IN VIVO STIMULUS-INDUCED RELEASE OF BRAIN CATECHOLAMINES

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ABSTRACT

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By

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There is extensive evidence to indicate that the catecholamines, dopamine and norepinephrine, serve a neurotransmitter function in the central nervous system. To function as a neurotransmitter, a substance must be released upon nerve depolarization. Therefore, before assigning neurotransmitter roles to these catecholamines, it is necessary to demonstrate their release upon appropriate stimulation. It is the purpose of this study to describe a method for monitoring the release of catecholamines from the central nervous system during depolarizing electrical stimulation.

Throughout the course of these experiments intraventricular injections of H^3-catecholamines (5 uc) were utilized to label the catecholamine stores in the striatum and hypothalamus of spinal cats. After 1 hour artificial cerebrospinal fluid was infused into the lateral ventricle and collected from a cannula placed in the cerebral aqueduct. After 2 hours of washout, 1 ml perfusates were collected and either the caudate nucleus or substantia nigra pars compacta were stimulated electrically. The perfusates were analyzed for their content of H^3-catecholamines and H^3-metabolites by alumina adsorption chromatography and ion exchange chromatography. These compounds were then

quantified by liquid scintillation spectrometry.

Consistent increases in ventricular perfusate concentrations of H^3-catecholamines were observed when the caudate nucleus was stimulated. The metabolites of H^3-catecholamines were present in the perfusate, but they did not increase in concentration during or following direct caudate stimulation. By comparing the effect of stimulation at various frequencies (12.5, 25, 50 and 100 hz) it was determined that 50 hz stimulation provided the greatest increase in H^3-norepinephrine release. Caudate nucleus stimulation was, however, ineffective in increasing the release of C^{14}-urea. The results of these studies demonstrated that direct electrical stimulation of the caudate nucleus causes the release of putative neurotransmitters (catecholamines) but not of a non-transmitter substance (urea). It would appear, therefore, that the release of H^3-catecholamines is specifically related to the depolarization of nerve terminals. The failure of the efflux of O-methylated-metabolites to increase upon direct caudate stimulation suggests that H^3-catecholamines may not be released exclusively from dopaminergic neurons.

Stimulation of the substantia nigra pars compacta increased the release of H^3-dopamine and H^3-3-methoxy-tyramine. Examination of the frequency-response relationships of this release revealed that the greatest release was evoked by 30 hz. An intensity-response curve indicated

that increasing the peak current of stimulation up to 400 uA caused a progressive increase in the release of H^3-dopamine. These results suggest that stimulation of the substantia nigra pars compacta causes the release of H^3-catecholamines from specific dopaminergic neurons in the caudate nucleus since the efflux of O-methylated-metabolites also increases with stimulation.

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Philip Friedrich Von Voigtlander

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to my parents

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INTRODUCTION

There is increasing evidence to indicate that dopamine as well as norepinephrine may serve as neurotransmitters in the central nervous system. This evidence is based upon: the regional and subcellular localization of dopamine and norepinephrine, the presence of specific amine transport systems and of enzymes for the synthesis and the metabolism of catecholamines, the ability of these compounds to alter the firing of neurons when applied microelectrophoretically, and the release of catecholamines upon appropriate electrical stimulation. It is the purpose of this thesis to describe a method for detecting the *in vivo* release of catecholamines from the central nervous system upon electrical stimulation. The results will be discussed in reference to the functional significance of dopamine in the central nervous system.

1. Localization of brain catecholamines

The regional localization of norepinephrine was first studied by Vogt (1954). By carefully dissecting various nuclei and white matter tracts, separating the norepinephrine of these areas by paper chromatography, and quantifying the norepinephrine by biological assay, she found that, contrary to previous thought, the localization

of norepinephrine was not strictly related to the vascularity of the specific brain areas but rather was concentrated in the brainstem. These results suggested that norepinephrine could have functions within the brain proper as well as being involved as a postganglionic transmitter in the cerebral vasculature. Somewhat later dopamine was identified in the brains of several mammalian species by utilizing a fluorometric assay (Carlsson, 1959). Dissection studies revealed dopamine to be concentrated in the striatum. More recently, Carr and Moore (1969[a]) have mapped the endogenous catecholamine concentrations of various periventricular areas of the cat brain. Their results, agreeing with those of Vogt and Carlsson, demonstrated that dopamine is concentrated in the caudate nucleus and norepinephrine in the hypothalamus.

