffer was replaced with an osmotically equivalent concentration of sucrose (Fig. 8a). (Sucrose was chosen to substitute for NaCl since previous experiments have indicated that cations such as choline or Tris, often used as Na^+ substitutes, may

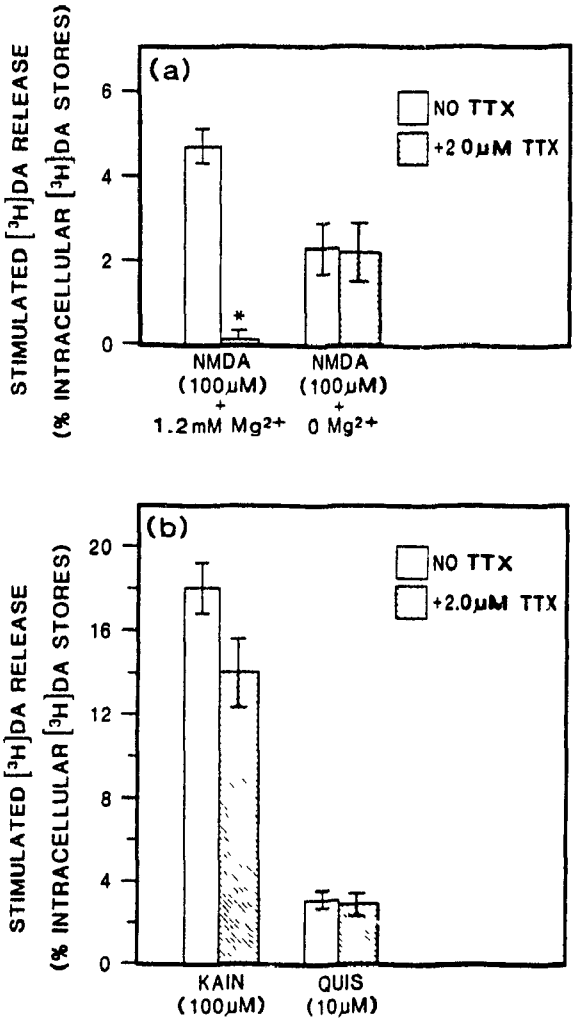


Fig 7 Effects of tetrodotoxin (TTX) on a [^{3}H]DA release stimulated by NMDA in the presence or absence of extracellular Mg^{2+} and on b [^{3}H]DA release stimulated by KAIN or QUIS in the presence of extracellular Mg^{2+}. Cells were loaded with [^{3}H]DA in control KRH buffer (1.2 mM $MgSO_4$). Subsequent rinses, spontaneous release and release in the presence of agonists (NMDA, 100 μM; KAIN, 100 μM; QUIS, 10 μM) were collected either in control KRH or in Mg^{2+}-free KRH, in the presence or absence of TTX (2 μM). Spontaneous [^{3}H]DA release has been subtracted from agonist-stimulated release. Results are the means ± SEM from 6–13 cultures. In a, NMDA-stimulated [^{3}H]DA release was significantly (*P< 001) inhibited by TTX in the presence of Mg^{2+}. TTX had no effect on the NMDA response in the absence of Mg^{2+}. In the absence of TTX, Mg^{2+} (1.2 mM) significantly increased NMDA-evoked [^{3}H]DA release (compare columns 3 and 1, P< 001). In the presence of TTX, Mg^{2+} (1.2 mM) significantly inhibited NMDA-stimulated [^{3}H]DA release (compare columns 4 and 2, P< 05). In b, [^{3}H]DA release stimulated by either KAIN or QUIS was not significantly altered by TTX.

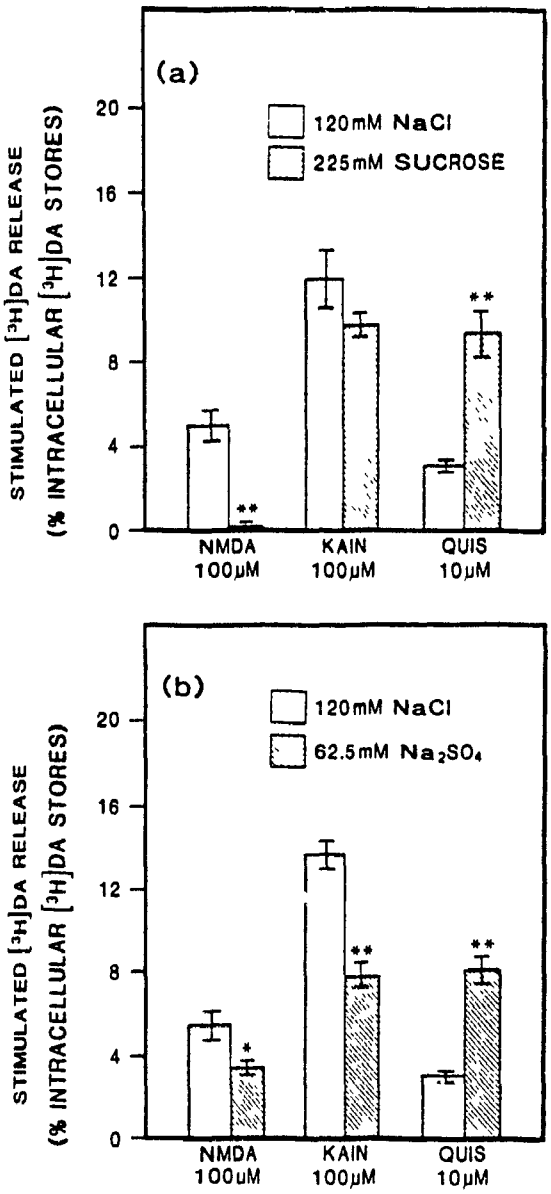


Fig 8 Effects of extracellular NaCl substitutions on [^{3}H]DA release stimulated by NMDA, KAIN and QUIS. Cells were loaded with [^{3}H]DA in control KRH buffer (containing 125 mM NaCl). Subsequent rinses, spontaneous [^{3}H]DA release and release evoked by agonists (NMDA, 100 μM; KAIN, 100 μM, QUIS, 10 μM) were collected either in control KRH or in KRH in which NaCl was replaced by a 225 mM sucrose or b 62.5 mM Na_2SO_4. Spontaneous [^{3}H]DA release has been subtracted from agonist-stimulated [^{3}H]DA release. Results are the means ± SEM from 5–20 cultures. Substitution of NaCl by sucrose or Na_2SO_4 did not significantly alter spontaneous [^{3}H]DA release (data not shown). In a, NMDA-evoked [^{3}H]DA release was significantly inhibited (**P< 001) when sucrose replaced NaCl, while KAIN-stimulated [^{3}H]DA release was unaffected and the response to QUIS was significantly (**P< 001) increased. In b, replacement of NaCl by Na_2SO_4 inhibited NMDA-evoked [^{3}H]DA release (*P< 05) and KAIN-evoked release (**P< 001), but increased QUIS-stimulated [^{3}H]DA release (**P< 001).

inhibit EAA-mediated responses [Mayer and Westbrook, 1987; Mount et al., 1989]). KAIN-induced release was unaffected in the sucrose buffer. However, QUIS-stimulated [^{3}H]DA release was enhanced more than 300% when NaCl was replaced by sucrose. To investigate whether effects of NaCl substitution with sucrose might be attributed to the absence of Cl^-, rather than to omission of Na^-, NaCl was substituted by Na_2SO_4. In the Na_2SO_4-buffer (Fig. 8b), NMDA- and KAIN-stimulated release were partially attenuated, while the response to QUIS was enhanced by more than 260%.

DISCUSSION

The major finding in this study is that EAAs stimulate Ca^{2+}-dependent release of [^{3}H]DA from mesencephalic cells in culture by activation of NMDA, and QUIS/KAIN receptor subtypes.

Responses to NMDA were antagonized by the broad-spectrum EAA antagonists, KYN and PDA. NMDA responses were also potently antagonized by the selective competitive NMDA antagonists, CPP and APV, and by noncompetitive NMDA inhibitors, PCP and MK-801. NMDA-stimulated release of [^{3}H]DA was completely inhibited or greatly reduced by each of these four NMDA antagonists, at concentrations having no effect on KAIN or QUIS responses. This pattern of antagonist selectivity clearly suggests involvement of the NMDA subtype of EAA receptor.

Another well-described characteristic of the NMDA receptor is that activity at the receptor can be inhibited by low millimolar concentrations of Mg^{2+} in the extracellular environment (Ault et al., 1980; Davies and Watkins, 1980; Mayer and Westbrook, 1985; Nowak et al., 1984). This Mg^{2+}-inhibition is voltage-dependent (i.e., Mg^{2+} does not inhibit NMDA responses in partially depolarized neurons) and does not occur at KAIN or QUIS receptors. Exploiting this property of NMDA receptors, it was reasoned that removal of Mg^{2+} from the Mg^{2+} (1.2 mM)-containing KRH buffer should increase NMDA-stimulated [^{3}H]DA release from cultures. However, removal of Mg^{2+} failed to increase NMDA-stimulated [^{3}H]DA release from the mesencephalic cell cultures, but actually decreased the response.

Such apparent insensitivity of an NMDA receptor-mediated effect to blockade by Mg^{2+} is unusual, but not unprecedented in the literature. For example, NMDA-receptor-mediated [^{3}H]GABA release (Weiss, 1988) and phosphatidyl inositol turnover (Schmidt et al., 1987) in primary cell cultures from striatum have been reported to be unaffected by low millimolar concentrations of Mg^{2+}. To explain these results, it has been suggested that a sustained depolarization of the neurons bearing NMDA receptors in these preparations might have relieved the Mg^{2+}-blockade (Weiss, 1988). To test the possibility that depolarization of the mesencephalic cells was somehow overcoming a Mg^{2+}-blockade of the NMDA receptor, ongoing electrical activity in the cultures was dampened with the local anaesthetic, lidocaine. In the presence of lidocaine, NMDA-evoked [^{3}H]DA release was not observed in standard release buffer (containing 1.2 mM Mg^{2+}), but was seen in Mg^{2+}-free buffer. Similar observations were made in the presence of the voltage-sensitive Na^+ channel blocker, TTX. Thus, it appears that depolarization via voltage-regulated Na^--channels can relieve a Mg^{2+}-blockade of the NMDA receptor and that dampening electrical activity with either lidocaine or TTX may be sufficient to restore the blockade. Because of this mechanism, there is an apparent TTX-sensitivity of the NMDA response when this sensitivity is tested in the presence of Mg^{2+} (i.e., TTX dampens ongoing electrical activity allowing Mg^{2+} blockade of the response). However, when the test is made in the absence of Mg^{2+}, it is clear that the NMDA response is, in fact, not TTX-sensitive. This indicates that the effect of NMDA on [^{3}H]DA release, in the absence of Mg^{2+}, does not require the propagation of Na^+ action potentials. In the presence of Mg^{2+}, NMDA was able to stimulate [^{3}H]DA release when extracellular NaCl was replaced by Na_2SO_4, but not when sucrose was substituted for NaCl. These observations indicate an absolute requirement for Na^-, but not Cl^-, and are consistent with the TTX results.

In view of these results, the presence or absence of Mg^{2+} in the release buffer may explain discrepancies between previous studies with regard to the TTX-sensitivity of NMDA receptor stimulated DA release from the rat striatum. NMDA receptor mediated DA release was found to be TTX-sensitive in trans-striatal dialysis experiments (Carter et al., 1988), in the presence of 1.19 mM Mg^{2+}, but TTX-insensitive (Clow and Jhamandas, 1989; Marien et al., 1983), or partially TTX-sensitive (Jhamandas and Marien, 1987; Snell and Johnson, 1986) in striatal slices superfused with Mg^{2+}-free buffer. For other brain areas, NMDA receptor mediated DA release in Mg^{2+}-free buffer was found to be TTX-insensitive (Marien et al., 1983) or partially TTX-sensitive (Jones et al., 1987) in slices of nucleus accumbens, and partially TTX-sensitive in substantia nigra (Marien et al., 1983). It is possible that the partial TTX-sensitivity of NMDA-evoked DA release in studies with brain slices in Mg^{2+}-free buffer was observed because extracellular Mg^{2+} was not completely eliminated from some superfused slice preparations. In this respect, cells in culture may be exposed to an environment that is closer to being Mg^{2+}-free, allowing more accurate analysis of TTX-sensitivity. Alternatively, synaptic connections present in vivo and in the intact slice may be lost in dissociated cell culture, so that direct, but not indirect interactions between EAAs and [^{3}H]DA-releasing neurons are operative in the culture system.

Both KAIN and QUIS stimulated release of [^{3}H]DA that was antagonized by the broad-spectrum competitive EAA antagonists, PDA and KYN, but was insensitive to the selective competitive NMDA antagonists, APV and CPP, and to the noncompetive NMDA antagonists, PCP and MK-801. It has previously been reported that PCP and Mg^{2+} may partially block KAIN- and QUIS-evoked [^{3}H]DA release from striatal slices (Snell and Johnson, 1986), but not from slices of nucleus accumbens (Jones et al., 1987). These results were interpreted as evidence that responses to KAIN and QUIS were mediated by both non-NMDA and NMDA receptors in striatum, but only by non-NMDA receptors in nucleus accumbens. In the mesencephalic cell cultures, it is apparent that only non-NMDA receptor activation underlies the effects of these agonists, at least at the concentrations used in this study.

Discrimination between receptor mechanisms underlying the actions of QUIS and KAIN has been hampered by the lack of highly selective QUIS or KAIN receptor

antagonists. However, in cat spinal cord (Davies and Watkins, 1985) and cultured rat cortical neurons (Drejer et al., 1987), the non-NMDA antagonist GAMS has been shown to inhibit KAIN responses without blocking those at the QUIS receptor. Similarly, in certain systems, KYN and PDA have been shown to be more selective inhibitors of responses stimulated by KAIN than those evoked by QUIS (Coleman et al., 1986; Gallo et al., 1987a,b, 1989). In the mesencephalic cell cultures, GAMS inhibited KAIN-stimulated [^{3}H]DA release at concentrations that did not alter QUIS- or NMDA-evoked [^{3}H]DA release. KYN had a preferential antagonistic effect on KAIN-stimulated [^{3}H]DA release although higher concentrations also blocked responses to QUIS. Differences in potency between KAIN and QUIS, together with the differential effects of GAMS and KYN on responses to KAIN and QUIS, are consistent with the suggestion that separate KAIN and QUIS receptors mediate [^{3}H]DA release from the cultures. However, it should be noted that for practical reasons (i.e., the small size of the QUIS response) it was necessary to use a maximally saturating concentration of QUIS in the antagonist studies, while a half-maximal concentration of KAIN could be used. Thus, the interpretation of the antagonist data may be confounded by these differences in the relative position of the test concentrations of KAIN and QUIS on their respective agonist concentration-response curves.

Whether or not distinct KAIN and QUIS receptors mediate the responses to these EAAs, further differences in the effects of KAIN and QUIS on the [^{3}H]DA release process were observed when responses to these agonists were analyzed after replacing extracellular NaCl with sucrose or Na_2SO_4. The response to KAIN was unchanged in the absence of both Na^+ and Cl^-, and was only partially attenuated when Cl^- was replaced by SO_4^{2-}. However, QUIS-evoked release was greatly enhanced by replacement of extracellular NaCl, or replacement of Cl^-, alone. The reason for the enhanced QUIS response was not investigated. QUIS is a substrate for the Cl^--dependent EAA uptake mechanism (Sawada et al., 1986; Zaczek et al., 1987). Thus, it is possible that QUIS uptake was reduced in the absence of Cl^-, allowing higher agonist concentrations at membrane receptors to enhance release. However, if this mechanism were operative, one might expect high concentrations of QUIS (100 μM or 1 mM) to produce greater release of [^{3}H]DA than would 10 μM QUIS. In fact, the converse was observed.

As expected of responses that do not require Na^+, KAIN-, or QUIS-stimulated [^{3}H]DA release was TTX-insensitive and thus did not require propagated Na^+ action potentials. This finding differs from previous reports in which KAIN- and QUIS-, but not NMDA-evoked DA release from striatal slices was partially inhibited by TTX (Clow and Jhamandas, 1989) and trans-striatal dialysis experiments, in which the release elicited by KAIN was partially inhibited by TTX (Carter et al., 1988). The observed TTX-insensitivity of [^{3}H]DA release from mesencephalic cell cultures, stimulated by NMDA (in the absence of Mg^{2+}), KAIN and QUIS suggests that EAA-stimulated release of [^{3}H]DA, in this preparation, may be due to a direct interaction of EAAs with receptors on dopaminergic neurons. However, it remains possible that EAAs act indirectly to release [^{3}H]DA through liberation of an intermediate transmitter into the extracellular environment, via a mechanism that does not require Na^+ action potentials. Thus, interneuronal involvement in EAA-evoked [^{3}H]DA release, although unlikely, cannot be completely ruled out. In addition to neurons, mesencephalic cell cultures contain a high percentage of glial cells, that are also capable of taking up [^{3}H]DA (Pelton et al., 1981; Barochovsky and Bradford, 1987). Stimulation of DA release from glial cells by EAAs appears unlikely in the present study, since it has been demonstrated that glial cell cultures do not release accumulated [^{3}H]DA in response to depolarizing stimuli (Barochovsky and Bradford, 1987). However, since EAA receptors have been identified on glial cells (Gallo et al., 1987b, 1989), stimulation of EAA receptors on these cells could conceivably release a secondary transmitter capable of stimulating DA release from the DA neurons.

In conclusion, results of this study indicate that separate NMDA and QUIS/KAIN receptor subtypes may mediate EAA-evoked release of [^{3}H]DA from dissociated mesencephalic cell cultures, via mechanisms that do not rely on regenerative action potentials. Differences in antagonist selectivity patterns and ionic requirements have permitted an initial discrimination between responses at these receptors. We are now examining characteristics of responses to naturally occurring EAA agonists, with a view to elucidating the roles of these EAA receptor subtypes in the regulation of dopaminergic transmission by naturally occurring EAAs.

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Stimulation of Dopamine Release from Cultured Rat Mesencephalic Cells by Naturally Occurring Excitatory Amino Acids: Involvement of Both *N*-Methyl-D-Aspartate (NMDA) and Non-NMDA Receptor Subtypes

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Abstract: In rat mesencephalic cell cultures, L-glutamate at concentrations ranging from 100 μM to 1 mM stimulated release of [^{3}H]dopamine that was attenuated by the non-*N*-methyl-D-aspartate (non-NMDA) receptor antagonist 6,7-dinitroquinoxalinedione, but not by the selective NMDA receptor antagonists (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK-801; 10 μM) and 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonate (300 μM). Even at 1 mM glutamate, this release was Ca^{2+} dependent. These observations suggest that the release was mediated by a non-NMDA receptor. Only release stimulated by a lower concentration (10 μM) of glutamate was inhibited by MK-801 (10 μM), indicating that glutamate at this concentration activates the NMDA receptor. By contrast, L-aspartate at concentrations of 10 μM to 1 mM evoked [^{3}H]dopamine release that was completely inhibited by MK-801 (10 μM) and was also Ca^{2+} dependent (tested at 1 and 10 mM aspartate). Thus, effects of aspartate involved activation of the NMDA receptor. Sulfur-containing amino acids (L-homocysteate, L-homocysteine sulfinate, L-cysteate, L-cysteine sulfinate) also evoked [^{3}H]dopamine release. Release evoked by submillimolar concentrations of these amino acids was attenuated by MK-801 (10 μM), indicating involvement of the NMDA receptor. Higher concentrations of the sulfur-containing amino acids (≥1 mM L-homocysteate, ≥1 mM L-homocysteine sulfinate, ≥10 mM L-cysteate, ≥10 mM L-cysteine sulfinate) evoked [^{3}H]dopamine release that was Ca^{2+} dependent (largely Ca^{2+} dependent for 10 mM L-cysteine sulfinate) and inhibited by 6,7-dinitroquinoxalinedione (100 μM), but unaffected by MK-801 (10 or 100 μM). Thus, like glutamate, higher concentrations of the sulfur-containing amino acids interact with non-NMDA receptors, while non-NMDA receptor involvement was not observed with aspartate. **Key Words:** Aspartate—Dopamine release—Excitatory amino acid receptors—Excitatory sulfur amino acids—Glutamate—Tissue culture. **Mount H. et al.** Stimulation of dopamine release from cultured rat mesencephalic cells by naturally occurring excitatory amino acids: Involvement of both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor subtypes. *J Neurochem* **55**, 268–275 (1990)

It is now well established that L-glutamate (Glu) and L-aspartate (Asp) are major excitatory amino acid (EAA) transmitters in corticocortical neurons and in many corticofugal pathways (reviewed by Fagg and Foster, 1983; Fonnum, 1984; Cotman et al., 1987) in the mammalian CNS, including projections to brain regions containing dopaminergic cell bodies (Carter, 1980, 1982; Fonnum et al., 1981; Nieoullon and Dusticier, 1983; Kornhuber et al., 1984; Christie et al., 1985) and nerve terminals (Reubi and Cuénod, 1979; Walaas, 1981; Girault et al., 1986). Stimulatory effects of Glu and/or Asp on release of dopamine (DA) have been observed in tissue slices (Roberts and Sharif, 1978; Roberts and Anderson, 1979; Marien et al., 1983; Snell and Johnson, 1986; Jhamandas and Marien, 1987; Clow and Jhamandas, 1989) and in vivo (Chéramy et al., 1986). There is also emerging evidence that naturally occurring amino acids other than Glu and Asp

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Abbreviations used Asp, L-aspartate; CA, L-cysteate; CPP, 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonate; CSA, L-cysteine sulfinate; DA, dopamine; DNQX, 6,7-dinitroquinoxalinedione; EAA, excitatory amino acid; GABA, γ-aminobutyric acid; Glu, L-glutamate; HCA, L-homocysteate; HCSA, L-homocysteine sulfinate; KRH, Krebs-Ringer-HEPES buffer; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate

may play a role in excitatory transmission in the CNS. The sulfur-containing amino acids L-cysteine sulfinate (CSA), L-cysteate (CA), L-homocysteine sulfinate (HCSA), and L-homocysteate (HCA) have been shown to be present in and released from rat brain slices upon depolarization (Do et al., 1986*a*,*b*, 1988, Keller et al., 1989). These sulfur-containing amino acids are electrophysiologically active (Mewett et al., 1983) and have been shown to evoke release of [^{3}H]Asp from primary cultures of cortical neurons (Dunlop et al., 1989) and of γ-[^{3}H]aminobutyrate ([^{3}H]GABA) from striatal (Weiss, 1988) and cerebellar granule (Dunlop et al., 1989) neurons. It has been reported that a single concentration of HCA or CA has a stimulatory effect on DA release from striatal slices (Roberts and Sharif, 1978; Roberts and Anderson, 1979). However, effects of other sulfur-containing amino acids on DA release have not been reported and the subtypes of EAA receptors involved in the effects of naturally occurring amino acids on DA release have not been characterized in detail.

We have previously reported that Glu evokes a Ca^{2+}-dependent release of [^{3}H]DA from dissociated cell cultures of the fetal rat mesencephalon (Mount et al., 1989), an area of brain containing cell bodies of origin that give rise to nigrostriatal, mesocortical, and mesolimbic dopaminergic pathways (Lindvall and Bjorklund, 1978). EAAs may act at one or more of (at least) three EAA receptor subtypes. These subtypes are named after the preferred agonists, *N*-methyl-D-aspartate (NMDA), quisqualate, and kainate, or are more broadly classified as NMDA or non-NMDA (quisqualate/kainate) receptor subtypes (see reviews by Watkins and Evans, 1981; McLennan, 1982; Mayer and Westbrook, 1987). Each of these preferred agonists was found to stimulate [^{3}H]DA release from mesencephalic cultures (Boksa et al., 1989; Mount et al., 1990). The pattern of antagonist selectivity and ionic sensitivity of release stimulated by NMDA, quisqualate, and kainate suggested that the responses were mediated by at least two distinct receptors. Thus, the system provides a good model for assessing receptor subtype involvement in the effects of EAAs on DA release.

To more fully elucidate how EAAs might interact with dopaminergic neurons, the present experiments were designed (a) to test the effects of a series of naturally occurring EAAs, including the sulfur-containing amino acids, on [^{3}H]DA release from mesencephalic cultures; (b) to assess the Ca^{2+} dependence of the EAA-evoked release; and (c) to characterize the EAA receptor subtype(s) (NMDA versus non-NMDA receptors) involved in these responses.

MATERIALS AND METHODS

Materials

3,4-[7,8-^{3}H(N)]Dihydroxyphenylethylamine ([^{3}H]DA; 18.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). HCSA and 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonate (CPP) came from Tocris Neuramin (Buckhurst Hill, U.K.). NMDA, quisqualate, kainate, Asp, CA, CSA, HCA, desipramine hydrochloride, and pargyline hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) was a gift from Dr. L. Iversen (Merck, Sharp & Dohme, Harlow, U.K.). Other chemicals were reagent grade from regular commercial sources.

Methods

Dissociated cell cultures were prepared from anterior ventral mesencephalic tissue of Sprague–Dawley rat fetuses (gestation day 15), as previously described by Mount et al. (1989). Cultures were grown in Dulbecco's Modified Eagle Medium supplemented with 10% (vol/vol) fetal calf serum and 20 m*M* KCl.

After 6–7 days in culture, cells were rinsed with Krebs-Ringer-HEPES buffer [KRH; pH 7.4, composed of the following (m*M*): NaCl, 125; KCl, 4.8; HEPES, 25; NaOH, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; D-glucose, 5.6; $CaCl_2$, 2.2; pargyline, 0.1; ascorbate, 1.0] and loaded with [^{3}H]DA (50 n*M*, 20-min incubation) in the presence of desipramine (50 μ*M*). Uptake of [^{3}H]DA into mesencephalic cultures has previously been shown to be predominantly into dopaminergic neurons, as the uptake was blocked by benztropine, a DA uptake inhibitor, was unaffected by fluoxetine, a serotonin uptake inhibitor, and was only slightly attenuated by desipramine, a noradrenaline uptake inhibitor (Mount et al., 1989). After [^{3}H]DA loading, each well was rinsed four times with KRH. KRH from a 5-min incubation (spontaneous release period), followed immediately by a 5-min incubation in the presence of an EAA agonist (stimulated release period), was collected, and [^{3}H]DA release was quantitated by measuring radioactivity in each aliquot of buffer collected. In earlier work, >95% of the radioactivity detected in buffer collected after spontaneous and Glu-stimulated release periods was determined by HPLC to be [^{3}H]DA (Mount et al., 1989). Also, >90% of the total cell content of tritiated species, assayed immediately before release collection periods, was found to be [^{3}H]DA. Following release incubations, radioactivity remaining in the cells was extracted by 30-min incubation with acidified ethanol (95% ethanol/5% 0.1 *M* HCl).

In Ca^{2+} dependence experiments, Ca^{2+}-free KRH was used for all rinses and incubations after [^{3}H]DA loading. When antagonists were tested, cells were exposed to these during the spontaneous release incubation as well as during exposure to the agonist.

For data presentation, spontaneous [^{3}H]DA release (typically 4–6% of total intracellular [^{3}H]DA stores) has been subtracted from release stimulated by the EAA agonist. The net release evoked by the agonist is expressed as a percentage of the total [^{3}H]DA uptake into cells. Statistical analyses involved unpaired *t* tests or one-way analysis of variance and the Newman-Keuls test for multiple comparisons, as appropriate. The accepted level of statistical significance was $\alpha = 0.05$.

RESULTS

MK-801 is a compound reported to act as an uncompetitive antagonist selective for EAA receptors of the NMDA subtype and to have little activity at non-NMDA receptor subtypes (Wong et al., 1986; Huettner and Bean, 1988). To test whether MK-801 could be

used to distinguish between NMDA and non-NMDA receptor subtypes mediating release of [^{3}H]DA from mesencephalic cell cultures, its effects on [^{3}H]DA release evoked by a range of concentrations of NMDA, quisqualate, or kainate were examined. From Table 1 it can be seen that 10 μM MK-801 completely antagonized the release stimulated by NMDA concentrations as high as 1 mM. However, 10 μM MK-801 failed to antagonize [^{3}H]DA release evoked by a wide range of concentrations of quisqualate (1 μM to 1 mM) or kainate (30 μM to 1 mM). Thus, 10 μM MK-801 can be used to selectively inhibit the component of [^{3}H]DA release induced by NMDA receptor activation in mesencephalic cell cultures.

As we have previously reported (Mount et al., 1989), Glu (10 μM to 1 mM) stimulated [^{3}H]DA release from the cell cultures (Fig. 1a). MK-801 (10 μM) abolished release evoked by 10 μM Glu, but had no significant effect on the release stimulated by higher Glu concentrations. At a concentration of MK-801 400 times the IC_{50} for MK-801 antagonism of [^{3}H]DA release evoked by 100 μM NMDA (Boksa et al., 1989; Mount et al., 1990), MK-801 (100 μM) only marginally attenuated release stimulated by 1 mM Glu (Table 2). Similarly, Table 3 shows that [^{3}H]DA release elicited by 10 μM Glu was completely abolished, while that evoked by 100 μM Glu was unaffected by 300 μM CPP (Davies et al., 1986; Lehman et al., 1987), a concentration of this selectively competitive NMDA receptor antagonist previously found to completely inhibit [^{3}H]DA release stimulated by 100 μM NMDA (IC_{50} = 3 μM) without affecting release evoked by 10 μM quisqualate or 100 μM kainate (Boksa et al., 1989; Mount et al., 1990).

TABLE 1. *Sensitivity of NMDA-, quisqualate-, and kainate-evoked [^{3}H]DA release to 10 μM MK-801*

EAA agonist	Concentration	Stimulated [^{3}H]DA release (% intracellular stores) 0 MK-801	+MK-801 (10 μM)
NMDA	100 μM	6.8 ± 0.9	0.0 ± 0.2[a]
NMDA	300 μM	7.2 ± 0.7	0.1 ± 0.3[a]
NMDA	1 mM	5.6 ± 0.8	−0.3 ± 0.1[a]
Quisqualate	1 μM	1.7 ± 0.3	1.8 ± 0.2[b]
Quisqualate	10 μM	2.6 ± 0.3	2.4 ± 0.3[b]
Quisqualate	30 μM	2.4 ± 0.4	2.2 ± 0.2[b]
Quisqualate	100 μM	1.9 ± 0.3	1.9 ± 0.2[b]
Kainate	30 μM	3.5 ± 0.3	3.8 ± 0.3[b]
Kainate	100 μM	8.8 ± 0.4	8.5 ± 0.2[b]
Kainate	1 mM	19.1 ± 1.6	17.0 ± 1.2[b]

Cells were exposed to the uncompetitive NMDA receptor antagonist MK-801 (10 μM) during the spontaneous incubation and in the presence of the EAA agonists NMDA, quisqualate, and kainate in the indicated concentrations. Spontaneous [^{3}H]DA release has been subtracted from EAA-stimulated [^{3}H]DA release. Results are the means ± SEM from four to six cultures

[a] $p < 0.001$ versus EAA-evoked release in 0 MK-801

[b] Not significantly different from EAA-evoked release in 0 MK-801

Asp also stimulated [^{3}H]DA release from mesencephalic cell cultures (Fig. 1b). Release was evoked by Asp concentrations as low as 10 μM and the release stimulated by 100 μM Asp was greater than that by 1 mM or 10 mM Asp. MK-801 at 10 μM abolished [^{3}H]DA release evoked by Asp at all concentrations tested.

[^{3}H]DA release was evoked by each of the four sulfur-containing amino acids, HCA (Fig. 2a), HCSA (Fig. 2b), CA (Fig. 3a), and CSA (Fig. 3b). The threshold concentration for stimulation of [^{3}H]DA release by HCA or HCSA was 10 μM, although release was marginal at this agonist concentration. For CA and CSA, 100 μM was required to evoke [^{3}H]DA release. The patterns of NMDA versus non-NMDA receptor activation by these amino acids were demonstrated, by use of MK-801 (10 μM), to be intermediate between those exhibited by Glu and Asp. [^{3}H]DA release evoked by both HCA and HCSA was completely inhibited by MK-801 at agonist concentrations up to 100 μM (Fig. 2). However, [^{3}H]DA release stimulated by 1 mM HCA or HCSA was only partially attenuated by 10 μM MK-801 and the response to 3 mM HCSA was unaffected by MK-801 (Fig. 2). Even 100 μM MK-801 failed to abolish [^{3}H]DA release evoked by 1 mM HCA or HCSA (Table 2). [^{3}H]DA release stimulated by concentrations of up to 1 mM CA or CSA was completely inhibited by 10 μM MK-801 (Fig. 3). Ten millimolar CA-stimulated [^{3}H]DA release (Fig. 3a) was partially (~40%) attenuated by 10 μM MK-801; although 100 μM MK-801 (Table 2) showed a similar tendency to attenuate release evoked by 10 mM CA, the inhibition was not statistically significant. Ten millimolar CSA was unaffected by 10 (Fig. 3b) or 100 (Table 2) μM MK-801.

To assess whether the MK-801-insensitive component of responses to Glu, HCA, HCSA, CA, and CSA reflected receptor-mediated release, we investigated whether MK-801-insensitive release evoked by high concentrations of these agonists was Ca^{2+} dependent or sensitive to inhibition by the potent non-NMDA receptor antagonist 6,7-dinitroquinoxalinedione (DNQX) (Honoré et al., 1988). As shown in Table 2, release stimulated by high concentrations (1 or 10 mM) of each EAA agonist tested was largely Ca^{2+} dependent. However, removal of Ca^{2+} did not completely abolish [^{3}H]DA release evoked by 10 mM CSA, but reduced the response to 33% of release in the presence of Ca^{2+}. Asp-stimulated [^{3}H]DA release was also clearly Ca^{2+} dependent. DNQX (100 μM) abolished the MK-801-insensitive [^{3}H]DA release stimulated by HCA, HCSA, CA, and CSA. The response to 1 mM Glu was strongly attenuated by 100 μM DNQX.

DISCUSSION

The present study demonstrates that the naturally occurring amino acids Glu, Asp, HCA, HCSA, CA,

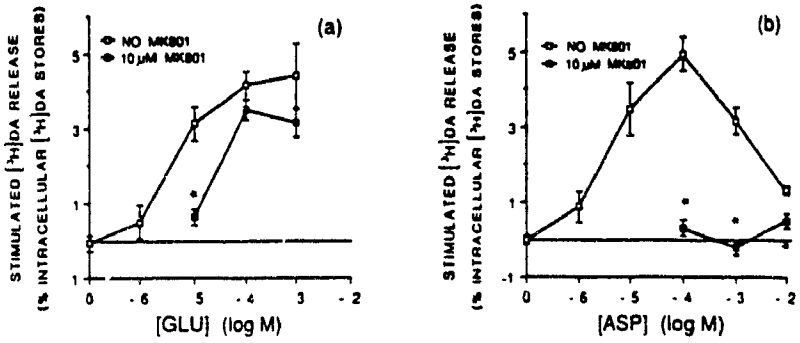

FIG. 1. Effects of the uncompetitive NMDA receptor antagonist MK-801 (10 μM) on [^{3}H]DA release evoked by Glu (a) and Asp (b). Cells were exposed to MK-801 during the spontaneous incubation and in the presence of Glu or Asp. Spontaneous [^{3}H]DA release has been subtracted from EAA-stimulated [^{3}H]DA release. Results are the means ± SEM from at least seven cultures. a: Glu evoked [^{3}H]DA release ($F = 32.6$, $df = 4,119$, $p < 0.001$), in the absence of MK-801, that was significant at Glu concentrations of ≥10 μM ($p < 0.05$). In the presence of MK-801, Glu also stimulated [^{3}H]DA release ($F = 48.5$, $df = 3,77$, $p < 0.001$). However, the release evoked by 10 μM Glu was abolished by MK-801. b: Asp evoked [^{3}H]DA release ($F = 18.0$, $df = 5,74$, $p < 0.001$), in the absence of MK-801, that was significant for 10 μM to 1 mM Asp, was maximal at 100 μM Asp, and was reduced to an insignificant response at 10 mM Asp ($p > 0.05$). In the presence of MK-801, Asp (100 μM to 10 mM) did not stimulate significant release of [^{3}H]DA ($F = 2.7$, $df = 3,54$, $p = 0.06$). *$p < 0.05$ compared with release in the absence of MK-801.

and CSA can evoke [^{3}H]DA release from mesencephalic cell cultures. The concentrations required to cause [^{3}H]DA release compare favorably with those found active in other studies on EAA-evoked transmitter release from dissociated cell cultures of other brain areas (e.g., Drejer et al., 1987; Weiss, 1988; Dunlop et al., 1989; Harris and Miller, 1989) and are equivalent to or less than those required to elicit DA release from tissue slices (e.g., 1 mM) (Jhamandas and Marien, 1987) or during in vivo dialysis (e.g., 100 μM to 10 mM) (Carter et al., 1988).

Previous evidence from our laboratory has indicated that distinct NMDA and non-NMDA receptors mediate EAA-evoked release of [^{3}H]DA from mesencephalic cultures. This was supported by observations that NMDA, quisqualate, and kainate induced Ca^{2+}-dependent release of [^{3}H]DA that was inhibited by the broad-spectrum antagonist kynurenic acid (Boksa et al., 1989; Mount et al., 1990). [^{3}H]DA release evoked by NMDA, but not by quisqualate or kainate, was also inhibited by competitive (CPP and 2-amino-5-phosphonovaleric acid) and uncompetitive (phencyclidine and MK-801) NMDA receptor antagonists (Mount et al., 1990).