Subcellular distribution studies lend support to the idea of the intracellular localization of catecholamines. Synaptosomes (pinched-off nerve endings) can be prepared by gentle homogenization of brain tissue in isotonic sucrose. The bulbar nerve endings disassociate from the slender axons and reheal in the isotonic solution with little loss of their contents. These synaptosomes may then be separated by continuous density gradient sucrose centrifugation. Comparison of the catecholamine content of the synaptosomes to that of the supernatant indicates that these amines are intracellular in location. (Green et al.,1969)

Within the synaptosomes, catecholamines appear to be located in storage granules or vesicles. Maynert et al. (1964) demonstrated that norepinephrine-containing vesicles could be separated from disrupted synaptosomes; they appear similar to those seen by electron microscopy in presynaptic terminals of brain sections. Hornykiewicz (1966[a]), however, cites evidence that the subcellular localization of dopamine is mostly cytoplasmic. In contrast, Gutman and Weil-Malherbe (1967) suggested that the apparent cytoplasmic localization of dopamine may be an artifact and that within a given region of the brain the subcellular localization of dopamine and norepinephrine are the same. They suggest that the low vesicular dopamine to cytoplasmic dopamine ratios characteristic of the striatum may be related to the fine dopaminergic terminals of the striatum; these terminals contain fragile vesicles that rupture during isolation and release their contents into the supernatant. Likewise, recent experiments (Philippu and Heyd, 1970) utilizing striatal subcellular vesicles, indicate that 46% of the dopamine of this region is contained within the vesicles.

2. Transport of brain catecholamines

The presence of specific amine transport systems in the brain has been demonstrated using brain slices, homogenates and subcellular fractions. Tissari et al. (1969) observed an active accumulation of norepinephrine

into synaptosomes. Of particular interest was the inhibition by ouabain of this accumulation of norepinephrine after a five minute lag period. The authors suggest that the lag period represents the time necessary for the dissolution of the transmembranal Na^+ gradient. They further suggest that amine transport may be coupled to the Na^+K^+ATPase pump by the Na^+ gradient. In this way, the transport of the catecholamines would be similar to that of glycine (Vidaver, 1968).

The result of the specific amine transport systems, namely the uptake of exogenous catecholamines into areas of the brain with high endogenous catecholamine concentrations, has been studied from several points of view. Snyder et al. (1969) have demonstrated that blocks of monkey brain tissue accumulate exogenous norepinephrine at rates proportional to their endogenous concentrations. Likewise, in the cat, Carr and Moore (1969[a]) have shown that intraventricularly administered norepinephrine is accumulated into nuclear areas of high endogenous norepinephrine concentrations but not into nuclear areas of low concentrations or into white matter. The autoradiographic study of Schubert and Labisich (1969) further confirms the previous studies; these workers injected H^3-norepinephrine intracisternally in rats and observed accumulations of H^3 throughout the ventricular system and the subarachnoid space, but with much greater concentrations in the catecholaminergic areas of the hypothalamus

and striatum. Fuxe and Ungerstedt (1968), in a histochemical study, localized the accumulation of intraventricularly administered norepinephrine and dopamine to cell bodies and terminals of catecholaminergic areas within 200-400 microns of the ventricular surface. The accumulation of exogenous norepinephrine has been localized subcellularly by Green et al. (1969). Following the in vivo administration of H^3-norepinephrine, these workers separated synaptosomes from various brain regions and demonstrated a coincidence of the peak H^3-norepinephrine band with the peak endogenous norepinephrine band. They concluded that exogenously administered norepinephrine is localized in noradrenergic synaptosomes.

3. Enzymes involved in catecholamine synthesis and metabolism

Enzymes for the synthesis of norepinephrine and dopamine have been identified in the brain; specifically the tyrosine hydroxylase and dopa decarboxylase activity of the striatum has been studied before and after lesions of dopaminergic neurons supplying this area (Goldstein et al., 1970). Lesions caused a decrease in the activity of both enzymes, suggesting that both are intraneuronal and dependent on the integrity of the neuron. Furthermore, monoamine oxidase and catechol-O-methyltransferase are present in the brain (Anden et al., 1969). The products of norepinephrine and dopamine metabolism by these enzymes have been widely studied. Carr and Moore (1969^a) have

shown that after intraventricular administration of H^3-norepinephrine in the cat, the primary metabolite in the hypothalamus and caudate nucleus is normetanephrine, the catechol-O-methyltransferase product. However, in the rat, the neutral deaminated metabolites predominate after H^3-norepinephrine (Glowinski et al., 1965) and H^3-dopamine administration (Taylor, 1969). Taylor also demonstrated that four to six hours after intraventricular H^3-dopamine only 10% of the striatal H^3 was H^3-norepinephrine. Likewise, Glowinski and Iversen (1966) were unable to find significant amounts of H^3-norepinephrine in the striatum after H^3-dopamine administration. This result suggests that dopamine-β-hydroxylase activity in the striatum is low.

4. Activity of microelectrophoretically applied catecholamines

The ability of catecholamines to directly alter nerve activity has been demonstrated by microelectrophoresis. This technique involves the placement of a microelectrode loaded with the substance to be tested extracellularly next to a neuron. A second barrel of the microelectrode is used to record the activity of the neuron as minute amounts of current are passed through the first electrode, carrying and depositing a small amount of the compound on the neuron. With such an apparatus Bloom et al.(1965) were able to demonstrate inhibitory as well as excitatory actions of norepinephrine

and dopamine on caudate neurons. Salmoiraghi (1966) observed that all brain areas tested showed some neurons that reacted to norepinephrine. McLennan (1967) further studied the action of microelectrophoretically administered dopamine on caudate neurons; he observed that dopamine inhibited firing of 60% of the tested neurons and demonstrated that phenoxybenzamine, but not dichloroisoproteronal, blocked this inhibition. Unfortunately, investigators cannot always identify precisely the neuronal regions under study so that results of experiments with this technique are not necessarily indicative of postsynaptic faciliation or inhibition by the putative neurotransmitter (Curtis and Crawford, 1969). Results obtained with this technique do not appear to be very specific since several amino acids that don't fulfill other criteria as transmitters may also act in an excitatory or inhibitory manner when applied microelectrophoretically (Curtis and Watkins, 1965).