A well-described characteristic of the NMDA receptor is that activity at the receptor can be inhibited by low millimolar concentrations of Mg^{2+} in a voltage-dependent manner (Ault et al., 1980; Mayer and Westbrook, 1985). In mesencephalic cell cultures under conditions used in the present study, [^{3}H]DA release is not inhibited by 1.2 mM Mg^{2+} (Mount et al., 1990). However, when ongoing electrical activity in the cultures is damped by the local anesthetic lidocaine or by the voltage-sensitive Na^+ channel blocker tetrodotoxin, the NMDA response does become Mg^{2+} sensitive (Mount et al., 1990). This suggests that the NMDA receptor modulating [^{3}H]DA release is a typical Mg^{2+}-sensitive receptor, but that tonic depolarization of cells

TABLE 2. *Calcium dependence of EAA-evoked [^{3}H]DA release and effects of 100 μM DNQX and 100 μM MK-801*

		Stimulated [^{3}H]DA release (% intracellular [^{3}H]DA stores)			
EAA agonist	Concentration (mM)	+Ca^{2+}	0 Ca^{2+}	+DNQX	+MK-801
Glu	1	5.8 ± 0.4	0.8 ± 0.4[a,b]	1.2 ± 0.5[b]	4.6 ± 0.3[c]
HCA	1	5.8 ± 0.3	0.8 ± 0.4[a,b]	0.2 ± 0.3[a,b]	5.3 ± 0.2[d]
HCSA	1	4.5 ± 0.2	0.5 ± 0.4[a,b]	−0.1 ± 0.3[a,b]	4.3 ± 0.7[d]
CA	10	2.7 ± 0.2	0.5 ± 0.2[a,b]	0.4 ± 0.4[a,b]	2.0 ± 0.4[d]
CSA	1	2.6 ± 0.5	0.3 ± 0.1[a,b]	—	—
CSA	10	3.3 ± 0.3	1.0 ± 0.2[b]	0.1 ± 0.2[a,b]	2.7 ± 0.3[d]
Asp	1	4.2 ± 0.5	0.2 ± 0.2[a,b]	—	—
Asp	10	1.4 ± 0.4	0.1 ± 0.2[a,b]	—	—

Cell cultures were loaded with [^{3}H]DA in standard KRH (2.2 mM Ca^{2+}). Subsequent rinses, spontaneous release, and release in the presence of EAAs were with standard KRH (+Ca^{2+}), Ca^{2+}-free KRH (0 Ca^{2+}), or standard KRH in the presence of DNQX (100 μM) or MK-801 (100 μM). Spontaneous [^{3}H]DA release has been subtracted from EAA-evoked release. The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells, as described in Materials and Methods. Each value is the mean ± SEM from at least six cultures.

[a] Not significantly different from spontaneous [^{3}H]DA release.

[b] $p < 0.001$ versus EAA-evoked release in +Ca^{2+}.

[c] $p < 0.05$ versus EAA-evoked release in +Ca^{2+}.

[d] Not significantly different from EAA-evoked release in +Ca^{2+}.

TABLE 3. *Effects of CPP on [^{3}H]DA release evoked by 10 and 100 μM Glu*

Glu concentration (μM)	Stimulated [^{3}H]DA release (% intracellular stores)	
	0 CPP	+CPP (300 μM)
10	4.4 ± 0.5	0.2 ± 0.3[a,b]
100	6.1 ± 0.2	5.4 ± 0.4[c]

Cells were exposed to the competitive NMDA receptor antagonist CPP (300 μM) during the spontaneous incubation and in the presence of 10 or 100 μM Glu. Spontaneous [^{3}H]DA release has been subtracted from Glu-stimulated release. Each value is the mean ± SEM from at least six cultures.

[a] Not significantly different from spontaneous [^{3}H]DA release.
[b] $p < 0.001$ versus EAA-evoked release in 0 CPP.
[c] Not significantly different from EAA-evoked release in 0 CPP.

in the cultures overcomes a Mg^{2+} blockade of the NMDA response.

Our earlier observations that [^{3}H]DA release from mesencephalic cultures stimulated by either NMDA (in the absence of Mg^{2+}), kainate, or quisqualate is tetrodotoxin insensitive (Mount et al., 1990) are consistent with localization of NMDA and non-NMDA receptors directly on the [^{3}H]DA-releasing cells. However, it remains unclear whether the EAA receptors are located on dendritic processes or axonal terminal fields, since the entire neuron is present in the cultures. It should also be kept in mind that expression of EAA receptors in vitro may differ from their expression in vivo. Development of functional EAA responses (release of [^{3}H]GABA) in vitro, which parallels EAA receptor development in the intact brain, has previously been reported for mouse cortex (Drejer et al., 1987). Studies are now underway in our laboratory comparing the ontogeny of EAA receptors in mesencephalic and striatal regions of the intact rat brain to the development, over days in vitro, of EAA-evoked [^{3}H]DA release from mesencephalic cell cultures.

In our previous studies, MK-801 was found to inhibit [^{3}H]DA release evoked by 100 μM NMDA with an IC_{50} of 0.25 μM and was the most potent and selective NMDA antagonist tested (Mount et al., 1990). In the present study, [^{3}H]DA release elicited by NMDA concentrations as high as 1 mM was completely inhibited by 10 μM MK-801. However, 10 μM MK-801 had no effect on release stimulated by quisqualate or kainate over a wide range of concentrations (up to 1 mM). Thus, it seems that MK-801 sensitivity provides a useful tool to elucidate the involvement of NMDA receptors in responses to naturally occurring EAAs.

Glu and Asp have been found to stimulate release of DA from tissue slices of striatum (Roberts and Shanf, 1978, Roberts and Anderson, 1979; Marien et al., 1983; Snell and Johnson, 1986, Jhamandas and Marien, 1987; Clow and Jhamandas, 1989), nucleus accumbens (Roberts and Anderson, 1979; Marien et al., 1983), olfactory tubercule (Marien et al., 1983), and substantia nigra (Roberts and Anderson, 1979, Marien et al., 1983). To the extent that these studies have characterized the involvement of EAA receptor subtypes, it has been observed that Glu-evoked DA release from striatum, nucleus accumbens, olfactory tubercle, and substantia nigra is mediated predominantly by NMDA receptors. In the earlier studies (Roberts and Anderson, 1979; Marien et al., 1983), these conclusions were based on the antagonism of Glu-evoked [^{3}H]DA release by Mg^{2+}. The more recent studies relied additionally on sensitivity to the competitive NMDA antagonist 2-amino-7-phosphonoheptanoic acid (Jhamandas and Marien, 1987; Clow and Jhamandas, 1989) or to 2-amino-5-phosphonovaleric acid and phencyclidine (Snell and Johnson, 1986) to establish the role of NMDA receptors in Glu-evoked DA release from striatum. In these studies, sensitivity of Glu-stimulated DA release to NMDA receptor antagonists was tested at single Glu concentrations. This may account for the common conclusion that non-NMDA receptors contribute less to Glu-evoked DA release than do NMDA receptors.

In the mesencephalic cell culture model, much of the effect of Glu on [^{3}H]DA release is clearly not de-

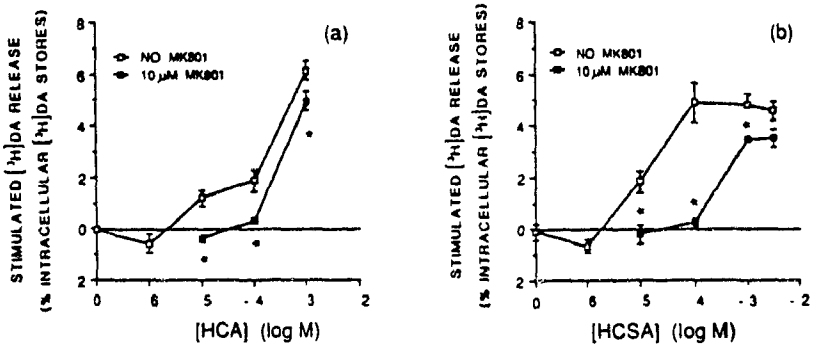

FIG. 2. Effects of the uncompetitive NMDA receptor antagonist MK-801 (10 μM) on [^{3}H]DA release evoked by HCA (a) and HCSA (b). Cells were exposed to MK-801 during the spontaneous incubation and in the presence of HCA or HCSA. Spontaneous [^{3}H]DA release has been subtracted from EAA-stimulated [^{3}H]DA release. Results are the means ± SEM from at least six cultures (SEMs not shown are within limits of the symbol for that value). a. HCA evoked [^{3}H]DA release ($F = 60.7$, $df = 4,81$, $p < 0.001$), in the absence of MK-801, that was significant at HCA concentrations of ≥10 μM ($p < 0.05$). In the presence of MK-801, HCA also stimulated [^{3}H]DA release ($F = 130.6$, $df = 3,54$, $p < 0.001$), but only at the highest concentration of HCA tested (1 mM). b. HCSA evoked [^{3}H]DA release ($F = 27.9$, $df = 5,32$, $p < 0.001$), in the absence of MK-801 that was significant for 10 μM to 3 mM HCSA ($p < 0.05$). In the presence of MK-801, the effect of HCSA ($F = 48.2$, $df = 4,26$, $p < 0.001$) was significant only for 1 to 3 mM ($p < 0.05$). *$p < 0.05$ compared with release in the absence of MK-801.

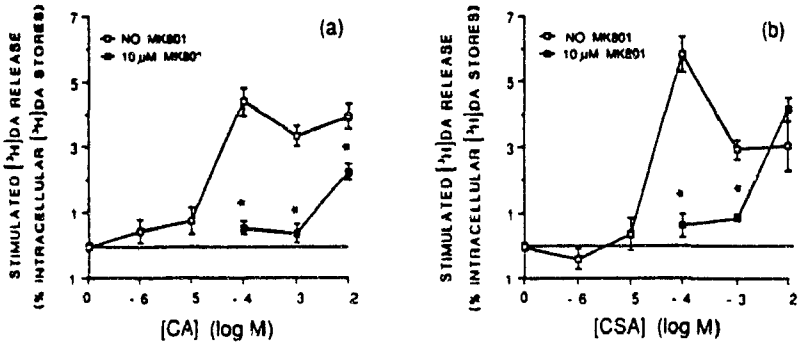

FIG. 3. Effects of the uncompetitive NMDA receptor antagonist MK-801 (10 μM) on [^{3}H]DA release evoked by CA (a) and CSA (b) Cells were exposed to MK-801 during the spontaneous incubation and in the presence of CA or CSA Spontaneous [^{3}H]DA release has been subtracted from EAA-stimulated [^{3}H]DA release Results are the means ± SEM from at least four cultures (SEMs not shown are within limits of the symbol for that value) a. CA evoked [^{3}H]DA release (F = 18 8, df = 5,74, $p < 0.001$), in the absence of MK-801, that was significant at CA concentrations ≥100 μM ($p < 0.05$) In the presence of MK-801, CA stimulated [^{3}H]DA release (F = 16 5, df = 3,59, $p < 0.001$), but only at the highest concentration tested (10 mM) b· CSA evoked [^{3}H]DA release (F = 42 8, df = 5 56, $p < 0.001$), in the absence of MK-801, that was significant for concentrations of ≥100 μM CSA ($p < 0.05$) In the presence of MK-801, the effect of CSA (F = 24 7, df = 3,36, $p < 0.001$) was significant only at 10 mM ($p < 0.05$) *$p < 0.05$ compared with release in the absence of MK-801

pencent upon activation of the NMDA receptor. A low concentration of Glu (10 μM) evoked [^{3}H]DA release that was abolished by 10 μM MK-801, indicating that this concentration of Glu activates the NMDA receptor. However, at 100 μM and 1 mM, Glu-evoked [^{3}H]DA release was overwhelmingly MK-801 insensitive, suggesting that activation of non-NMDA receptors mediates the effects of higher Glu concentrations Further, Glu was the most potent naturally occurring EAA at non-NMDA receptors. The release evoked by even 1 mM Glu reflected EAA receptor-mediated stimulus-secretion coupling, since it was Ca^{2+} dependent and largely inhibited by DNQX (100 μM), a competitive non-NMDA receptor antagonist [with non-competitive antagonist activity at NMDA receptors in certain systems (see review by Foster, 1988)]. The observed mixed agonist effects of Glu at NMDA and non-NMDA receptors correspond to the reported behavior of comparable Glu concentrations in whole-cell current noise analysis studies (Mayer and Westbrook, 1984; Ascher et al., 1988).

Effects of Asp on [^{3}H]DA release were mediated completely by the NMDA receptor, since 10 μM MK-801 inhibited release stimulated by all Asp concentrations tested (100 μM to 10 mM). Selectivity of Asp for the NMDA receptor is consistent with the finding by Snell and Johnson (1986) that Asp-evoked DA release from striatal tissue slices was abolished in the presence of Mg^{2+}, phencyclidine, or 2-amino-5-phosphonovaleric acid. The concentration-response profile pattern for Asp suggests that high agonist concentrations may cause desensitization of the NMDA receptor. This explanation for the decreased response at high Asp concentrations receives support from the reported desensitization of Asp responses in rat olfactory cortex (Braitman, 1986, Collins and Surtees, 1986) and hippocampus (Baudry et al., 1983).

Endogenous sulfur-containing EAAs have recently been identified in brain and have been shown to be released in response to depolarizing stimuli from brain regions containing DA cell bodies (mesodiencephalon) or terminal fields (basal ganglia, cortex) (Do et al., 1986*a*,*b*, 1988; Keller et al., 1989). Previous studies have also demonstrated that HCA, HCSA, CA, and CSA evoke release of [^{3}H]GABA and [^{3}H]Asp from cultured cerebral cortical neurons and cerebellar granule cells (Dunlop et al., 1989), while HCA, CA, and CSA also stimulate release of [^{3}H]GABA from striatal neurons in primary culture (Weiss, 1988). The present experiments demonstrate that these sulfur-containing amino acids can also stimulate DA release

The concentration-response curves for [^{3}H]DA release evoked by sulfur-containing amino acids in the absence and presence of 10 μM MK-801 reveal patterns of EAA receptor subtype involvement intermediate between those observed for Glu and Asp. For HCA and HCSA, [^{3}H]DA release stimulated by agonist concentrations from 10 to 100 μM was MK-801 sensitive and thus NMDA receptor mediated, while release evoked by higher concentrations of the compounds was MK-801 insensitive. For CA and CSA, release induced by concentrations from 100 μM to 1 mM was MK-801 sensitive, while that evoked by 10 mM was predominantly MK-801 insensitive We also observed that MK-801-insensitive [^{3}H]DA release evoked by high concentrations of the sulfur-containing EAAs (1–10 mM) was largely Ca^{2+} dependent and was antagonized by the EAA receptor blocker DNQX (100 μM). Together, these observations indicate that higher concentrations of the compounds evoke [^{3}H]DA release by activating non-NMDA receptors These findings are consistent with results from EAA receptor binding assays. HCA and HCSA are reported to bind with high affinities at the NMDA receptor subtype and with affinities an order of magnitude lower at quisqualate and kainate receptors (Pullan et al., 1987) CA and CSA have binding affinities at NMDA and kainate receptors that are approximately equivalent to those of HCA and HCSA at quisqualate and kainate sites (Pullan et al., 1987). However, CA and CSA are less potent inhibitors of binding (again by an order of magnitude) at the quisqualate receptor (Pullan et al., 1987).

In summary, naturally occurring EAAs can evoke [^{3}H]DA release from mesencephalic cell cultures by

mechanisms that involve both NMDA and non-NMDA receptor subtypes. All EAAs tested were found to interact with NMDA receptors. Glu and Asp are clearly the most potent ligands at the NMDA receptor, followed by HCA and HCSA, and then by CA and CSA. Non-NMDA receptors are involved in mediating the effects of Glu at concentrations of ≥100 μM and also of HCA and HCSA at concentrations of ≥1 mM. It is unlikely that CA and CSA act at non-NMDA receptors under physiological conditions since the receptors were activated only by very high (10 mM) concentrations of these amino acids. Non-NMDA receptors play no apparent role in the effects of Asp. It remains to be established which (if any) of these EAAs act as endogenous regulators of specific dopaminergic pathways in the intact rat brain. The present study, showing that six naturally occurring EAAs can stimulate DA release via both NMDA and non-NMDA receptors in vitro, together with observations that these same EAAs are present in and released from brain areas containing DA cell bodies and/or terminals (Do et al., 1986*a*,*b*; Keller et al., 1989), make these compounds likely candidates for this role.

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Phencyclidine and related compounds evoke [^{3}H]dopamine release from rat mesencephalic cell cultures by a mechanism independent of the phencyclidine receptor, sigma binding site, or dopamine uptake site

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At concentrations ≥ 100 μM, phencyclidine (PCP), *N*-(1-(2-thienyl)-cyclohexyl)piperidine (TCP), and MK-801 induced [^{3}H]dopamine release from dissociated cell cultures of rat mesencephalon. This release was Ca^{2+} independent and tetrodotoxin insensitive. Tetrodotoxin (2 μM) itself had no effect on spontaneous release of [^{3}H]dopamine [^{3}H]Dopamine release was induced by 1,3-di(2-tolyl)guanidine, a sigma ligand, and by 4-aminopyridine (1–3 mM), a K^+ channel blocker. No stereoselectivity was observed for [^{3}H]dopamine release evoked by the dioxadrol enantiomers, dexoxadrol, and levoxadrol, or by enantiomers of *N*-allylnormetazocine (SKF 10,047). The selective dopamine uptake inhibitor 1-(2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (GBR 12909) did not affect spontaneous or TCP-evoked [^{3}H]dopamine release. Together, these data suggest that the dopamine-releasing effects of PCP-like compounds on the mesencephalic cells were not mediated by actions at the PCP receptor or sigma binding site, Ca^{2+}, or Na^+ channels, or at the high affinity dopamine uptake site. It remains conceivable that blocking actions of PCP-like compounds at voltage-regulated K^+ channels may at least partly explain the response. These results are discussed in comparison with findings in intact brain.

Key words: dopamine, phencyclidine, cell culture, mesencephalon.

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A des concentrations ≥ 100 μM, la phencyclidine (PCP), *N*-(1-(2-thienyl)-cyclohexyl)piperidine (TCP), et MK-801 induisent la libération de la [^{3}H]dopamine à partir de cellules dissociées de mésencéphale de rat, en culture. Cette libération est indépendante du Ca^{2+} et insensible à la tétrodotoxine. Par elle-même, la tétrodotoxine (2 μM) n'a aucun effet sur la libération spontanée de la [^{3}H]dopamine. Le 1,3-di(2-tolyl)guanidine, un ligand du récepteur sigma, et la 4-aminopyridine (1–3 mM), un bloqueur du canal K^+, induisent la libération de la [^{3}H]dopamine. Les énantiomères du dioxadrol, le déxoxadrol et le lévoxadrol, et les énantiomères de la *N*-allylnormétazocine (SKF 10,047) induisent la libération de la [^{3}H]dopamine de façon non stéréosélective. L'inhibiteur sélectif du transport de la dopamine, le 1-(2-[bis(4-fluorophényl)methoxy]éthyl)-4-(3-phénylpropyl)pipérazine dihydrochloride (GBR 12909) n'affecte ni la libération spontanée de la [^{3}H]dopamine, ni celle évoquée par la TCP. L'ensemble de ces données suggère que les effets des composés de type PCP sur la libération de dopamine, à partir de cellules mésencéphaliques en culture, ne peuvent être expliqués par une interaction avec les sites typiques PCP ou sigma, les canaux Ca^{2+} ou Na^+ ou encore avec le site de transport de haute affinité dopaminergique. Il paraît cependant possible que le blocage des canaux K^+ voltage dépendants par les composés PCP puisse en partie expliquer ces effets. Ces résultats sont aussi discutés en relation avec les données obtenues sur des cerveaux intacts.