5. Evidence for a dopaminergic pathway

Careful anatomical studies delineating catecholaminergic pathways in the central nervous system have provided additional evidence for the functional role of catecholamines. Anden et al. (1964) were able, by fluorescence microscopy, to trace a tract of dopamine-containing fibers from the substantia nigra pars compacta to the neostriatum (caudate nucleus and putamen). This tract passed through the cerebral peduncle and the internal capsule.

The cell bodies giving rise to these fibers were specifically localized in the substantia nigra pars compacta, the pars lateralis and the pars reticularis of the substantia nigra contained few dopaminergic cell bodies. As an extension of this work, Hökfelt and Ungerstedt (1969) demonstrated by histofluorescence that lesions to the nigro-neostriatal neurons caused a loss of amine accumulating ability in the neostriatum on the ipsilateral side. These changes were concurrent with ultrastructural degeneration of the terminals as observed by electron-microscopy. On the other hand, earlier workers (Afifi and Kaelber, 1965) were unable to demonstrate degeneration in the caudate nucleus after substantia nigral lesions and Nauta staining for degenerating fibers. Their negative results could be due to the small diameter of the fibers involved (Adinolfi, 1967) or to the strict topographical relationships between areas of the substantia nigra and the striatum. Indeed, Afifi and Kaelber may not have been lesioning the proper area of the substantia nigra. Likewise, Adinolfi's (1967) failure to observe more than slight astrocyte infiltration of the caudate after substantia nigral lesions may have been due to failure to lesion the substantia nigra pars compacta.

Additional lesion studies have, however, confirmed Hökfelt and Ungerstedt's results. Poirier and Sourkes (1965) demonstrated that lesions of the nigrostriatal fibers depleted norepinephrine and dopamine from the

ipsilateral striatum and also decreased the tyrosine hydroxylase activity in that area. Likewise, Goldstein et al. (1970) demonstrated that unilateral substantia nigral lesions in the monkey caused a decrease in endogenous dopamine, a decrease in ability to accumulate dopamine, a decrease in tyrosine hydroxylase activity, and a decreased dopa decarboxylase activity in the ipsilateral caudate. Similar results were obtained by Faull and Laverty (1969), although these authors were unable to alter striatal dopamine levels by lesions of the nucleus ventralis lateralis of the thalamus. They concluded that the nigro-striatal fibers were dopaminergic whereas the fibers from the nucleus ventralis lateralis of the thalamus to the striatum were not.

Retrograde degeneration studies (Bedard et al., 1969) have further supported the idea of a nigro-striatal pathway and have localized the substantia nigra pars compacta cell bodies that supply various striatal areas. These workers demonstrated that lesions in the striatum caused selective cell loss in the ipsilateral substantia nigra pars compacta. They confirmed that the effect was retrograde degeneration rather than a transneuronal effect by lesioning the strio-nigral fibers. Lesions of these fiber tracts produced no substantia nigral degeneration; therefore, they concluded that substantia nigral cell body degeneration seen after striatal lesions must be retrograde. Furthermore, these authors demonstrated a

strict topographical relationship between areas in the striatum and areas in the substantia nigra pars compacta; lesions of the putamen in the cat caused degeneration of cell bodies in the lateral-caudal substantia nigra pars compacta, whereas, lesions of the caudate nucleus caused degeneration in the rostral-medial substantia nigra pars compacta. By means of extensive striatal lesions, Bedard et al. (1969) were also able to demonstrate that most of the substantia nigra pars compacta neurons degenerated; hence, most project to the striatum.

In addition to extensive biochemical and histological evidence, electrophysiological studies suggest the existence of a nigro-striatal pathway. Connor (1968) recorded the responses of single units in the caudate following substantia nigra pars compacta stimulation. He observed that 44% of the units tested were depressed by substantia nigra pars compacta stimulation, whereas 14% were facilitated. This study and the microelectrophoretic studies suggest an inhibitory function for the dopaminergic nigro-striatal fibers.