Mots clés : dopamine, phencyclidine, cellules en culture, mésencéphale

Introduction

Phencyclidine (*N*-(1-phenylcyclohexyl)piperidine, PCP) belongs to a class of compounds that are thought to produce psychotomimetic effects through interaction with brain dopaminergic transmission (Domino and Luby 1981). PCP-induced dopamine (DA) release has been demonstrated *in vitro* from striatal slices and synaptosomes (Ary and Komiskey 1982; Vickroy and Johnson 1982; Bowyer et al. 1984; Snell et al. 1984; Snell and Johnson 1986) but not from the intact striatum (Deutch et al. 1987, Gerhardt et al. 1987; Rao et al. 1989). In contrast with results reported for striatum, PCP and related compounds have been shown to stimulate DA release from terminal fields of mesocorticolimbic DA systems both *in vitro* and *ex vivo* (Gratton et al. 1987, Deutch et al. 1987; Carboni et al. 1989; Rao et al. 1989; Wood and Rao 1989) and to induce dose-dependent increases in the firing rates of A_{10} neurons *in vivo* (French 1989; French and Ceci 1989). The order of potency for PCP-like compounds in stimulating DA release from the pyriform cortex (Wood and Rao 1989) suggested an interaction with the PCP receptor (Quirion et al. 1987), within the channel of the *N*-methyl-D-aspartate (NMDA) receptor complex. However, the competitive NMDA receptor antagonist CGS 19755 did not increase DA release (Wood and Rao 1989). These results have been interpreted as evidence that PCP may interact with an "NMDA-uncoupled form of the PCP receptor" (Wood and Rao 1989).

In a previous study, we have shown that PCP and the potent PCP-like NMDA channel blocker (+)-5-methyl-10,11-dihy-

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ABBREVIATIONS: 4-AP, 4-aminopyridine; DA, dopamine; D,L-APV, D,L-2-amino-5-phosphonovalerate; DMEM, Dulbecco's modified Eagle's medium; DTG, 1,3-di(2-tolyl)guanidine; GBR 12909, 1-(2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride, KRH, Krebs–Ringer–HEPES buffer; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; PCP, *N*-(1-phenylcyclohexyl)-piperidine (phencyclidine), SKF 10,047, *N*-allylnormetazocine; TCP, *N*-(1-(2-thienyl)-cyclohexyl)piperidine, TTX, tetrodotoxin.

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dro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) inhibited NMDA-stimulated release of [^{3}H]DA (IC_{50} = 2.3 and 0.3 μM, respectively) from dissociated primary cell cultures of fetal rat anterior ventral mesencephalon (Mount et al. 1990). At higher concentrations ($\geq$100 μM), MK-801 and PCP produced a significant enhancement of spontaneous [^{3}H]DA release that resembled stimulatory effects of PCP on DA release in the intact brain.

In addition to the NMDA–PCP receptor complex, there are numerous other target sites for actions of PCP in the central nervous system. These include the sigma binding site (Quirion et al. 1987), as well as various ion channels and the neuronal high affinity DA uptake site (see Lazdunski et al. 1983). To determine what neurochemical mechanism(s) might underly PCP-induced enhancement of dopaminergic transmission, the effects of PCP on [^{3}H]DA release from mesencephali cell cultures have been evaluated as a model for interactions with dopaminergic systems in the intact brain. Use of dissociated cell cultures facilitates the study of compounds with stimulatory effects on dopaminergic transmission, since entire neurons (neuronal soma and processes) from mesocorticolimbic and nigrostriatal pathways are simultaneously accessible and exposed. Normal neural connectivity is disrupted in the cultures. Thus, the model allows assessment of direct effects on dopaminergic neurons, but it reduces the opportunity for indirect, polysynaptic responses to test compounds.

Some preliminary results from this study have been presented at a meeting (Mount et al. 1989*a*).

Methodology

Materials

3,4-[8-^{3}H(N)]Dihydroxyphenylethylamine ([^{3}H]DA, 20 Ci/mmol) (1 Ci = 37 GBq) was purchased from New England Nuclear (Boston, MA), 3-(2-carboxypiperazine-yl)propyl-1-phosphonate (CPP) was purchased from Tocris Neuramin (Buckhurst Hill, U.K.), and 1,3-di(2-tolyl)guanidine (DTG) was purchased from Aldrich Chemical Co (Milwaukee, WI). 1-(2-[Bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (GBR 12909) was ordered from Research Biochemicals Inc. (Natick, MA) and dexoxadrol and levoxadrol were from Upjohn Co (Kalamazoo, MI). NMDA, D,L-2-amino-5-phosphonovalerate (D,L-APV), 4-aminopyridine (4-AP), desipramine hydrochloride, and pargyline hydrochloride were ordered from Sigma Chemical Co (St. Louis, MO). Phencyclidine and *N*-(1-(2-thienyl)cyclohexyl)piperidine (TCP) were gifts from the National Institute on Drug Abuse (Baltimore, MD). (+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) and the enantiomers of *N*-allylnormetazocine ((+)- and (−)-SKF 10,047) were gifts from Dr L. Iversen (Merck, Sharp & Dohme, Harlow, U.K.) and from Dr K. H. Jhamandas (Queen's University, Kingston, Ont.), respectively. Other chemicals were of reagent grade from regular commercial sources.

Methods

Dissociated cell cultures were prepared from anterior ventral mesencephalic tissue of gestation day-15 Sprague–Dawley rat embryos, as previously described (Mount et al. 1989*b*). Cultures were grown in Dulbecco's modified Eagle's medium, supplemented with 10% v/v fetal calf serum and 20 mM KCl.

After 6 days in culture, cells were rinsed with Krebs–Ringer–HEPES buffer (KRH, pH 7.4 composed of the following (mM): NaCl, 125; KCl, 4.8; HEPES, 25; NaOH, 5; $MgSO_4$, 1.2, KH_2PO_4, 1.2; D-glucose, 5.6, $CaCl_2$, 2.2; pargyline, 0.1; ascorbate, 1.0.) and loaded with [^{3}H]DA (50 nM) in a 20-min incubation at 37°C, in the presence of desipramine (50 μM). Uptake of [^{3}H]DA into cultures has previously been shown to be predominantly into dopaminergic neurons, since it was blocked by a DA uptake inhibitor (5 μM benztropine), was only marginally attenuated by a noradrenaline uptake inhibitor (5 μM desipramine), and was unaffected by a serotonin uptake inhibitor (1 μM fluoxetine) (Mount et al. 1989*b*). After [^{3}H]DA loading, each culture was rinsed four times with KRH. KRH buffer from a 5-min incubation (spontaneous release period), followed immediately by a 5-min incubation in the presence of the test compound (stimulated release period), were collected, and [^{3}H]DA release was quantitated by measuring radioactivity in each aliquot of buffer collected. In earlier work, more than 90% of the cell content of ^{3}H, assayed just prior to the time at which release was normally tested, was determined by HPLC to be [^{3}H]DA (Mount et al. 1989*b*). Following release incubations, radioactivity remaining in the cells was extracted by 30 min incubation with acidified ethanol (95% ethanol – 5% 0.1 M HCl).

When examining the dependence of test compound-evoked [^{3}H]DA release on extracellular Ca^{2+}, modified KRH (0 Ca^{2+}) was used for all rinses and incubations after [^{3}H]DA loading. When antagonists were tested, cells were exposed to the antagonists during the spontaneous release incubation as well as during exposure to the test compound. For data presentation, spontaneous [^{3}H]DA release (typically 4–6% of intracellular [^{3}H]DA stores) was subtracted from release stimulated by the test compound. The net evoked [^{3}H]DA release was expressed as a percent of the total [^{3}H]DA uptake into cells. Statistical analyses involved unpaired *t*-tests or one-way analysis of variance and *post hoc* Newman–Keuls test for multiple comparisons.

Results

At concentrations $\geq$100 μM, PCP receptor ligands (including MK-801; PCP itself; and TCP, a thienyl-substituted PCP analog) and the sigma ligand DTG (Weber et al. 1986) evoked dose-dependent [^{3}H]DA release (Fig. 1). TCP evoked [^{3}H]DA release with greater efficacy than did PCP or MK-801 (Fig. 1) and was therefore used as a prototypic PCP ligand to characterize the pharmacological specificity of PCP-evoked [^{3}H]DA release.

To determine whether the [^{3}H]DA-releasing effect of PCP-like compounds might be mediated by blockade of the NMDA receptor, the effect of a competitive NMDA-receptor antagonist (D,L-APV, Evans et al. 1982; Davies and Watkins 1982) was also assessed. However, D,L-APV (100 μM – 3 mM) failed to alter spontaneous [^{3}H]DA release (Table 1).

To indicate whether effects of TCP might involve an interaction with the [^{3}H]DA uptake site, rather than stimulation of release, activity of the potent DA uptake inhibitor GBR 12909 (Heikkila and Manzino 1984) was examined. GBR 12909 (100 μM – 1 mM) had no effects on spontaneous [^{3}H]DA release (Table 1) or on release evoked by TCP (Table 2).

It was reasoned that direct blocking actions of TCP at one or more ion channels might lead to the [^{3}H]DA release. However, [^{3}H]DA release evoked by TCP was not reduced when Ca^{2+} was omitted from the KRH buffer (Table 2). Also, both spontaneous (Table 1) and TCP-evoked [^{3}H]DA release (Table 2) were unaffected by 2 μM tetrodotoxin (TTX), a concentration of the potent voltage-sensitive Na^+-channel inhibitor previously found to completely inhibit release evoked by 10 μM veratridine (Mount et al. 1989*b*). At concentrations of 1 and 3 mM, the K^+ channel blocker 4-aminopyridine (4-AP, Bowman and Savage 1981; Glover 1982) stimulated [^{3}H]DA release from cell cultures (Fig. 1). This result suggests that K^+ channel blockade may indeed lead to increased [^{3}H]DA release.

Dexoxadrol has been reported to be more potent than its stereoisomer levoxadrol at the PCP receptor (e.g., Jacobson et al. 1987; ffrench-Mullen and Rogawski 1989) and as a K^+

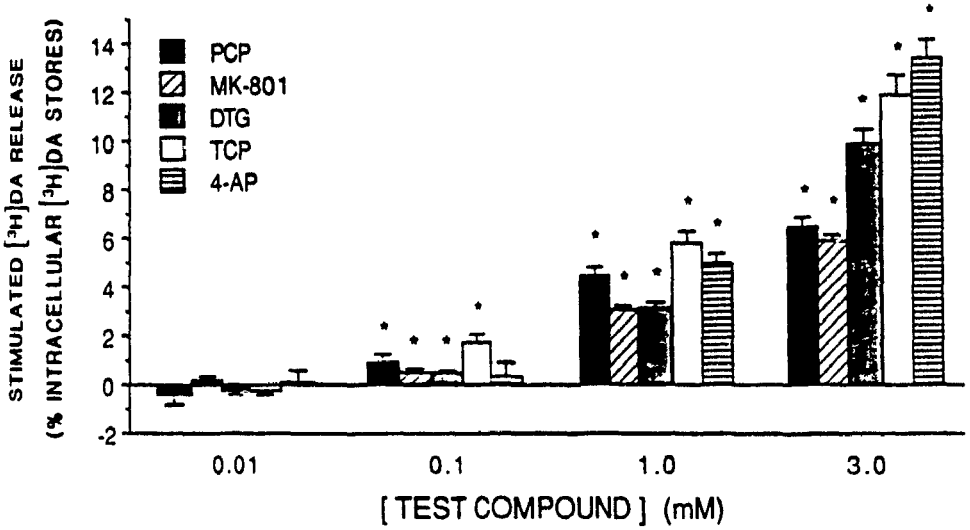

FIG. 1. Effects of PCP, MK-801, DTG, TCP, and 4-AP on [³H]DA release. Spontaneous [³H]DA release has been subtracted from release evoked by the test compound. The resultant stimulated release is expressed as a percentage of total [³H]DA uptake into cells. Results are the means ± SEM of 4–16 cultures. *$p < 0.05$ versus spontaneous [³H]DA release.

TABLE 1. Effects of D,L-APV, TTX, and GBR 12909 on [³H]DA release

Test compound and concentration	Stimulated [³H]DA release (% intracellular stores)
D,L-APV	
100 μM	−0.1±0.2
1 mM	−0.2±0.2
3 mM	−0.7±0.2
TTX	
2 μM	0.0±0.1
GBR 12909	
10 μM	−0.6±0.2
100 μM	−0.5±0.3
1 mM	0.5±0.2

NOTE: Spontaneous [³H]DA release has been subtracted from [³H]DA release evoked by the test compound. The resultant stimulated release is expressed as a percentage of total [³H]DA uptake into cells. None of the test compounds stimulated [³H]DA release that was significantly different from spontaneous release ($p > 0.05$). Each value is the mean ± SEM of five to six cultures.

channel blocker (Sorensen and Blaustein 1988; ffrench-Mullen and Rogawski 1989). However, no differences were observed between the [³H]DA-releasing effects of dexoxadrol and levoxadrol (Fig. 2). Ligand binding affinity at both PCP and sigma sites is also characterized by marked stereoselectivity for (+)-SKF 10,047 over (−)-SKF 10,047 (Contreras et al. 1988; Itzhak 1988). However, the enantiomers were equally effective in stimulating [³H]DA release (Table 3). Since (−)-SKF 10,047 may activate mu or kappa opioid receptors, effects of SKF 10,047 enantiomers were also tested in the presence of the opioid receptor antagonist naloxone. No differences were observed between effects of the enantiomers in the presence of 100 μM naloxone (Table 3).

Discussion

The principal finding of this study is that high concentrations (≥100 μM) of PCP and PCP-like compounds evoke dose-dependent [³H]DA release from mesencephalic cell cultures. At lower concentrations, it is well known that PCP is a potent inhibitor of DA release stimulated by the excitatory amino acid NMDA from tissue slices (Snell and Johnson 1986; Jones et al. 1987) and intact brain (Carter et al. 1988). Also, we have previously shown that low concentrations (1–10 μM) of PCP and MK-801 antagonize Ca^{2+}-dependent release of [³H]DA from dissociated primary mesencephalic cell cultures evoked by NMDA (100 μM) (Mount et al. 1990). The possibility that higher concentrations of these compounds might cause release through effects at the same receptor (NMDA–PCP receptor complex) was assessed. However, even high concentrations of the competitive NMDA antagonist D,L-APV (100 μM – 3 mM) failed to have any effect on spontaneous [³H]DA release. These data indicate that NMDA receptor blockade cannot underly the [³H]DA-releasing effects of PCP. In this respect, results in cell culture resemble the failure of competitive NMDA antagonists to evoke DA release from intact brain (Rao et al. 1989; Wood and Rao 1989).

The dioxadrol optical isomers dexoxadrol and levoxadrol were equally effective in evoking [³H]DA release from the cell cultures. These results distinguish PCP-evoked [³H]DA release in cell cultures from the reported stereoselective effects of the compounds on mesocortical DA turnover in intact brain (Wood and Rao 1989) and [³H]DA release from striatal slices (Snell et al. 1984). These observations also differ from the stereoselective blockade by dexoxadrol of the NMDA receptor ionophore (ffrench-Mullen and Rogawski 1989). It thus appears unlikely that the [³H]DA-releasing effect of PCP in mesencephalic cell cultures was linked to effects at the PCP–NMDA receptor complex.

To test whether PCP-like compounds evoked [³H]DA release through actions at the sigma site, effects of DTG, a potent sigma ligand (Weber et al. 1986), were assessed. DTG stimulated [³H]DA release with a potency and efficacy comparable with that of MK-801, PCP, and TCP. This finding was unexpected, since DA release from the intact rat brain (Wood and Rao 1989) has previously been found to be insensitive to DTG. Other sigma ligands have also been found inactive. For

TABLE 2. Effects of extracellular calcium removal and of TTX and GBR 12909 on TCP-evoked [^{3}H]DA release

Test compounds and concentration	Stimulated [^{3}H]DA release (% intracellular [^{3}H]DA stores)
TCP (1 mM)	6.2±0.4
TCP (1 mM) in 0 Ca^{2+} KRH	6.4±0.3[a]
TCP (1 mM) + TTX (2 μM)	6.6±0.6[a]
TCP (1 mM) + GBR 12909 (100 μM)	5.7±0.4[a]
TCP (1 mM) + GBR 12909 (1 mM)	5.3±0.6[a]

NOTE Cell cultures were loaded with [^{3}H]DA in standard Krebs–Ringer–HEPES buffer (KRH) (2.2 mM Ca^{2+}) Spontaneous release and release in the presence of the test compound were collected either in standard KRH (+Ca^{2+}), in Ca^{2+}-free KRH (0 Ca^{2+}), or in standard KRH in the presence of TTX (2 μM) or GBR 12909 (100 μM or 1 mM) Spontaneous [^{3}H]DA release has been subtracted from release evoked by the test compound The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells Each value is the mean ± SEM of at least six cultures

[a]No difference from [^{3}H]DA release evoked by 1 mM TCP (in +Ca^{2+} KRH)

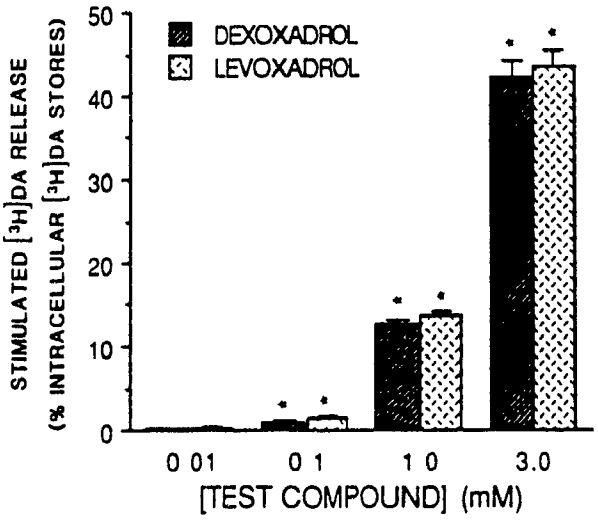

FIG. 2. Effects of the stereoisomeric dioxadrol compounds, dexoxadrol, and levoxadrol on [^{3}H]DA release Spontaneous [^{3}H]DA release has been subtracted from dioxadrol-evoked release The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells Results are the means ± SEM of five to six cultures. *$p < 0.05$ versus spontaneous [^{3}H]DA release.

TABLE 3 Effects of (+)-SKF 10,047 and of (−)-SKF 10,047 on [^{3}H]DA release

Test compound and concentration	Stimulated [^{3}H]DA release (% intracellular stores) 0 naloxone	100 μM naloxone
(+)-SKF 10,047 (30 μM)	−0.3±0.3	0.0±0.2[c]
(+)-SKF 10,047 (100 μM)	0.9±0.2[a]	1.2±0.3[ac]
(+)-SKF 10,047 (300 μM)	2.9±0.3[a]	3.0±0.2[ac]
(+)-SKF 10,047 (1 mM)	6.2±0.4[a]	6.1±0.2[ac]
(−)-SKF 10,047 (30 μM)	0.1±0.2[b]	0.2±0.2[bc]
(−)-SKF 10,047 (100 μM)	0.8±0.3[ab]	1.0±0.2[abc]
(−)-SKF 10,047 (300 μM)	3.0+0.1[ab]	3.1±0.2[abc]
(−)-SKF 10,047 (1 mM)	6.4+0.3[ab]	6.7±0.4[abc]

NOTE Cells were exposed to the opioid receptor antagonist naloxone (100 μM) during the spontaneous incubation and in the presence of indicated concentrations of the enantiomers (+)-SKF 10,047 or (−)-SKF 10,047 Spontaneous [^{3}H]DA release has been subtracted from SKF 10,047 stimulated release Each value is the mean ± SEM of five to six cultures

[a]$p < 0.05$ versus spontaneous [^{3}H]DA release

[b]No different from [^{3}H]DA release evoked by the corresponding concentration of (+)-SKF 10,047

[c]No different from [^{3}H]DA release evoked by the corresponding concentration of (+)- or (−)-SKF 10,047 in 0 naloxone

example, rimcazole had no effect on DA release (Wood and Rao 1989), pentazocine failed to mimic the PCP-induced increase in firing rate of A_{10} neurons (French and Ceci 1989), and haloperidol did not affect MK-801-induced speeding of A_{10} neurons (French and Ceci 1989).

The role of the PCP receptor and sigma site may be assessed by a comparison between effects of the benzomorphan enantiomers (+)-SKF 10,047 and (−)-SKF 10,047. Both sites have high affinity for the (+)-enantiomer relative to the (−)-compound (Contreras et al. 1988; Itzhak 1988). However, SKF 10,047 enantiomers showed no selectivity in their effects on [^{3}H]DA release from cell cultures. Mu and kappa opioid receptors have a reversed stereoselectivity for (−)-SKF 10,047 over (+)-SKF 10,047 (Zukin et al. 1984; Martin et al. 1984). Thus, actions of the (−)-enantiomer of SKF 10,047 at naloxone-sensitive opioid receptors might mask stereoselective interaction of the (+)-isomer at the sigma site. An unmasking of stereoselective sigma responses to benzomorphans in the presence of naloxone was recently described for inhibition of the serotonin-induced twitch responses in guinea pig ileum (Campbell et al. 1989). In the present study, when effects of the enantiomers were tested in the presence of 100 μM naloxone, no stereoselectivity was detected for (+)-SKF 10,047 evoked [^{3}H]DA release. Thus, effects of (−)-SKF 10,047 on mu or kappa opioid receptors cannot account for the lack of stereoselectivity. The lack of stereoselectivity for (+)-SKF 10,047 indicates that the [^{3}H]DA-releasing effects of both PCP and sigma ligands were mediated neither at the typical PCP receptor nor at the sigma site.

The high concentrations of DTG and PCP-like compounds required to evoke [^{3}H]DA release from cell cultures suggests that the responses may be mediated by anaesthetic actions of the drugs. Indeed, at concentrations ≥100 μM, PCP, DTG, and the sigma ligand 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)-3-PPP) all caused increases in membrane resistance and action potential duration and a reduction in spike height in CA1 hippocampal pyramidal slices (Malouf et al. 1988). Such anaesthetic effects (Catterall 1987) may be explained by known interactions of PCP and PCP-like compounds with various ion channels. The general sequence of efficiency of PCP on these channels is K^+ channel > Na^+ channel >

Ca^{2+}-dependent K^+ channel ≫ Ca^{2+} channel (Lazdunski et al. 1983).

In several preparations, PCP has been found to block K^+ channels (Albuquerque et al. 1981; Tourneur et al. 1982; Lazdunski et al. 1983; Blaustein and Ickowicz 1983; Aguayo and Albuquerque 1987). Blockade of the K^+ channel delays repolarization, resulting in a prolongation of the action potential. This would be expected to cause an enhancement of transmitter release. Nanomolar concentrations of PCP were reported to inhibit K^+ channels in rat brain synaptosomes (Blaustein and Ickowicz 1983). However, much higher concentrations of PCP were required to block the slowly activating outward K^+ current (10–100 μM) or the rapidly activating and inactivating voltage-dependent K^+ current (50–100 μM) in cultured hippocampal neurons (ffrench-Mullen et al. 1988), and in cultures of spinal neurons, K^+ channel blockade was only observed at PCP concentrations >200 μM (Aguayo and Albuquerque 1987). This is the range of PCP concentrations found to evoke [^{3}H]DA release in the present study. Similarly, the K^+ channel blocker 4-AP evoked [^{3}H]DA release from the mesencephalic cell cultures at concentrations (1–3 mM) reported to cause blockade of the transient fast-activating and inactivating K^+ channel (Rogawski et al. 1988).

Although it has been reported that voltage-gated K^+ channels are blocked stereoselectively by dexoxadrol (Sorensen and Blaustein 1988; ffrench-Mullen and Rogawski 1989), there is also evidence that dexoxadrol and levoxadrol may be equally effective blockers of K^+ channels in certain cell culture systems (Rogawski et al. 1988). Thus, the observed lack of stereoselectivity of the dioxadrol stereoisomers in the present study rules out activation of the NMDA–PCP receptor complex, but it does not preclude the possibility that blockade of K^+ channels underlies the responses. It is significant that PCP has been reported to release DA from striatal slices (Vickroy and Johnson 1982) or synaptosomes (Raiteri et al. 1979) in either the absence or presence of extracellular K^+. These findings indicate that while K^+ channel blockade may be an important mechanism of action for PCP-like compounds, other mechanisms must also be considered.

Depolarization-induced catecholamine release from central nerve endings is Ca^{2+} dependent (Baldessarini and Kopin 1967; Blaustein 1975; Cotman et al. 1976). However, similar to results reported for PCP-induced [^{3}H]DA release from striatal slices (Vickroy and Johnson 1982), removing Ca^{2+} from the external medium did not affect the [^{3}H]DA release evoked from cell cultures by TCP. This observation indicates that the mechanism of action for TCP involves neither classical stimulation–secretion coupling nor blockade of Ca^{2+}-dependent K^+ channels or Ca^{2+} channels.

PCP can block Na^+ channels in various systems (Allaoua and Chicheportiche 1989), causing a reduction in spike amplitude following depolarization. However, in the mesencephalic cells, when voltage-regulated Na^+ channels were blocked by 2 μM TTX, spontaneous [^{3}H]DA release and release stimulated by 1 mM TCP were unaffected. Thus, effects of TCP on [^{3}H]DA release were not related to Na^+ channel blockade.

Mechanisms other than ion channel blockade might also play a role, particularly in the high concentration effects of PCP on mesencephalic cultures. For example, PCP-like compounds inhibit [^{3}H]DA uptake in synaptosomes (Lazdunski et al. 1983; Vignon and Lazdunski 1984; Johnson and Snell 1985; Vignon et al. 1988) and primary cultures of dissociated monoaminergic neurons (Marlier et al. 1987), with IC_{50}s ranging from 0.5 to 100 μM. To test the possibility that apparent [^{3}H]DA release evoked by PCP and PCP-like compounds were caused by inhibition of [^{3}H]DA uptake, we assessed the effects of the selective inhibitor of the neuronal DA uptake site (GBR 12909) (Heikkila and Manzino 1984) on [^{3}H]DA release. This compound failed to alter spontaneous [^{3}H]DA release, or release stimulated by 1 mM TCP, over a broad range of concentrations (10 nM – 1 mM). Thus, by itself, inhibition of the DA uptake carrier cannot account for effects of TCP (or PCP) in cultures. Further, although MK-801 has been reported to be 250-fold less potent than PCP as an inhibitor of [^{3}H]DA uptake into striatal synaptosomes (Snell et al. 1988), the two compounds had similar potency in evoking [^{3}H]DA release from the cell cultures. This finding also argues against an action at the DA uptake site.

The amphetamine-like class of stimulants can induce DA release from tissue slices by a counter-transport process (reversal of the DA uptake carrier). This amphetamine-evoked release can be blocked by the DA uptake blocker nomifensine (Vickroy and Johnson 1982). However, a similar mechanism may not satisfactorily explain DA-releasing effects of the PCP-like compounds, since PCP-induced DA release from striatal slices was not blocked by nomifensine (Vickroy and Johnson 1982). In the mesencephalic cell cultures, the DA uptake blocker GBR 12909 also failed to block TCP-evoked [^{3}H]DA release. Thus, it is unlikely that DA-releasing effects of the PCP-like compounds are mediated by a counter-transport mechanism.

From previous reports, it has not been clear whether the DA-releasing effect of PCP-like compounds in studies on intact brain was related to a direct effect of the ligands on the dopaminergic neuron or to activation of a pathway afferent to the DA-releasing cell. The mesencephalic cultures provide a model for assessing direct effects of compounds on dopaminergic neurons. We have previously shown that low concentrations (1–10 μM) of PCP and MK-801 inhibit NMDA-evoked [^{3}H]DA release (Mount et al. 1990); in the present study, low concentrations of these PCP-like drugs had no effect on spontaneous [^{3}H]DA release from cell cultures. Higher (≥100 μM), anaesthetic concentrations of PCP-like drugs caused [^{3}H]DA release from the cells. This effect on spontaneous release was not inhibited by TTX and was therefore likely mediated by a direct action on the dopaminergic neurons themselves. Although the neurochemical mechanism underlying PCP-induced [^{3}H]DA release from cell cultures remains unclear, its pharmacological profile does not resemble known effects of PCP at the PCP receptor or sigma binding site, DA uptake sites or voltage-regulated Na^+, Ca^{2+}, or Ca^{2+}-activated K^+ channels. At least part of the actions of these compounds in mesencephalic cell cultures may involve blockade of voltage-regulated K^+ channels. The relatively large concentrations required to evoke the release in cell culture and the pharmacological profile of this release (i.e., activity of DTG, lack of stereoselectivity for dioxadrol enantiomers, TTX insensitivity) differ from the characteristics of the potent DA-releasing effects of PCP observed in the intact rat brain (Deutch et al. 1987; French and Ceci 1989; Rao et al. 1989; Wood and Rao 1989). This would suggest that polysynaptic mechanisms, rather than direct effects of PCP on dopaminergic neurons, may be responsible for PCP modula-

tion of dopaminergic transmission in the intact brain.

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7. INHIBITORY AND POTENTIATING INFLUENCES OF GLYCINE ON NMDA-EVOKED DOPAMINE RELEASE FROM CULTURED RAT MESENCEPHALIC CELLS

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SUMMARY

In the presence of 1.2 mM Mg^{2+}, glycine (30-100 μM) inhibited [^{3}H]dopamine ([^{3}H]DA) release stimulated by *N*-methyl-D-aspartate (NMDA), from fetal rat mesencephalic cell cultures. Strychnine (1 μM) blocked the inhibitory effect of 100 μM glycine, indicating an action via strychnine-sensitive inhibitory glycine receptors. A higher concentration of strychnine (100 μM) by itself inhibited NMDA-evoked [^{3}H]DA release in the presence or absence of Mg^{2+}. Spontaneous [^{3}H]DA release and [^{3}H]DA release stimulated by kainate and quisqualate were unaffected by glycine ($\leq$ 100 μM) or strychnine ($\leq$ 100 μM), indicating that glycine and strychnine modulatory effects are only associated with the NMDA receptor subtype. [^{3}H]DA release evoked by K^+ (56 mM) was unaffected by glycine ($\leq$ 100 μM), but was attenuated by a high concentration of strychnine (100 μM).

In the absence of exogenous Mg^{2+}, glycine (30-100 μM) potentiated NMDA-evoked [^{3}H]DA release by a strychnine-insensitive mechanism. A selective antagonist of the NMDA-associated glycine receptor, 7-chlorokynurenate (10 μM), attenuated NMDA-evoked [^{3}H]DA release in the absence of Mg^{2+}. The effect of 10 μM 7-chlorokynurenate was overcome by 1 μM glycine. Also, when tested in the presence of 1.2 mM Mg^{2+} and 1 μM strychnine, 100 μM 7-chlorokynurenate inhibited NMDA-evoked [^{3}H]DA release and this antagonism was overcome by 30 to 100 μM glycine.

These results indicate that two distinct glycine receptors modulate NMDA-stimulated [^{3}H]DA-release from mesencephalic cells in culture. Manipulation of extracellular Mg^{2+} permits the differentiation of a strychnine-sensitive glycine response (inhibition of NMDA-evoked

[^{3}H]DA release) from a strychnine-insensitive glycine response (potentiation of NMDA-evoked [^{3}H]DA release). It is suggested that voltage-dependent Mg^{2+} blockade of the NMDA response may allow for the expression of these opposing effects of glycine.

INTRODUCTION

The *N*-methyl-D-aspartate (NMDA) receptor is under the control of various modulators, including the divalent cation, Mg^{2+}, and the amino acid, glycine. Activity at the receptor is subject to voltage-dependent inhibition by low millimolar concentrations of Mg^{2+} (1-4). Thus, the blockade of NMDA responses may be alleviated in partially depolarized neurons (5). Glycine acts at a strychnine-insensitive site allosterically associated with the NMDA receptor to potentiate NMDA receptor-mediated responses (6, also reviewed in 7)

NMDA receptors have been found to mediate release of dopamine (DA) or [^{3}H]DA from rat brain slices of substantia nigra (8,9), striatum (10,8,11-14) and nucleus accumbens (8,15), from cultured cells of fetal rat ventral mesencephalon (16) and in trans-striatal dialysis experiments (17). In striatal slices, NMDA receptor activation resulted in DA release that was consistently inhibited by Mg^{2+} (10,8,11-13). However, exogenous glycine failed to potentiate NMDA-evoked release of [^{3}H]DA (18) or endogenous DA (14). Despite this observation, kynurenate, an agent that blocks NMDA responses through competitive inhibition of glycine binding at the strychnine-insensitive glycine site

(19-21) did antagonize NMDA-stimulated [^{3}H]DA release from the slices (18). It is therefore plausible that in the absence of exogenous glycine and kynurenate, a potentiating effect of glycine on NMDA-stimulated [^{3}H]DA release is produced by endogenous glycine, present in the striatal slice at levels sufficient to maximally saturate this glycine binding site.

Dopaminergic transmission may also be modulated by the actions of glycine at an inhibitory (hyperpolarizing) glycine receptor, selectively blocked by 1 to 10 μM strychnine (22,23). Strychnine-sensitive glycine receptors have been demonstrated in brain regions enriched in dopaminergic cell bodies and nerve terminals (24-27) and have been implicated in inhibition of DA neuron firing in the substantia nigra zona compacta (28,29) and the modulation of DA release from striatum (30,10,31, but see 14), substantia nigra (32,9) and ventral tegmentum (33).

We previously reported (16) that NMDA, quisqualate and kainate each evoked Ca^{2+}-dependent release of [^{3}H]DA from dissociated cell cultures of fetal rat mesencephalon. The pattern of antagonist selectivity and ionic sensitivity of release stimulated by NMDA, quisqualate and kainate suggested that the responses were mediated by distinct NMDA and non-NMDA excitatory amino acid (EAA) receptors.

In this study, we tested effects of glycine on spontaneous and EAA-evoked [^{3}H]DA release from mesencephalic cell cultures. We show that either inhibitory or potentiating effects of glycine on NMDA-evoked [^{3}H]DA release may be demonstrated through manipulation of extracellular Mg^{2+} concentrations. We suggest that it is the unique property of the

NMDA receptor to exhibit voltage-dependent Mg^{2+} blockade that allows glycine to produce these opposing effects on the NMDA response.

MATERIALS AND METHODS

Materials

3,4-[8-^{3}H(N)]Dihydroxyphenylethylamine ([^{3}H]DA, 20 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). *N*-methyl-D-aspartate (NMDA), quisqualate, kainate, desipramine hydrochloride and pargyline hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glycine, strychnine phosphate and 7-chlorokynurenate came from Fisher Scientific (Fair Lawn, New Jersey), the New York Quinine Co. (New York, N.Y.) and Research Biochemicals Inc. (Natick, MA), respectively. Other chemicals were reagent grade from regular commercial sources.

Methods

Dissociated cell cultures were prepared from anterior ventral mesencephalic tissue of Sprague-Dawley rat fetuses (gestation day 15), as previously described (34). Cell cultures were grown in 24-well Multiwell plates (fetal tissue from a single dam was divided between 24 to 36 culture wells) in 0.5 ml Dulbecco's modified Eagle's medium, supplemented with 10% v/v fetal calf serum and 20 mM KCl.

After 6-7 days in culture, cells were rinsed with Krebs-Ringer-HEPES buffer (KRH: pH 7.4 composed of (mM): NaCl, 125; KCl, 4.8; HEPES, 25; NaOH, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; D-glucose, 5.6; $CaCl_2$, 2.2; pargyline, 0.1; ascorbate, 1.0.) and loaded with [^{3}H]DA (50 nM, 20 min incubation), in the presence of desipramine (50 μM). After [^{3}H]DA loading, each well was rinsed 4 times with KRH. KRH buffer from a 5 min incubation (spontaneous release period), followed immediately by a 5 min incubation in the presence of an EAA agonist (stimulated release period), were collected, and [^{3}H]DA release was quantitated by measuring radioactivity in each aliquot of buffer collected. In earlier work, more than 95% of the radioactivity detected in buffer collected after spontaneous and glutamate-stimulated release periods was determined by HPLC to be [^{3}H]DA (34). Also, more than 90% of the total cell content of tritiated species, assayed immediately before release collection periods, was found to be [^{3}H]DA. Following release incubations, radioactivity remaining in the cells was extracted by 30 min incubation with acidified ethanol (95% ethanol/5% 0.1 M HCl). When antagonists were tested, cells were exposed to these during the spontaneous release incubation as well as during exposure to the agonist. In experiments performed in the absence of Mg^{2+}, $MgSO_4$ was omitted from the KRH buffer.

For data presentation, spontaneous [^{3}H]DA release (typically 3-5% of total intracellular [^{3}H]DA stores) was subtracted from release stimulated by the EAA agonist. The net release evoked by the agonist was expressed as a % of the total [^{3}H]DA uptake into cells. Reported values are means of at least six determinations (six cell culture wells from fetal tissue of four or more pregnant dams). Statistically significant differences between means were determined by Student's *t*-

test for unpaired observations, or by one-way analysis of variance and post-hoc Newman-Keuls test for multiple comparisons, as appropriate. The accepted level of statistical significance was α=0.05.

RESULTS

Effects of glycine on EAA- and K^+-evoked [^{3}H]DA release in the presence of 1.2 mM Mg^{2+}

As we have previously reported (34,16), NMDA (100 μM) (Fig. 1), quisqualate (10 μM), kainate (100 μM) and K^+ (56 mM) (Table 1) stimulated [^{3}H]DA release from mesencephalic cell cultures. In the presence of 1.2 mM Mg^{2+}, exogenous glycine (30-100 μM) selectively blocked the NMDA response (Fig. 1) without affecting spontaneous [^{3}H]DA release, or [^{3}H]DA release stimulated by quisqualate, kainate, or K^+ (Table 1). Strychnine (0.1 to 1 μM) induced a concentration-dependent blockade of this inhibitory effect of glycine (Fig. 2A).