6. Function and dysfunction of the striatum and substantia nigra

The function of the nigro-striatal pathway, or for that matter of the caudate nucleus, is obscure. It seems to function in the regulation and inhibition of movement; caudate stimulation inhibits stretch reflexes (Eyzaguirre, 1969). Other inhibitory properties have been attributed to the

caudate. Amato et al.(1969) observed that caudate stimulation blocks cerebral cortical seizure discharges induced by pentamethylenetetrazol or picrotoxin. Likewise, Mutani and Fariello (1969) showed that caudate stimulation could block seizure discharges from a focus in the cruciate cortex. Behavioral inhibitory responses to caudate stimulation have also been observed (Ursin et al., 1969). These authors describe a cessation of ongoing movement, searching eye movements and alerting upon caudate stimulation in freely-moving cats. Repeated stimulation caused habituation of this response. Presentation of a novel sound dishabituated this orienting response, implying that the caudate may be involved in an animal's response to a novel environment. Other behavioral observations relating to the function of the nigro-striatal pathway are provided by Goldstein et al. (1970). Monkeys with substantia nigral lesions exhibited fine rapid tremors of the contralateral limbs.

The experimental observations of Goldstein et al.(1970) are of particular interest in light of recent findings relating to Parkinsonism in man. Hornykiewicz (1966[a]) observed that Parkinsonism, a chronic neurological disorder involving muscular rigidity and resting tremor, is accompanied by a gross depletion of dopamine from the basal ganglia. He also points out that degeneration of the substantia nigra is often associated with this condition. Hornykiewicz (1966[b]) further suggested there is a causal relationship

between depressed basal ganglia dopamine concentrations and the symptoms of Parkinsonism. That is, degeneration in the substantia nigra and basal ganglia results in the loss of dopamine which may be involved in the inhibitory extrapyramidal control of movement. Loss of this inhibitory control is then responsible for the motor dysfunctions associated with the disease.

Other workers invoke a cholinergic mechanism of Parkinsonism. Duvoisin (1967) observed that physostigmine, a cholinesterase inhibitor, exacerbated Parkinsonian tremor. This tremor could be quickly reversed by the anti-Parkinsonian drug, benztropine, which has cholinergic blocking actions. He deduced that Parkinsonism involved either an overabundance of acetylcholine in some brain region, or an inbalance between acetylcholine and dopamine. Indeed, the belladonna alkaloids have been used to treat Parkinsonism for years (Goodman and Gilman, 1965). Other investigators (Farguharson and Johnston, 1959), however, demonstrated an apparent lack of correlation between the antitremor effect and anticholinergic, antihistaminic, and local anesthetic properties of several anti-Parkinsonian compounds. These results suggest that some other property of these drugs must be responsible for their efficacy in treating Parkinsonism. In this regard, Coyle and Snyder (1969) recently demonstrated that low concentrations of several anti-Parkinsonian drugs (benztropine, diphenhydramine, and diethazine) noncompetitively inhibit

the uptake of dopamine into striatal synaptosomes. Whether this effect also occurs *in vivo* and is the mechanism of the therapeutic effects of these drugs remains to be seen. If this is the mechanism of action of these drugs in Parkinsonism it would provide further support for the dopaminergic theory; a blockage of dopamine uptake into the presynaptic terminal would increase the effective concentration of dopamine in the striatum thereby counteracting the depletion of dopamine, which Hornykiewicz feels is responsible for the symptoms.

Recent developments in Parkinsonian therapy further support the dopaminergic theory for the etiology of this disease. L-Dopa, the precursor of dopamine, has been shown to be effective in the relief of many of the symptoms associated with Parkinsonism (Cotzias *et al.*, 1969; Klawans and Erlich, 1969). The administration of L-dopa increases the brain concentration of dopamine but not norepinephrine (Everett, 1970); therefore, it seems likely that the therapeutic effects of this amino acid are related to dopamine concentrations. Sourkes (1970), however, points out that another active metabolite of L-dopa, such as its O-methylated product, may be responsible for the effects of the compound. At the present time the dopamine replacement theory of the effect of L-dopa seems tenable and agrees with the dopaminergic theory of Parkinsonism.

Attempts have been made to develop an animal model for Parkinsonism. In the past tremorine has been used to

screen for the antitremor activity of various anti- Parkinsonian compounds (George et al., 1962; Ahmed and Marshall, 1962). This system, however, is nonspecific and open to considerable criticism (Duvoisin, 1967). Neff et al. (1969) have suggested a different model based upon the toxicity of manganese. The administration of MnO_2 to monkeys for several months produced muscular rigidity, tremors and flexion posturing coincident with a depression of caudate concentrations of dopamine and 5-hydroxytryptamine. However, the animals under study were moribund; this obviously limits the usefulness of this system for studying Parkinsonism.

What is necessary at this point is an animal model which tests some of the assumptions of the dopaminergic theory of Parkinsonism. Is dopamine released upon depolarization of terminals in the striatum? If it is, is it a specific neuron mediated effect? Is the nigro-striatal dopaminergic pathway involved in this release? Such a model might be useful to study the effects of established and potential anti-Parkinsonian drugs.