Control experiments showed that in the absence of exogenous glycine, the NMDA response was unaffected by 1 or 10 μM strychnine, but was blocked by 100 μM strychnine (Table 2). This confirms that 1 μM is an appropriate concentration of strychnine to test strychnine-sensitivity of glycine's inhibitory effect on the NMDA response, as in Fig. 2A. Quisqualate- and kainate-evoked [^{3}H]DA release were unaffected by strychnine (1 μM [data not shown] to 100 μM) (Table 2). K^+-induced [^{3}H]DA release was unaffected by 1 μM strychnine (data not shown), but was partially attenuated by 100 μM strychnine (Table 2).

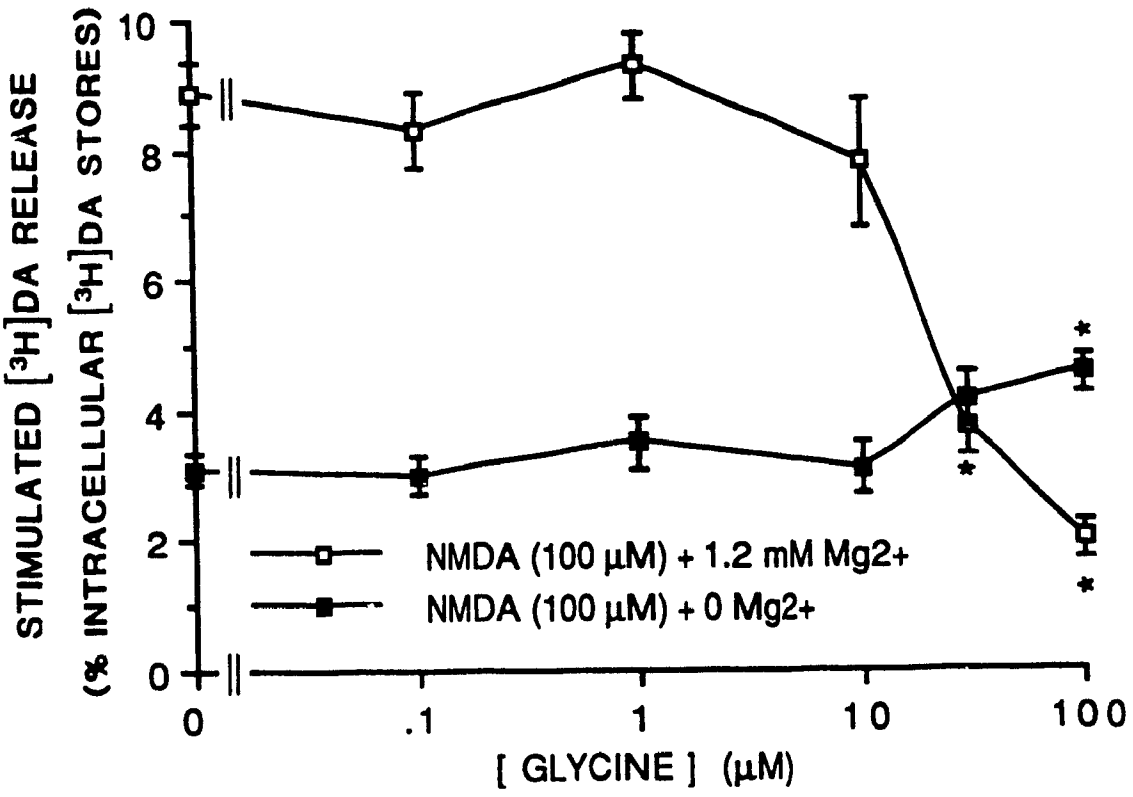

Fig. 1. Effect of glycine on [^{3}H]DA release evoked by NMDA, in the presence of 1.2 mM Mg^{2+} and under nominally Mg^{2+}-free conditions. Cells were exposed to glycine (0.1-100 μM) during the spontaneous incubation and in the presence of NMDA (100 μM). Spontaneous [^{3}H]DA release has been subtracted from [^{3}H]DA release stimulated by NMDA. Results are the means ± SEM from six to twelve cultures.
* *p<.05, compared to control NMDA-evoked [^{3}H]DA release.*

TABLE 1

Effect of glycine on spontaneous [^{3}H]DA release and on [^{3}H]DA release evoked by quisqualate, kainate and K^+

Spontaneous [^{3}H]DA release and release in the presence of glycine, quisqualate, kainate or K^+ were collected in standard KRH, or in KRH containing 100 μM glycine. Spontaneous [^{3}H]DA release has been subtracted from release evoked by the test compound. The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells. Each value is the mean $\pm$ SEM from six to eleven cultures.

Test compounds	Stimulated [^{3}H]DA release (% intracellular [^{3}H]DA stores)
Glycine (100 μM)	-0.15 $\pm$ 0.3[a]
Quisqualate (10 μM)	4.3 $\pm$ 0.2
Quisqualate (10 μM) + glycine (100 μM)	3.8 $\pm$ 0.3[b]
Kainate (100 μM)	11.7 $\pm$ 0.9
Kainate (100 μM) + glycine (100 μM)	11.7 $\pm$ 1.0[b]
K^+ (56 mM)	26.8 $\pm$ 0.9
K^+ (56 mM) + glycine (100 μM)	28.8 $\pm$ 1.6[b]

[a] No different from spontaneous [^{3}H]DA release

[b] No different from [^{3}H]DA release evoked by the test compound in the absence of glycine

Release stimulated by NMDA in the presence of 1.2 mM Mg^{2+} was antagonized by 100 μM 7-chlorokynurenate, a selective antagonist of the glycine binding site associated with the NMDA receptor (35,36) (Fig. 2B). Exogenous glycine reversed the inhibition of NMDA-evoked [^{3}H]DA release by 7-chlorokynurenate, when the effect of 30 to 100 μM glycine was assessed in the presence of 1 μM strychnine (Fig. 2B).

Effect of glycine on NMDA-evoked [^{3}H]DA release in the absence of Mg^{2+}

When tested in the absence of Mg^{2+}, rather than being inhibited by glycine, the NMDA response was potentiated by 30-100 μM glycine (Fig. 1). [As we have previously found (16), removal of Mg^{2+}, by itself reduced the control NMDA response (compare control NMDA-stimulated [^{3}H]DA release in the presence and absence of added Mg^{2+} in Fig. 1)]. Strychnine (1 μM) failed to alter the potentiating effect of 100 μM glycine on NMDA-stimulated [^{3}H]DA release in the absence of Mg^{2+} (Fig. 3A). As observed in the presence of Mg^{2+}, a higher concentration of strychnine (100 μM), when tested by itself, also attenuated NMDA-evoked [^{3}H]DA release in the absence of Mg^{2+} (Fig. 3A).

In the absence of both exogenous Mg^{2+} and strychnine, 10 μM 7-chlorokynurenate attenuated the [^{3}H]DA release evoked by NMDA (Fig. 3B). This inhibition was overcome by 1 μM glycine.

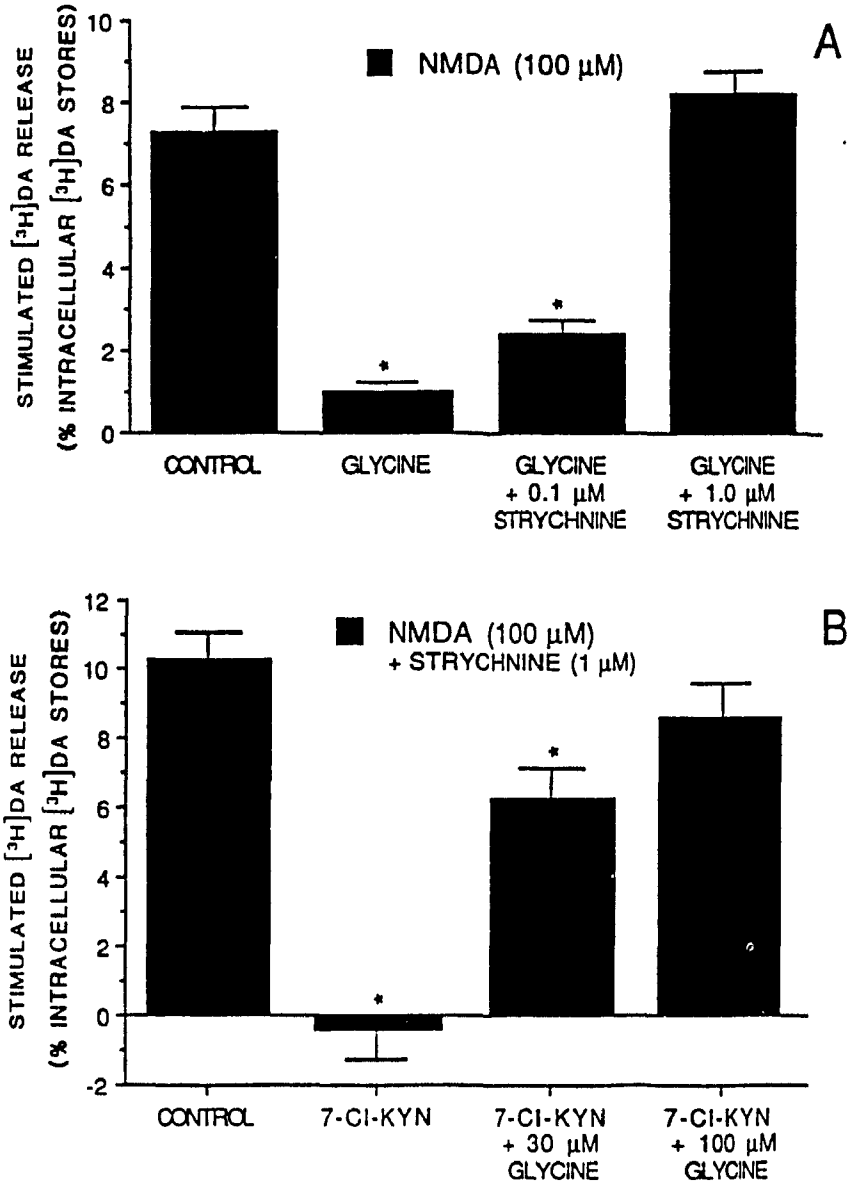

Fig. 2. Effect of glycine +/- strychnine on [^{3}H]DA release evoked by NMDA (A) and effect of 7-chlorokynurenate (7-Cl-KYN) (+ strychnine) on the potentiation by endogenous glycine of the NMDA response (B), in the presence of 1.2 mM Mg^{2+}. To assess the inhibitory effect of glycine on NMDA-evoked [^{3}H]DA release, cells were exposed to glycine (100 µM) +/- strychnine (0.1 or 1 µM) during the spontaneous incubation and in the presence of NMDA (100 µM). To assess competitive inhibitory effects of 7-Cl-KYN on the potentiation of NMDA-evoked [^{3}H]DA release by endogenous glycine, cells were exposed to strychnine (1 µM) and 7-Cl-KYN (100 µM) +/- glycine (30 or 100 µM) during the spontaneous incubation and in the presence of NMDA (100 µM). Spontaneous [^{3}H]DA release has been subtracted from [^{3}H]DA release stimulated by NMDA. Results are the means ± SEM from nine to twenty one cultures.

* *$p<.05$, compared to control NMDA-evoked [^{3}H]DA release.*

TABLE 2

Effect of strychnine on spontaneous [^{3}H]DA release and [^{3}H]DA release evoked by NMDA, quisqualate, kainate and K^+

Spontaneous [^{3}H]DA release and release in the presence of strychnine (1 or 100 μM), NMDA, quisqualate, kainate or K^+ were collected in standard KRH, or in KRH containing the indicated concentration of strychnine. Spontaneous [^{3}H]DA release has been subtracted from release evoked by the test compound. The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells. Each value is the mean ± SEM from six to twelve cultures.

Test compounds	Stimulated [^{3}H]DA release (% intracellular [^{3}H]DA stores)
strychnine (1 μM)	-0.3 ± 0.4[a]
strychnine (100 μM)	1.0 ± 0.5
NMDA (100 μM)	7.5 ± 0.8
NMDA (100 μM) + strychnine (1 μM)	8.0 ± 1.3
NMDA (100 μM) + strychnine (10 μM)	5.8 ± 0.9
NMDA (100 μM) + strychnine (100 μM)	0.8 ± 0.2[a,b]
Quisqualate (10 μM)	4.3 ± 0.2
Quisqualate (10 μM) + strychnine (100 μM)	4.3 ± 0.2
Kainate (100 μM)	11.7 ± 0.9
Kainate (100 μM) + strychnine (100 μM)	10.6 ± 0.7
K^+ (56 mM)	26.4 ± 0.7
K^+ (56 mM) + strychnine (100 μM)	18.9 ± 0.9[b]

[a] No different from spontaneous [^{3}H]DA release.

[b] $p<.05$ versus [^{3}H]DA release stimulated by the test compound in the absence of strychnine.

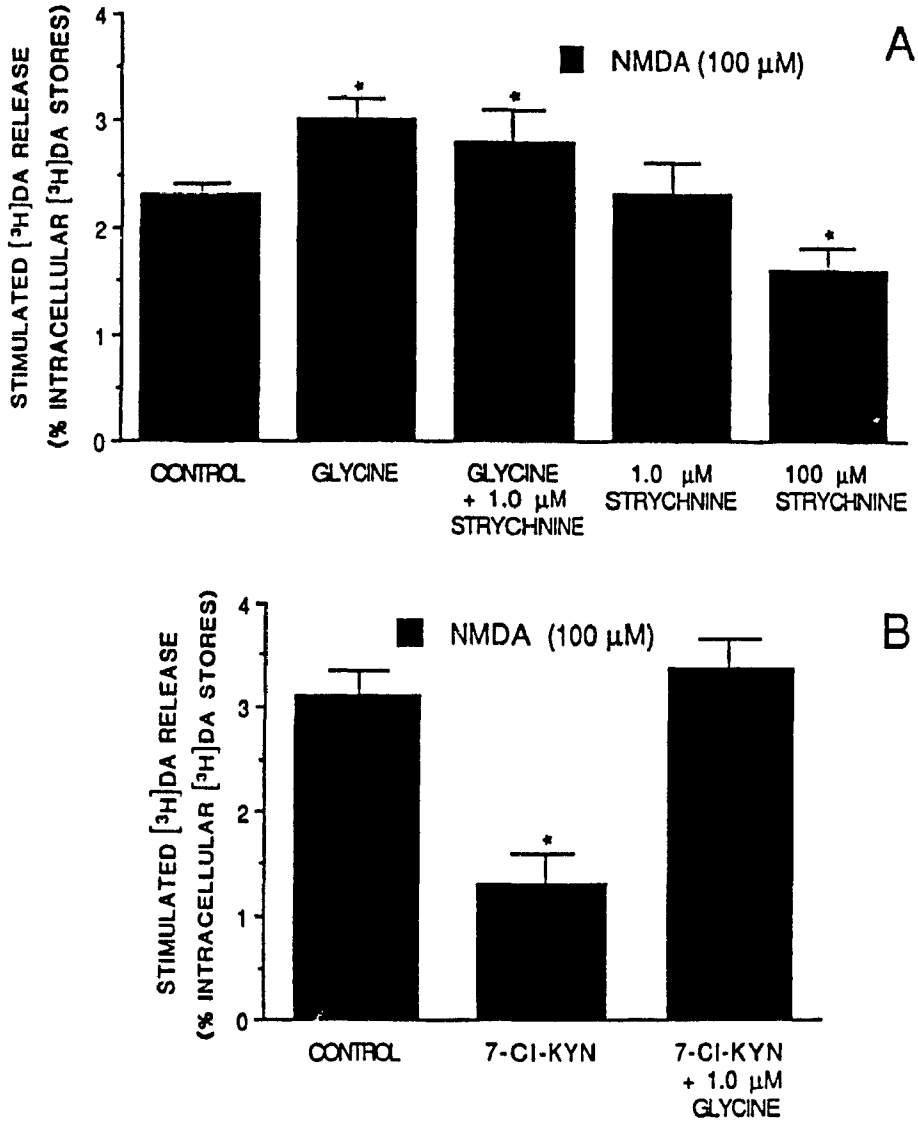

Fig. 3. Effect of glycine +/- strychnine on [^{3}H]DA release evoked by NMDA (A) and effect of 7-chlorokynurenate (7-Cl-KYN) on the potentiation by endogenous glycine of the NMDA response (B), under nominally Mg^{2+}-free conditions. To assess the potentiating effect of glycine on NMDA-evoked [^{3}H]DA release, cells were exposed to glycine (100 μM) +/- strychnine (1 or 100 μM) during the spontaneous incubation and in the presence of NMDA (100 μM). To assess competitive effects of 7-Cl-KYN on the potentiation of NMDA-evoked [^{3}H]DA release by exogenous glycine, cells were exposed to 7-Cl-KYN (10 μM) +/- glycine (1 μM) during the spontaneous incubation and in the presence of NMDA (100 μM). Spontaneous [^{3}H]DA release has been subtracted from [^{3}H]DA release stimulated by NMDA. Results are the means ± SEM from nine to twelve cultures.

* *$p<.05$, compared to control NMDA-evoked [^{3}H]DA release.*

DISCUSSION

Results from this study indicate that two distinct glycine receptors may modulate NMDA-stimulated [^{3}H]DA release from fetal rat mesencephalic cells in culture. Manipulation of extracellular Mg^{2+} permitted the differentiation of a strychnine-sensitive glycine response (inhibition of NMDA-evoked [^{3}H]DA release) from a strychnine-insensitive glycine response (potentiation of NMDA-evoked [^{3}H]DA release). By itself, glycine did not affect spontaneous [^{3}H]DA release.

In the presence of Mg^{2+}, the antagonism of NMDA-stimulated [^{3}H]DA release by 100 μM glycine was probably mediated by the classical inhibitory glycine receptor, as the effect of glycine was blocked in a concentration-dependent manner by low concentrations of strychnine (0.1 or 1 μM). The inhibitory effect of 100 μM glycine was selective for the release evoked by NMDA. It was not observed for quisqualate-, kainate- or K^+-stimulated [^{3}H]DA release. This observation suggests that the NMDA response may be selectively sensitive to hyperpolarizing effects of inhibitory glycine receptor activation on membrane potential. A less likely possibility is that glycine might interact with the NMDA receptor itself to produce the observed inhibition.