7. Release of catecholamines from the brain

In the past a variety of drugs have been used to release putative neurotransmitters, including dopamine, from the brain. Philippu and Heyd (1970) demonstrated a temperature-dependent release of dopamine from striatal subcellular particles which is enhanced by Ca^{++}. Acetylcholine, however, failed to affect this release. Using

tissue slices, Besson et al. (1969) were able to increase the release of exogenous H^3-dopamine by the addition of d-amphetamine or certain monoamine oxidase inhibitors. McKenzie and Szerb (1968) perfused high doses of d-amphetamine directly into the caudate nucleus and observed barely detectable amounts of dopamine in the perfusate. Utilizing the technique of ventricular perfusion, Carr and Moore (1969^b) demonstrated that the efflux of H^3-norepinephrine and H^3-dopamine could be significantly increased by d-amphetamine.

Extensive use has also been made of in vitro systems to study the evoked release of putative neurotransmitters. Baldessarini and Kopin (1966) demonstrated that by using high current electrical field stimulation of brain slices, they could increase the release of H^3-norepinephrine into the bathing media. Later, Katz and Kopin (1969) showed that the release of norepinephrine was dependent on Ca^{++} and blocked by Li^{++}. They also demonstrated a block of norepinephrine uptake by ouabain. Recently, McIlwain and Snyder (1970) observed the uptake of H^3-norepinephrine, H^3-5-hydroxytryptamine and H^3-glycine into brain tissue slices. However, only H^3-norepinephrine and H^3-5-hydroxytryptamine could be released by field stimulation. These investigators also studied the effects that field stimulation had upon the lactate and K^+ concentrations of the tissue. Tissue lactate was found to be increased and K^+ concentrations decreased by stimulation. McIlwain (1966) earlier reported that field stimulation of brain greatly increased the O_2 uptake of the tissue. The cause and effect relationship

of these metabolic alterations with the release of norepinephrine have not been elucidated. It is not known if the norepinephrine effluxes from the slice as a consequence of a nonspecific shift in metabolism and/or membrane function, or whether these metabolic effects are indicative of the normal physiological sequence of events leading to the release of norepinephrine. Further doubt as to the specificity of the field stimulation technique was indicated by the results of Katz *et al.* (1969) who demonstrated that field stimulation released several amino acids (lysine, cyloleucine and leucine). Since these amino acids lack neuroactivity (Curtis and Watkins, 1965) and do not have the characteristic subcellular distribution of neurotransmitters, their release from this *in vitro* system suggests a nonspecific efflux of intracellular or membrane-bound substance.

In *in vivo* studies, attempts have been made to equate alterations in steady state levels of amines with nervous activity. For example, 3 hours of direct electrical stimulation of the amygdaloid nucleus depleted the adrenal and brainstem of norepinephrine (Gunne and Reis, 1963) and reduced norepinephrine fluorescence in forebrain terminals (Fuxe and Gunne, 1964). These results suggest that nerve stimulation releases norepinephrine but, in fact, they may indicate only an alteration in norepinephrine turnover.

In vivo experiments have also been devised to monitor the efflux of transmitter from the brain. The push-pull

cannula technique has been utilized in an attempt to study the release of dopamine from the caudate nucleus (McLennan, 1964). This perfusion method utilizes a large and a small hypodermic needle fitted together concentrically such that the smaller needle protrudes slightly. This assembly is implanted into the brain region to be studied. The perfusion fluid is forced into the small center needle, forming an artificial space in the tissue, and then collected from the larger cannula and analyzed. McLennan reported an increase in dopamine release from the caudate when the nucleus centromedianus of the thalamus, but not when the substantia nigra or the caudate nucleus were stimulated electrically. In subsequent experiments, McLennan (1965) found that stimulation of substantia nigra increased dopamine outflow from the putamen. Since other investigators (McKenzie and Szerb, 1968) were unable to detect any baseline efflux of dopamine from a push-pull cannula in the caudate, McLennan may not have been measuring dopamine in the perfusates. Stein and Wise (1969) combined the use of a push-pull cannula in the hypothalamus and amygala, intraventricular injections of H^3-norepinephrine, and median forebrain bundle electrical stimulation in rats. In 50% of the rats, up to $1\frac{1}{2}$ hours of stimulation increased the H^3 efflux, most of which consisted of deaminated O-methylated products. The authors attempted to correlate the rewarding value of the stimulation and the amount of catecholamine metabolites effluxing into the the cannula.

However, the magnitude of the cannula-induced brain lesion made such correlations irrelevent. Roth et al. (1969) have also attempted to utilize the push-pull cannula. They were unable to demonstrate an efflux of labeled compounds from the monkey caudate after the termination of an infusion of C^{14}-dopa. Likewise, during an hour of electrical stimulation, using the cannula as a monopolar electrode, no labeled compounds appeared in the perfusate. Once again the lack of positive results may be the result of the damage created by the cannula.

Despite recent modification in push-pull cannula techniques (Myers, 1970), the basic fact remains that the cannula damages the tissue which are being perfused. This damage is sufficient in most cases to make the results questionable (Bloom and Giarman, 1968; Vogt, 1969). In fact, Schubert and Labisich (1969) produced autoradiographic evidence that lesions, which resemble those caused by a push-pull cannula, alter the binding of H^3-norepinephrine to the tissue. This nonspecific binding by traumatized tissue could cause artifactual results. Indeed, Chase and Kopin (1968) demonstrated that simultaneous with the evoked efflux of norepinephrine from the olfactory bulb of the rat there was an efflux of other non-transmitter substances such an inulin and urea into the cannula. The push-pull cannula system, therefore, seems to have serious limitations for studying the in vivo release of neurotransmitters.

Ventricular perfusion, a technique which causes virtually no tissue damage to the perfused area, has been utilized to study neurotransmitter release *in vivo*. Portig and Vogt (1966) reported that dopamine was detectable in minute amounts in ventricular perfusates. These authors were unable, however, to observe any consistent changes in dopamine release in conscious cats following substantia nigral stimulation, sciatic nerve stimulation or loud noises. Later these same workers (Portig and Vogt, 1968) observed that homovanillic acid, a metabolite of dopamine, was present in ventricular perfusates in higher concentrations than dopamine and that sciatic nerve stimulation caused consistent and repeatable increases in the efflux of homovanillic acid. This suggested that sciatic nerve stimulation caused the release of dopamine, which was subsequently metabolized to homovanillic acid. Vogt (1969) reported that difficulties with their perfusion system have produced errors that often make interpretaion of results difficult. The primary problem is the alteration of ventricular pressure associated with partial blockage of the outflow cannula. These pressure changes greatly alter the amount of dopamine or metabolite diffusing into the ventricles. Alternative systems which utilize the cerebral aquaduct for outflow have been described (Carmichael *et al.*, 1964). Philippu *et al.* (1970) recently utilized a technique of third ventricle to cerebral aquaduct perfusion to detect the release of H^3-norepinephrine upon

electrical stimulation of the hypothalamus. Due to the use of a slow perfusion rate, these investigators had to collect perfusates at 20 minute intervals, hence, their stimulation lasted for 20 minutes and was rather extreme. Further, hypothalamic stimulation may cause significant increases in blood pressure (Eyzaguirre, 1969). An increase in blood pressure could increase interstitial pressure which in turn could alter the efflux of H^3-norepinephrine from the tissue and thus be a source of error in their experiments. The ventricular perfusion technique has also been adapted to the rat (Palaic and Khairallah, 1968). These investigators stimulated the central end of a sectioned vagus nerve and were able to detect an increase in H^3 in their perfusates from rat previously injected with H^3-norepinephrine; angiotension perfused intraventricularly enhanced the H^3 efflux.

In the experiments reported in this thesis the technique of ventricular perfusion was utilized to detect electrically-evoked release of H^3-catecholamines and metabolites from striatal and hypothalamic areas. Stimulation was applied to the caudate nucleus in some experiments and to the substantia nigra pars compacta in others. An attempt was made to determine if the release of H^3-catecholamines was related to the depolarization of dopaminergic terminals in the caudate nucleus.

METHODS

1. Surgical methods

Throughout the course of these experiments, 2-3 kg. domestic cats (Felis catus) of either sex were utilized. The cats were anesthetized with methoxyflurane using a small polyethylene nose cone. After anesthetic induction a tracheal cannula was inserted. Since the cat was then breathing through the cannula, the nose cone was removed and a funnel containing a methoxyflurane dampened gauze was attached to the cannula to maintain anesthesia. In cats which were to be stimulated in the pars compacta of the substantia nigra, the vagosympathetic trunks were dissected free of the common carotid arteries and sectioned. The cat was then placed in a small stereotaxic instrument (David Kopf Instruments) with raised ear bars. A mid-dorsal skin incision was made from the level of the supra-orbital processes to the axis. The top of the skull was exposed by reflecting the temporal muscles back to the temporal line. The supraoccipital region and cisterna magna were exposed by separating the dorsal cervical muscles along their median raphe, reflecting them back, and cutting them away. The connective tissue and dura mater just above the atlas were then incised, exposing

the spinal cord. The cord was sectioned and hemorrhage quickly controlled by packing cotton into the wound. Since the cat was then unable to maintain respiration, the anesthetic was removed and the tracheal cannula attached to a Harvard small animal respiration pump adjusted to 20 breaths/min and an appropriate tidal volume. The incision areas and pressure points were then topically treated with hexylcaine.

Utilizing an electrode carrier (David Kopf Instruments) and cannula insertion equipment (David Kopf Instruments), burr holes followed by 1/8 in. holes were drilled in the skull at 16.5 mm anterior to the interaural line, 3.5 mm lateral to the midline (right and left). Stainless steel self-tapping screw cannulas (David Kopf Instruments) were cut to 14 mm and were guided stereotaxically through these holes and screwed into place so as to be in the right and left lateral ventricle at 16.5 anterior, 3.5 lateral + 8 deep (Snider and Niemer, 1961). Five uc of H^3-dopamine (9.8-10.6 c/mM, New England Nuclear) or H^3-norepinephrine (8.76-16.7 c/mM, New England Nuclear) in an effective volume of 10 ul, or 2.5 uc C^{14}-urea (.27 mc/mM, New England Nuclear) in an effective volume of 20 ul were then injected through the left lateral ventricular cannula. During the one hour period allowed for the absorption of the labeled compound, the supraoccipital area of the skull was removed by means of rongeur forceps so as to expose the cerebellum. Hemorrhage from the cut bone was controlled by packing bone