To determine which of these mechanisms might explain the selectivity for NMDA responses, effects of glycine were compared in the presence and absence of Mg^{2+}. Extracellular Mg^{2+} was manipulated because activity at the NMDA receptor is characteristically inhibited by low millimolar concentrations of Mg^{2+}, in a voltage-dependent manner (1-4). We have previously shown (16), and confirm in the present study, that under our culture conditions, NMDA-evoked [^{3}H]DA release is not

inhibited by Mg^{2+} (1.2 mM) (but is actually enhanced relative to NMDA-evoked [^{3}H]DA release in the absence of Mg^{2+}). However, when ongoing electrical activity in the cultures is dampened by lidocaine or tetrodotoxin, the NMDA response does become Mg^{2+}-sensitive (16). An explanation for the enhancement of the NMDA response in the presence of 1.2 mM Mg^{2+} (and absence of tetrodotoxin or lidocaine) remains elusive, but speculatively, may be related to the maturational state of cell cultures as described for cortical cell cultures by Frandsen et al. (37) or to protection by Mg^{2+} against receptor desensitization induced by tonic stimulation of NMDA receptors. The critical observation with respect to the present study is that the NMDA receptor modulating [^{3}H]DA release is sensitive to the blocking effect of Mg^{2+}, but that tonic depolarization of the cell cultures overcomes Mg^{2+} blockade of the NMDA response.

When tested in the absence of Mg^{2+}, glycine failed to inhibit the NMDA response. This observation indicates that the inhibition by glycine (in the presence of Mg^{2+}) was probably not mediated by a direct interaction of glycine with the NMDA receptor. Rather, it is consistent with the suggestion that when 1.2 mM Mg^{2+} is present, glycine hyperpolarizes NMDA-responsive neurons through activation of strychnine-sensitive glycine receptors, allowing Mg^{2+} blockade of the NMDA response. It appears that if the mechanism by which Mg^{2+} blocks the NMDA response is eliminated (e.g. by doing the experiment in 0 Mg^{2+}), inhibitory effects of glycine on the NMDA response are lost. In the intact brain, it is possible that convergent depolarizing inputs onto NMDA-responsive neurons may, under certain conditions, sufficiently counteract the hyperpolarizing effects of glycine so that the voltage-

dependent Mg^{2+} blockade, and hence the inhibitory effects of glycine, may be overcome. With alleviation of the Mg^{2+} blockade, the action of glycine at the strychnine-insensitive site would become evident. Thus, in the presence of glycine, the NMDA response may vary from a completely inhibited response to a glycine-potentiated response, depending on the presence or absence of other depolarizing inputs that may modulate the Mg^{2+} blockade.

In the absence of Mg^{2+}, glycine did not inhibit, but enhanced NMDA-evoked [^{3}H]DA release, through a strychnine-insensitive mechanism. This suggests that the NMDA receptor stimulating [^{3}H]DA release is associated with a strychnine-insensitive glycine site, capable of potentiating the NMDA response. Consistent with this notion and as previously reported in a similar experiment that tested the effect of kynurenate on NMDA-stimulated [^{3}H]DA release from striatal slices (18), we observed that 7-chlorokynurenate antagonized NMDA-evoked [^{3}H]DA release, in the absence of exogenous glycine, and that the 7-chlorokynurenate antagonism was reversed by addition of glycine (both in the presence and absence of 1.2 mM Mg^{2+}). It has been proposed that the potentiating action of glycine at the strychnine-insensitive allosteric site on the NMDA receptor may be an absolute requirement for NMDA receptor activation (38,39). Thus, in the absence of exogenous glycine, 7-chlorokynurenate presumably inhibits NMDA-evoked [^{3}H]DA release by antagonizing the action of endogenously released glycine at this site. Both spontaneous and EAA-evoked release of endogenous glycine have been observed from primary cultures of striatal neurons (40). It is conceivable that mesencephalic cells also release glycine at levels sufficient to allow NMDA receptor-mediated [^{3}H]DA release and blockade

of this release by 7-chlorokynurenate. Glycine appears to be a more potent agonist at the NMDA receptor-associated site than at the strychnine-sensitive glycine receptor, in this system. These results suggest that the tonic effect of glycine in this system is to potentiate the NMDA response. Stimulation of glycine release may be required to elicit the strychnine-sensitive effect of glycine-induced inhibition of the NMDA response. This hypothesis may explain the failure of strychnine alone to potentiate NMDA-evoked [^{3}H]DA release in the presence or absence of exogenous Mg^{2+}.

Strychnine by itself inhibited the NMDA-evoked [^{3}H]DA release from cell cultures, at a concentration of strychnine (100 μM) much higher than that needed to block the strychnine-sensitive glycine receptor (1 μM). Araneda and Bustos (9) have recently reported that NMDA-evoked [^{3}H]DA release from substantia nigra slices was both attenuated by 10 μM strychnine and tetrodotoxin-sensitive. They suggested, on this basis, that the DA releasing effect of NMDA in the substantia nigra may be mediated via a trans-synaptic mechanism involving glycinergic neurons. However, in mesencephalic cell cultures, we have shown that NMDA-induced [^{3}H]DA release is not tetrodotoxin-sensitive when tested in the absence of exogenous Mg^{2+} (16). Thus, in the present study, strychnine sensitivity of NMDA-evoked [^{3}H]DA release probably does not indicate involvement of glycinergic interneurons, but may instead be related to the voltage-dependent blockade of NMDA-activated cationic channels produced by higher concentrations (20 to 60 μM) of strychnine (41). The inhibition of K^+-stimulated [^{3}H]DA release by 100 μM strychnine indicates that while high concentrations of strychnine may not interact

with other EAA receptor subtypes, they do not interact selectively with the NMDA receptor.

In summary, the present data indicate that both strychnine-sensitive and strychnine-insensitive glycine receptors are present on rat mesencephalic cells in culture. Strychnine-sensitive glycine receptors inhibit NMDA-stimulated [^{3}H]DA release, while strychnine-insensitive glycine receptors potentiate NMDA-evoked [^{3}H]DA release. It has previously been observed that the ability for Mg^{2+} to produce a voltage-dependent blockade of the NMDA receptor allows the NMDA receptor to act as an "input-sensitive amplifier of excitatory synaptic responses" (i.e. a small depolarizing input may alleviate the Mg^{2+} blockade so as to permit activation of an NMDA response) (42). We suggest that in a system regulated by both inhibitory and potentiating glycine receptors, the dual influence of glycine could allow for even further input-sensitive amplification of the NMDA response.

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8. GENERAL DISCUSSION

Experiments in this thesis demonstrate EAA-regulation of DA release in a dissociated DA cell culture system. Prototypic EAA agonists, NMDA, quisqualate and kainate, as well as the naturally occurring EAAs glutamate, aspartate, HCA, HCSA, CA and CSA stimulated Ca^{2+}-dependent [^{3}H]DA release through actions at multiple EAA receptor subtypes. Pharmacological characteristics of these receptors were found comparable to those observed in other *in vitro* and *in vivo* studies on neural tissue. Differences in the *in vitro* ontogenic profile (see Appendix A) and antagonist-sensitivity of responses to NMDA, quisqualate and kainate favour the conclusion that distinct receptors for each agonist are present.

Aspartate induced [^{3}H]DA release exclusively through its actions at the NMDA receptor. However, glutamate and the sulfur-containing EAAs had mixed agonist effects. Each of these compounds activated NMDA receptors at low EAA concentrations. Non-NMDA receptors were recruited by higher concentrations. The results of this study are entirely consistent with a recent analysis of dose response curves for activation of EAA receptor subtypes on mouse cultured hippocampal neurons by these agonists (Patneau and Mayer 1990).

The observation that [^{3}H]DA release stimulated by each of the prototypic EAA agonists (including NMDA, when tested under nominally Mg^{2+}-free conditions) was TTX-insensitive is strong evidence for a localization of NMDA and non-NMDA receptors directly on the [^{3}H]DA releasing cells. Surprisingly, NMDA-evoked [^{3}H]DA release was TTX-sensitive when the test was made in the presence of 1.2 mM Mg^{2+}.

Together, these findings suggest a role for regenerative Na^+ action potentials in depolarizing the DA-releasing cell to relieve NMDA receptor blockade by Mg^{2+}. They also suggest that a residual presence of Mg^{2+} in tissue slice preparations might explain differences between *in vitro* studies on the TTX-sensitivity of NMDA responses.

A more physiologically relevant example of the role played by Mg^{2+} in regulating the NMDA response was revealed when NMDA-evoked [^{3}H]DA release was tested in the presence of exogenous glycine. Although glycine did not affect spontaneous, quisqualate, kainate or K^+-evoked [^{3}H]DA release, it either inhibited (through a strychnine-sensitive glycine receptor) or potentiated (through allosteric interaction with the NMDA receptor) NMDA-evoked [^{3}H]DA release, depending upon the extracellular Mg^{2+} concentration. The most parsimonious explanation for these data would be that in the presence of 1.2 mM Mg^{2+}, glycine hyperpolarized neurons sufficiently to ensure voltage-dependent blockade of the NMDA receptor by Mg^{2+}. Under nominally Mg^{2+}-free conditions, the NMDA receptor could no longer be blocked by Mg^{2+}, so only the potentiating effect of glycine at the NMDA receptor was observed.

Like other NMDA responses reported in the literature, NMDA-evoked [^{3}H]DA release was inhibited by a low millimolar concentration of Mg^{2+}. However, it required the presence of an agent that blocks action potentials (*e.g.* TTX or lidocaine) to reveal this property. A puzzling aspect of NMDA-evoked [^{3}H]DA release from the mesencephalic cell cultures is that in the absence of TTX, the response measured in the presence of 1.2 mM Mg^{2+} was greater than release in the absence of exogenous Mg^{2+}. The reason for this potentiation of the NMDA response is unclear. Frandsen et al. (1989) have observed that after four days

in vitro, 10 μM glutamate-elicited $^{45}Ca^{2+}$ uptake was enhanced in 0.5 mM Mg^{2+} relative to uptake in 0.1 mM Mg^{2+}, in immature neurons from rat cerebral cortex (Frandsen et al. 1989*a*). They found that Mg^{2+} inhibited NMDA responses in more mature (8 to 14 days *in vitro*) cultured neurons (Drejer et al. 1987; Frandsen et al. 1989*b*). In the mesencephalic cell cultures, no such maturational change in the sensitivity of NMDA responses to the blocking action of Mg^{2+} (in the absence of TTX or lidocaine) has been observed over the first 10 days in culture (unpublished observations). The possibility of subsequent changes in the NMDA response remains to be studied. To address this issue, it will be necessary to adopt long-term culture conditions (see section 8.2.2).

Finally, the present studies demonstrated that high concentrations (≥100 μM) of PCP and related compounds evoked [^{3}H]DA release from cultures by a TTX-sensitive mechanism, independent of actions at the PCP receptor, sigma binding site, dopamine uptake system, Ca^{2+} or Na^+ ion channels. The functional significance of this finding is unclear and an understanding of exactly how PCP and MK-801 stimulate [^{3}H]DA release from cell cultures remains elusive. However, the data in this thesis do not preclude blockade of K^+ channels (causing a delay in repolarization and hence prolongation of the action potential) as a contributing mechanism and the relative potencies of PCP and 4-AP in stimulating [^{3}H]DA release corresponds to their potencies as K^+ channel-blocking drugs. The pharmacological profile of PCP-induced [^{3}H]DA release from the cultures differs from moderate dose effects of PCP and its analogs on DA release *ex vivo* (Rao et al. 1988,1989,1990; Wood and Rao 1989) and on firing rate of A_{10} dopaminergic neurons *in vivo* (French 1989; French and Ceci 1989). PCP-evoked [^{3}H]DA release from cell cultures does not

model these effects in the intact brain. Our results suggest that the latter likely involve polysynaptic mechanisms, rather than direct actions of the compounds on dopaminergic neurons.

Sulzer and Rayport (1989) have also examined the effect of PCP on mesencephalic cell cultures. These authors hypothesized that PCP, amphetamine, and several other weak bases might enter the cell by diffusion and alter the equilibrium between vesicular and nonvesicular DA pools so as to favor its outward diffusion from vesicles. This would increase the pool of free intraneuronal DA accessible to diffusion from the cell. They noted that uptake of monoamine transmitters into synaptic vesicles is driven by an interior acidic pH gradient and they observed neutralization of granule transmembrane proton gradients and time-dependent alkalinization of acidic intracellular compartments in the presence of PCP.

This explanation is not fully satisfactory. Entry of stimulants into the cell by diffusion does cause increased free DA concentrations. However, subsequent diffusion of free intraneuronal DA out of the cell requires operation of the DA uptake carrier (in a reverse direction). Under conditions of carrier blockade, no increase in DA efflux is seen (Fisher and Cho 1979; present study). The high concentrations of PCP studied by Sulzer and Rayport (1989) and in this thesis are much higher than those required to block the DA uptake carrier (*i.e.* IC_{50} 2.4 μM; Boyd and Schwarz 1989). Also [^{3}H]DA release evoked by PCP analog, TCP was not affected by the potent DA uptake inhibitor, GBR 12909. Finally, high concentrations of GBR 12909 and naloxone, though weakly basic, did not share the [^{3}H]DA-releasing property of MK-801 and PCP.

The findings summarized above indicate that EAA-stimulated [^{3}H]DA release provides a functional measure of EAA receptor function as well as an *in vitro* model of EAA-DA interactions. The broader significance of specific results and some planned experiments with the mesencephalic cell culture model are discussed below.

8.1 Relevance of the present work

It is potentially hazardous to extrapolate from results in fetal rat cell culture to the *in vivo* mature brain. It raises further difficulties when an extrapolation is made to the human. However, the findings of the present study, in conjunction with the reports outlined in the introduction to this thesis (section 1.4.5 "Synaptic localization of EAA receptors"), reinforce behavioral indications that EAAs have stimulatory effects on DA systems (section 1.4.3 "Behavioral effects of EAAs on dopamine systems"). It is also likely that several EAA receptor subtypes mediate these effects and that the receptors are located, at least partly, on the DA neurons themselves.

A direct action of EAAs on DA neurons complicates the schematic organization of EAA-DA interactions recently hypothesized by Carlsson and Carlsson (1990). These authors have suggested, mainly on the basis of behavioral and psychotomimetic effects of uncompetitive NMDA receptor antagonists, that effects of EAAs at the level of DA cell bodies and within striatum are mediated indirectly through EAA receptors on GABAergic inhibitory interneurons. They proposed that GABAergic interneurons in the SN inhibit firing of DA neurons, while GABAergic interneurons in the striatum are in turn inhibited by the DA afferents

from SN. According to their model, the net effect of EAA agonists is to decrease DA transmission. They have suggested, partly on the basis of these neural relationships, that EAA agonists could have therapeutic potential in the treatment of schizophrenia (Carlsson and Carlsson 1989a, 1990). The actual mechanistic link (if any) between schizophrenia and disturbances of EAA-DA transmission remains a matter of speculation. However, there do appear to be major problems with a strategy that proposes the use of EAA agonists in schizophrenia. First, Carlsson and Carlsson (1990) argue that DA neurons do not receive EAA innervation (but a substantial body of evidence suggests that they do: see sections 1.4.1 to 1.4.5) and that any EAA receptors which may be present on DA neurons are not tonically stimulated under physiological conditions. Even if the latter assumption were justified, activation of any such extraneous receptors by an exogenous EAA agonist may be expected to depolarize the DA neuron and cause enhanced DA turnover. Second, the ubiquitous distribution of EAA receptors in the CNS makes it difficult to envisage how specific GABAergic neurons would be targeted. Third, a prolonged exposure of neurons to EAAs (or glycine) may be expected to have serious neurotoxic consequences.

The pharmacological profile of PCP-induced DA release *in situ* (*e.g.* Gratton et al. 1987; Deutch et al. 1987; Wood and Rao 1989; Rao et al. 1989, 1990) was not observed in cell cultures. However, at high concentrations ($\geq$100 μM) PCP and MK-801 induced [^{3}H]DA release by a mechanism (most likely blockade of K^+ channels, resulting in a delay of repolarization) that may have toxicological relevance in chronic users. PCP can be remarkably persistent in the body (it has been detected in the urine of chronic users up to 30 days after the last exposure) and

high brain concentrations may be achieved after repeated administrations (see review by Gallant and Mallott 1983). This property would limit the potential of therapy with PCP-related compounds in excitotoxic disorders that might benefit from prolonged EAA antagonist therapy. If K^+ channel blockade is achieved, continued drug exposure might conceivably exacerbate rather than protect against excitotoxic neuronal injury, as has been recently seen with MK-801 (McDonald et al. 1990).

Beyond questions of clinical relevance, the results from this thesis provide insights into fundamental issues concerning the regulation of EAA receptor activation. For example, the dual potentiating and inhibitory influence of glycine on NMDA-evoked [^{3}H]DA release may be important in regulating both synaptic specificity and neuronal plasticity. The voltage-dependent blockade of the NMDA receptor induced by physiological concentrations of Mg^{2+} allows the receptor to act as an "input-sensitive amplifier of excitatory synaptic responses" (Young and Fagg 1990) (*i.e.* a small depolarizing input can be magnified to elicit a large NMDA response through alleviation of the Mg^{2+} blockade). Both input-sensitivity and amplification of the NMDA responses of dopaminergic cells may be further enhanced by a convergent glycinergic innervation of the neuron. Thus, glycine may serve an important role in regulating the specificity of afferent fibre-DA neuron innervation in the ventral mesencephalic area, where neurons in tightly-packed nuclei maintain discrete projections to collectively vast terminal field regions. The presence of inhibitory glycine receptors on DA neurons could reduce the likelihood of inappropriate neuronal firing in response to a depolarizing trigger directed at adjacent neurons (*e.g.* firing induced by diffusion of a neurotransmitter or spreading

depolarization); the hyperpolarization produced by glycine would preserve the Mg^{2+} blockade of the NMDA response. Conversely, in the specifically targeted neuron (that receives sufficient depolarization to alleviate the Mg^{2+} blockade), the action of glycine at the NMDA receptor would serve to boost activation of the neuron through a potentiation of the response to tonically present or released EAA. An NMDA receptor conductance is elicited only when presynaptic activity (resulting in a postsynaptic depolarization) coincides with postsynaptic NMDA receptor activation. Thus, regulation of glycine release may present a locus for the control of activity-dependent neuronal plasticity (*e.g.* in long-term potentiation of synaptic activity, certain types of learning, or synaptic pruning during the development of neuronal circuitry in the neonate [see McDonald and Johnston (1990) for a recent discussion of the role of NMDA receptors in these processes]), particularly in DA neurons.

Finally, the demonstration of [^{3}H]DA-releasing effects of HCSA, CSA and CA suggests that these EAAs, found to be naturally occurring in the ventral mesencephalic region as well as in brain areas innervated by dopaminergic projections, may, like glutamate, aspartate and HCA, be important endogenous regulators of DA turnover *in vivo*. This finding has obvious relevance in the identification of transmitter candidates at excitatory synapses throughout the brain.