wax into the cut edge. After one hour, the dura mater over the cerebellum was incised and the cerebellum carefully lifted until the cerebral aquaduct could be visualized. A cannula made from 5 cm of 2 mm O.D. polyethylene tubing with .5 cm silastic cuff on one end, was then inserted so that the cuff fitted snugly into the cerebral aquaduct. The washout of residual isotope from the ventricular system was then initiated. Artificial cerebrospinal fluid (Pappenheimer et al., 1962) was infused into the lateral ventricle at a rate of 0.1 ml/min. by a Harvard compact infusion pump mounted with a 50 cc syringe. The syringe was connected to the left ventricular inflow cannula by a polyethylene cannula and 26 gauge needle. The outflow of the ventricular system was collected from the cerebral aquaduct for a period of two hours. During this period the right femoral artery was isolated and cannulated with P.E. 90 polyethylene tubing for blood pressure recording using a Statham physiological pressure transducer and Grass Model 7 polygraph. The rectal temperature of all cats was monitored with a Yellow Springs Telethermometer and maintained at 37.5 $\pm$ .5 degrees with a heating pad. During the two hour washout period, the stimulating electrode was positioned stereotaxically. In experiments involving direct caudate stimulation, the electrode assembly consisted of two bipolar electrodes (.5 mm separation, .5 mm exposed tip, David Kopf Instruments) spaced 5 mm apart at 18.0 anterior and 13.0 anterior,

4.0 lateral and + 5 deep (Snider and Niemer, 1961) with the anterior electrode cathodal to the posterior electrode. The caudate stimulating electrodes were angled into the caudate at 24 degrees so as to avoid puncturing the lateral ventricle. In experiments involving substantia nigra pars compacta stimulation, a single bipolar electrode (.5 mm separation, .5 mm exposed tip, David Kopf Instruments) was placed at 4.1 anterior, 2.8 lateral and -4.8 deep (Berman, 1968).

In experiments in which 2 min perfusates were collected, the washout rate was increased to .5 ml/min after 110 minutes of washout. After a total of 120 minutes of washout, the collection of perfusates began. In experiments using H^3-norepinephrine, the 5 ml conical glass collection tubes contained 0.1 ml 5 N acetic acid; in experiments using H^3-dopamine, 100 ug ascorbate in 10 ul H_2O was added to the acetic acid. When C^{14}-urea was used, the tubes contained no preservative. When the cerebrospinal fluid was infused at a rate of 0.1 ml/min, 1.0 ml perfusates were collected every 10 minutes; when the flow rate was increased to .5 ml/min, 1.0 ml perfusates were collected every 2 minutes. At various times during perfusate collection, constant current stimulation of various intensities and frequencies, and 1 msec duration was provided by a Grass S-4 stimulator. Constant current was maintained either with a Grass constant current unit or by wiring one million ohms of resistance in series with the electrodes

and monitoring the current with a Triplett Model 630 Microammeter with appropriate adjustments to the voltage output of the stimulator being made manually. The number of stimulation periods presented to a structure varied from one to four, and the length of a stimulation coincided with the length of one or two collection periods.

After the collection of perfusates had been completed, the entire process of isotope injection, absorption, washout, electrode placement, perfusate collection and stimulation was repeated on the opposite side. After the entire experiment was completed, the cat was euthanatized by the intra-arterial injection of 60 mg sodium pentobarbital and the termination of artificial respiration. Upon cardiac arrest, the brain was removed and fixed in 10% formalin.

2. Histological methods

After at least 48 hours of formalin fixation all brains were dissected to verify the cannula and electrode placement. In experiments involving direct caudate stimulation, gross horizontal sections were made of the brain and the verification of the position of the cannulas and caudate electrodes made directly. In the experiments involving substantia nigra pars compacta stimulation, position of the cannulas was also checked grossly. However, for the substantia nigra pars compacta electrode position verification, a frontal section of the brainstem about 5 mm thick containing the electrode tract was

dissected out. This tissue block was placed on an American Optical 880 sliding microtome and frozen with a Scientific Products Histofreeze unit. Twenty micron frontal sections were cut from the block until the electrode tracts were reached. The section containing the tracts was then placed on a clean microscope slide and stained. The slide was flooded with buffered cresyl violet stain (Humason, 1967) for twenty minutes, washed quickly with 70% and then with 90% ethanol. Dehydration was completed by flooding the slide with isopropanol for three minutes. This technique provided a quick and dependable method of electrode tracing. The major nuclei of the area of interest were clearly delineated and their relationship to the electrode tract apparent when examined under a 1.5-8x dissecting microscope.