8.2 Future perspectives

The mesencephalic cell culture model has been shown useful for examining acute effects of EAAs on dopaminergic transmission and may conceivably be used to study the effects of other putative

neurotransmitters and neuromodulators on DA release and/or EAA receptor function. For example, the characterization of EAA-regulated [^{3}H]DA release undertaken in the present work provided this laboratory with a functional system in which to investigate a possible role for protein kinase C as a modulator of NMDA responses (Chaudieu et al. 1990). Other uses of the mesencephalic cell culture model to study acute and chronic regulation of DA release and/or EAA receptor function are illustrated by the following examples, currently being studied by the author:

8.2.1 Acute modulation of DA release

8.2.1.1 Effects of neuropeptide Y (NPY) and peptide YY (PYY) on spontaneous and EAA-evoked [^{3}H]DA release

Our laboratory is currently using mesencephalic cell cultures to examine potential effects of neuropeptide Y (NPY) and peptide YY (PYY) on spontaneous and EAA-evoked DA release. There are conflicting reports that intrastriatal NPY or PYY injection augments striatal DA turnover (Beal et al. 1989), while intraventricular injection decreases DA turnover in striatum (Vallejo et al. 1987). Others have found stimulatory effects on DA release from hippocampus, but not from striatum, nucleus accumbens or SN (Drumheller et al. in press). In addition, intriguing recent reports suggest that NPY and PYY might be potent endogenous ligands for PCP and sigma binding sites (Roman et al. 1990; Monnett et al. 1990). Our experiments have tested the hypothesis that NPY and PYY have functionally significant effects on DA release through interactions at the PCP site within the NMDA/PCP receptor complex. Preliminary results indicate that NPY does not affect

spontaneous [^{3}H]DA release, but that high concentrations (≥1 μM) of both NPY and PYY attenuate NMDA and kainate receptor activation (Mount et al. 1990). Current efforts are directed at elucidating the specificity of this effect of NPY, through the use of other peptides, including NPY fragments with varying degrees of selectivity for NPY receptor subtypes.

8.2.2 Long-term modulation of DA release

The mesencephalic cell cultures also present an excellent *in vitro* system for investigating the molecular basis of neuronal sensitization and other consequences of chronic drug exposure on the function of DA neurons. However, in order to maintain neurons for a prolonged period of time (up to several weeks) modification of the culture techniques used in the present work is required.

To enhance long-term survival of cultures, it is necessary to limit the proliferation of glia and thereby increase the proportion of DA neurons relative to other cell types. In some studies, cell cultures have been grown in the presence of cytosine arabinoside, an antimitotic and cancer chemotherapeutic drug that competitively inhibits the incorporation of 2'-deoxycytidine into DNA (di Porzio et al. 1980; Barochovsky and Bradford 1985, 1987*a*,*b*). The object of cytosine arabinoside treatment is to selectively kill non-neuronal elements that are still proliferating such as fibroblasts and glial cells, so that they do not overrun cultures. However, cytosine arabinoside may also be toxic to post-mitotic neurons (Oorschot and Jones 1986; Smith and Orr 1987; Wallace and Johnson 1989; Martin et al. 1990). An alternative

antimitotic treatment of 5-flurodeoxyuridine + uridine has the advantage of not killing neurons (Wallace and Johnson 1989).

Another means of reducing glial proliferation is to grow cultures in serum-free conditions. Eliminating serum from the incubating medium demands precise characterization and replacement of the nutrients and trophic factors required for neuronal survival (Dal Toso et al. 1988). While serum-free conditions prolong the survival of neuronal cultures, the inclusion of fetal calf serum has been shown to produce functionally more mature cultures (Ahmed et al. 1983).

8.2.2.1 Sensitization in dopaminergic neurons: The development of a cell culture model for an enduring hyperdopaminergic state

In both rats and humans, repeated exposure to amphetamine has been shown to cause a sensitized behavioral response and progressive increase in DA turnover (measured in rats) in response to a test dose of amphetamine (reviewed by Robinson and Becker 1986). Because of behavioral and neurochemical parallels between behavioral sensitization (also clinically known as amphetamine-psychosis) and paranoid schizophrenia, there is great interest in elucidating the molecular mechanism(s) underlying these persistent functional changes. In addition, other stimuli that directly or indirectly activate nigrostriatal and/or mesocorticolimbic DA systems (*e.g.* neurotensin, opiate agonists and stress) also induce behavioral sensitization (reviewed by Kalivas et al. 1989a) and reports of cross-sensitization between stimuli raises the intriguing possibility of a common mechanism

and of an endogenous trigger for the development of sensitization (Antelman et al. 1980; Kalivas et al 1989*a*).

After appropriate modifications of culture techniques so as to permit the purification and long-term survival (>14 days) of mesencephalic neurons (as described above), it is conceivable that [^{3}H]DA turnover in cultures chronically treated with sensitizing stimuli may provide a simple *in vitro* model of this persistent hyperdopaminergic state. With such a model, the postulated involvement of specific neurochemical mechanisms (*e.g.* changes in DA receptor density, or increased calmodulin-dependent protein kinase activity) could be conveniently examined.

9. SUMMARY OF CONTRIBUTIONS TO THE LITERATURE

In this thesis, mesencephalic cell cultures were used to study EAA receptor-mediated acute regulation of dopaminergic transmission. Previous work with dissociated mesencephalic cell cultures has shown the cells capable of DA synthesis, uptake and release in response to high concentrations of K^+ or veratridine. The experiments reported here contribute to establishing a model of DA neuronal function that might be used to investigate effects of a wide number of transmitters, neuromodulators and second messengers that are suspected to alter dopaminergic transmission under precisely controlled experimental conditions. The present work may also lead to an appropriate model for the neurochemical *in vitro* examination of long-term changes in DA transmission (*e.g.* in behavioral sensitization, a syndrome that resembles certain types of schizophrenia).

The novel findings of this thesis that contribute most to the characterization of EAA-DA interactions in cell cultures and/or to EAA receptor function are listed below.

1. Glutamate-stimulated [^{3}H]DA release from cell cultures was found to resemble glutamate-evoked DA or [^{3}H]DA release *in vivo* or from tissue slices in that it was:
   a. Concentration-dependent.
   b. Ca^{2+}-dependent.
   b. inhibited by an EAA receptor antagonist.
   c. TTX-insensitive.

These studies provide evidence that EAA receptors regulating DA release are present directly on the [^{3}H]DA-accumulating cells. The response was not due to an acute excitotoxic mechanism whereby glutamate exposure might result in the osmotic lysis of these cells.

2. A detailed pharmacological characterization of [^{3}H]DA release evoked by the prototypic EAA receptor agonists (NMDA, quisqualate and kainate) was presented. These studies revealed that,

   a. multiple EAA receptor subtypes mediate the [^{3}H]DA releasing effects of EAAs. The pharmacological characteristics of responses to NMDA, quisqualate, and kainate (shape of concentration-response curves, sensitivities to EAA receptor antagonists, and to manipulations of extracellular ion concentrations) and the ontogenic profile of responses over 12 days *in vitro* (Appendix A) indicated that each of NMDA, quisqualate and kainate induced [^{3}H]DA release by a distinct EAA receptor subtype.

   b. different effects of TTX (or lidocaine) on NMDA-evoked [^{3}H]DA release were obtained in the presence or absence of physiological concentrations of Mg^{2+}. The most parsimonious explanation for these data is that when NMDA-evoked [^{3}H]DA release is tested in the presence of 1.2 mM Mg^{2+}, a regenerative Na^+ action potential is required to depolarize the DA-releasing cell sufficiently so as to overcome Mg^{2+} blockade of the NMDA receptor. TTX blocks the activation of voltage-sensitive Na^+ channels so that Mg^{2+} blockade cannot be relieved and hence, the NMDA response is prevented. In the absence of exogenous Mg^{2+}, the NMDA response was unaffected by TTX, since remaining Mg^{2+} concentrations were not sufficient to block the NMDA receptor. It was suggested that these results may, in part, explain differences between other reports in the literature on the TTX-sensitivity of NMDA responses (see section 1.4.5).

3. This thesis presents characterization of EAA receptor subtype involvement in a functional response (neurotransmitter release) for sulfur-containing EAAs (HCA, HCSA, CA and CSA) over a range of physiologically relevant concentrations. These data have been

presented alongside a comparable characterization of responses to glutamate and aspartate. Specific findings were:

a. concentration-response relationships and Ca^{2+}-dependence of [^{3}H]DA release evoked by HCA, HCSA, CSA and CA (in the case of HCA and CA, effects of single high concentrations had been previously studied in tissue slice experiments).

b. that aspartate stimulated [^{3}H]DA release exclusively through activation of the NMDA receptor. Glutamate, HCA, HCSA, CA, and CSA had mixed agonist effects. Lower concentrations of these EAAs induced responses that were blocked by an NMDA receptor-selective antagonist. Higher concentrations interacted preferentially with non-NMDA receptors.

Results from this study are entirely consistent with a subsequent electrophysiological analysis of receptor subtype involvement in responses evoked by these EAAs (Patneau and Mayer 1990).

4. PCP and related compounds both potently blocked NMDA-evoked [^{3}H]DA release and, at higher concentrations, stimulated DA release, as has been observed in other studies, both *in vitro* and *in vivo*. Studies reported in this thesis examined several hypothesized mechanisms for the PCP-induced enhancement of DA turnover. Blockade of DA reuptake, a proposed mechanism for PCP-enhanced DA turnover in recent work with tissue slices (Buxton et al. 1989), was not a factor in releasing effect seen in the cell culture model. Similarly, PCP failed to affect DA release by an "NMDA-uncoupled PCP receptor" as suggested on the basis of *in vivo* studies of mesocorticolimbic DA turnover (Rao et al. 1989, 1990; Wood and Rao 1990; Wood et al. 1990). It was concluded that high concentrations of PCP-like compounds ($\geq$ 100 μM) may cause TTX-insensitive DA release due to their K^+ channel-blocking actions. Results of the

present work suggest that *in situ* effects of PCP on DA turnover are probably not due to direct effects of PCP on the DA-releasing cells themselves.

5. Glycine either potentiated or inhibited NMDA-stimulated [^{3}H]DA release, depending on the extracellular Mg^{2+} concentration. Specifically,

   a. glycine reduced NMDA-evoked [^{3}H]DA release, through a strychnine-sensitive mechanism, when the test was made in the presence of 1.2. mM Mg^{2+}.

   b. NMDA-evoked [^{3}H]DA release was also reduced by 7-chlorokynurenate, a selective antagonist of the strychnine-insensitive glycine site. This antagonism was overcome by the addition of excess glycine, in the presence of strychnine. This observation suggested that glycine simultaneously has both potentiating and inhibitory effects on DA-releasing cells.

   c. in the absence of exogenous Mg^{2+}, the addition of glycine resulted in potentiation (strychnine-insensitive) of NMDA-evoked [^{3}H]DA release. However, when 1.2 mM Mg^{2+} was present, the inhibitory effect of added glycine predominated.

These data illustrate the functional implications of interactive effects of neurotransmitters and neuromodulators on DA transmission. More particularly, they demonstrate the input-sensitivity of NMDA receptor activation.

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APPENDIX A:

FUNCTIONAL ONTOGENY OF EAA RECEPTORS IN MESENCEPHALIC CELL CULTURES

In experiments of section 4, the pharmacological profiles of [^{3}H]DA release evoked by NMDA (100 μM), quisqualate (10 μM) and kainate (100 μM) were found to differ substantially from one another. Only NMDA-evoked release was sensitive to blockade by the NMDA receptor antagonists CPP, PCP, MK-801 and Mg^{2+} (in the presence of TTX). NMDA-evoked [^{3}H]DA release was also more potently blocked by APV and kynrenate than was the release induced by quisqualate or kainate. Kainate-stimulated [^{3}H]DA release was distinguished from the quisqualate response by a greater sensitivity to antagonism by kynurenate. In addition, GAMS blocked kainate-evoked [^{3}H]DA release but was without effect against both quisqualate and NMDA responses. Finally, under nominally Cl^--free conditions, quisqualate-stimulated [^{3}H]DA release was enhanced while both NMDA and kainate responses were attenuated.

Together, the evidence summarized above suggests that distinct receptors mediate the [^{3}H]DA release induced by NMDA, quisqualate and kainate. However, it remains possible in the case of quisqualate and kainate responses that differences in the relative position of the tested EAA agonist concentrations on their respective concentration-response curves may have contributed to the observed patterns of antagonist selectivity. To further test the notion that NMDA-, quisqualate- and kainate-stimulated [^{3}H]DA release involved three

distinct EAA receptor mechanisms, the *in vitro* development of responsiveness to EAAs was tracked over a 12 day period in culture.

In Fig. 1, different patterns of development are evident for functional responses to NMDA, quisqualate and kainate over days 2-12 in culture. Quisqualate-evoked [^{3}H]DA release was present from days 2-12. Kainate responses appeared early, peaked at 4 days *in vitro* and subsequently declined, while NMDA responses were late appearing (6 days *in vitro*). The development of the response to 100 μM glutamate most closely resembled that of kainate.

These results indicate that responses to EAAs develop as early as day 2 in culture. This finding is consistent with suggestions that EAAs may play a role in the early stages of neural development (see review by McDonald and Johnston 1990). Responses to quisqualate, NMDA and kainate develop on different days in culture. This provides strong support for the notion that separate receptors mediate the actions of these three agonists on [^{3}H]DA release. The developmental profile of glutamate-evoked [^{3}H]DA release suggests that this concentration of glutamate may interact predominantly with the kainate receptor. The decline of EAA-evoked [^{3}H]DA release after 10 days in culture likely reflects the glial proliferation and drop in neuronal survival, reported in studies of DA cells grown under similar culture conditions (Barochovsky and Bradford 1987). Glial cells are capable of high affinity [^{3}H]DA uptake (Pelton et al. 1981, Barochovsky and Bradford 1987) but do not release accumulated [^{3}H]DA in response to depolarizing stimuli (Barochovsky and Bradford 1987).

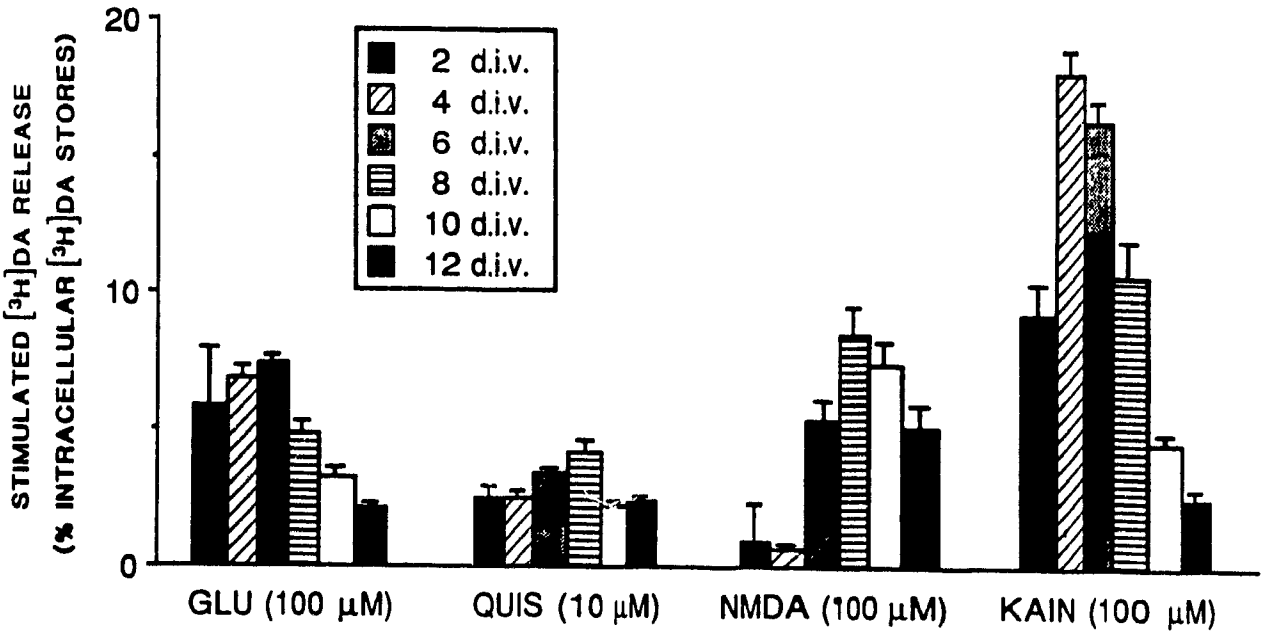

Fig. 1 Ontogenic development of [^{3}H]DA release stimulated by 100 μM NMDA, 10 μM quisqualate, 100 μM kainate and 100 μM glutamate, over 12 days in culture. Results are means from 10-20 cultures.

APPENDIX B:

LIST OF ABBREVIATIONS

ACPD	trans-1-aminocyclopentane-1,3-dicarboxylate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
α-mpt	α-methyl-*p*-tyrosine
4-AP	4-aminopyridine
APB	2-amino-4-phosphonobutyrate
APV	D,L-2-amino-5-phosphonovalerate
APH	D,L-2-amino-7-phosphonoheptanoate
Asp	L-aspartate (in section 5 only)
ATP	adenosine triphosphate
B_{max}	maximum number of ligand binding sites
CA	L-cysteate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
COMT	catechol-*O*-methyl transferase
CPP	3-(2-carboxypiperazine-4-yl)propyl-1-phosphonate
CSA	L-cysteine sulfinate
DA	dihydroxyphenylethylamine (dopamine)
DMEM	Dulbecco's modified Eagle's medium
DNQX	6,7-dinitroquinoxalinedione
DOPAC	3,4-dihydroxyphenylacetic acid
DTG	1,3-di(2-tolyl)guanidine
EAA	excitatory amino acid
GABA	γ-aminobutyric acid
GAMS	γ-D-glutamylaminomethyl-sulfonic acid

GBR 12909	1-[2-[*Bis*(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl] piperazine dihydorchloride
GDEE	glutamate diethyl ester
Glu	L-glutamate (in section 5 only)
HA-966	3-amino-1-hydroxy-2-pyrrolidone
HCA	L-homocysteate
HCSA	L-homocysteine sulfinate
HPLC	high performance liquid chromatography
5-HT	serotonin
HVA	homovanillic acid
IP_3	1,4,5-trisphosphate
KAIN	kainate (used in section 4 only)
K_m	Michaelis-Menten constant
KRH	Krebs-Ringer-HEPES buffer
KYN	kynurenic acid (also 4-hydroxyquinoline-2-carboxylic acid)(in section 4 only)
LAAD	L-aromatic amino acid decarboxylase
LDH	lactate dehydrogenase
L-DOPA	L-dihydroxyphenylacetic acid
MAO	monoamine oxidase
MK-801	(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (also dizocilpine)
MPTP	1-methyl-4-phenylpyridinium
3-MT	3-methoxytyramine
NA	noradrenaline (in section 3 only)
NMDA	*N*-methyl-D-aspartate
NPY	neuropeptide Y
6-OHDA	6-hydroxydopamine

PCP	phencyclidine
PDA	*cis*-2,3-piperidine dicarboxylic acid (also *cis*-PDA in section 3)
PK 26124	2-amino-6-trifluoromethoxybenzothiazole
PYY	peptide YY
QUIS	quisqualate (used in section 4 only)
SEM	standard error of the mean
SKF 10,047	*N*-allylnormetazocine
SN	substantia nigra (in section 1 only)
TCP	*N*-[1-(2-thienyl)-cyclohexyl]piperidine
TH	tyrosine hydroxylase
TTX	tetrodotoxin
V_{max}	maximum rate of enzyme activity
VTA	ventral tegmental area

APPENDIX C:

LETTERS OF PERMISSION TO REPRODUCE PUBLISHED MATERIAL