3. Biochemical methods

In many of the experiments, the total radioactivity of the perfusates was of interest. For this reason 100 ul of each perfusate were transferred into glass scintillation vials containing a toluene-ethanol-2,4-diphenyloxasole (7:3, 0.5% 2,5-diphenyloxasole) scintillator. The radioactivity was then determined in a Beckman LS-100 liquid scintillation counter with direct readout module. The counts were corrected for counting efficiency for these total perfusates as well as all other samples counted; hence, the data presented are in units of absolute radioactivity (DPMs or muc). The background was subtracted and, except for total radioactivity in the perfusates, a factor for recovery of a standard was applied to correct for losses

during the separation procedures.

The initial separation performed on the perfusates was the alumina extraction of the catechol compounds. For this purpose 0.1 ml of .2 M NaEDTA plus 6 drops of an alumina suspension (approximately 100 mg of aluminum oxide) were added to 5 ml centrifuge tubes containing the collected perfusates. The pH of the contents of each tube was then adjusted to 8.5-8.6 with 5 N, 1 N and 0.1 N KOH. The tubes were then shaken for five minutes in an Eberbach horizontal tube shaker followed by a five minute centrifugation at 1800 x gravity. The resulting supernatant fluid containing the non-catechol compounds was then aspirated and in some experiments saved for further analysis. The alumina containing the adsorbed catechols was then washed twice, once with 2 ml H_2O and once with 1 ml H_2O. These washes involved the same shaking and centrifuging steps as previously outlined. After the second wash, the catechols were eluted from the alumina with 1 ml 0.2 N acetic acid. The acid and alumina were shaken for ten minutes, centrifuged for five minutes and the eluate aspirated off and saved. In experiments with H^3-norepinephrine, and in some experiments with H^3-dopamine, 100 ul of this eluate were counted as previously described.

In a number of experiments the alumina supernatant (non-catechol fraction) was further separated into an amine fraction and a non-amine fraction by cationic exchange on Dowex 50 resin, H^+ form, 100-200 mesh. The

Dowex was formed into 6 mm x 40 mm free flowing columns and the alumina supernatants, after having been adjusted to pH 6 with 0.2 N acetic acid, were poured on the columns. After the sample had run through, 5 ml H_2O was run through; these two fractions contained the non-amine portion of the non-catechol fraction, the deaminated-O-methylated metabolites. In some experiments this fraction was saved and counted. The amines were then eluted from the ion exchange resin with 5 ml of a 1:1 solution of 95% ethanol and 6 N HCl. One ml of this fraction, containing the O-methylated amines, normetanephrine or 3-methoxytyramine, was added to empty scintillation vials and dried. The dried salts were then dissolved in 1 ml H_2O, 10 ml of modified Bray's solution (6 gm of 2,5-diphenyloxasole and 100 gm of naphthalene per liter of dioxane) was added, and the samples counted in the scintillation spectrophotometer.

In the initial experiments using H^3-norepinephrine, the alumina eluate was separated into an amine and a non-amine fraction to determine if significant quantities of deaminated catechol metabolites were present. This separation was carried out by cation exchange chromatography exactly as described for the alumina supernatant separation with the exception that the pH had to be raised to 6 with 1 N and 0.1 N KOH.

In initial experiments using H^3-dopamine the alumina eluate was separated into a H^3-dopamine fraction and a

H^3-norepinephrine fraction by selective elution from a 6 mm x 40 mm column of Dowex 50, 200-400 mesh. After the flow rates of the columns were adjusted to 5-7 drops per minute, the Dowex was changed to Na^+ form by passing 25 ml of 0.1 M NaH_2PO_4, pH 6.5 buffer through them, followed by 5 ml H_2O. The samples were prepared by the addition of 100 ug of Na ascorbate, 100 ug of norepinephrine and 100 ug of dopamine, each in a volume of 10 ul. Before being added to the columns, the samples were adjusted to pH 6 with 1.0 N and 0.1 N KOH. After the sample had run through the column, 5 ml of H_2O, 8ml 1.0 N HCl, 10.0 ml 1.0 N HCl and 4.0 ml 1:1 6.0 N HCl-95% ethanol solution were added in succession. The 10.0 ml 1.0 N HCl eluate contained H^3-norepinephrine peak and the 4.0 ml HCl-ethanol contained the H^3-dopamine; 2.0 ml of each of these fractions were transferred to empty scintillation vials, dried, redissolved in 10.0 ml toluene-95% ethanol-2,5-diphenyl-oxasole scintillator and counted.

4. Statistical methods

All results reported are the mean of at least four experiments. All calculations of means, standard errors, and statistical significance were performed on either an Olivetti-Underwood Programma 101 computer or a SCM Marchant Cogito 240 SR calculator. For the direct caudate stimulation experiments, statistical significance of the evoked release was calculated by comparing the differences of the two periods previous to stimulation and the period of, plus

the period following stimulation in a single-tailed paired Student's t-test. Similarly, for experiments involving substantia nigra pars compacta, a single-tailed paired Student's t-test was used to compare the differences between the period previous to stimulation and the period of stimulation. A P value of less than .05 was considered to indicate statistical significance.

RESULTS

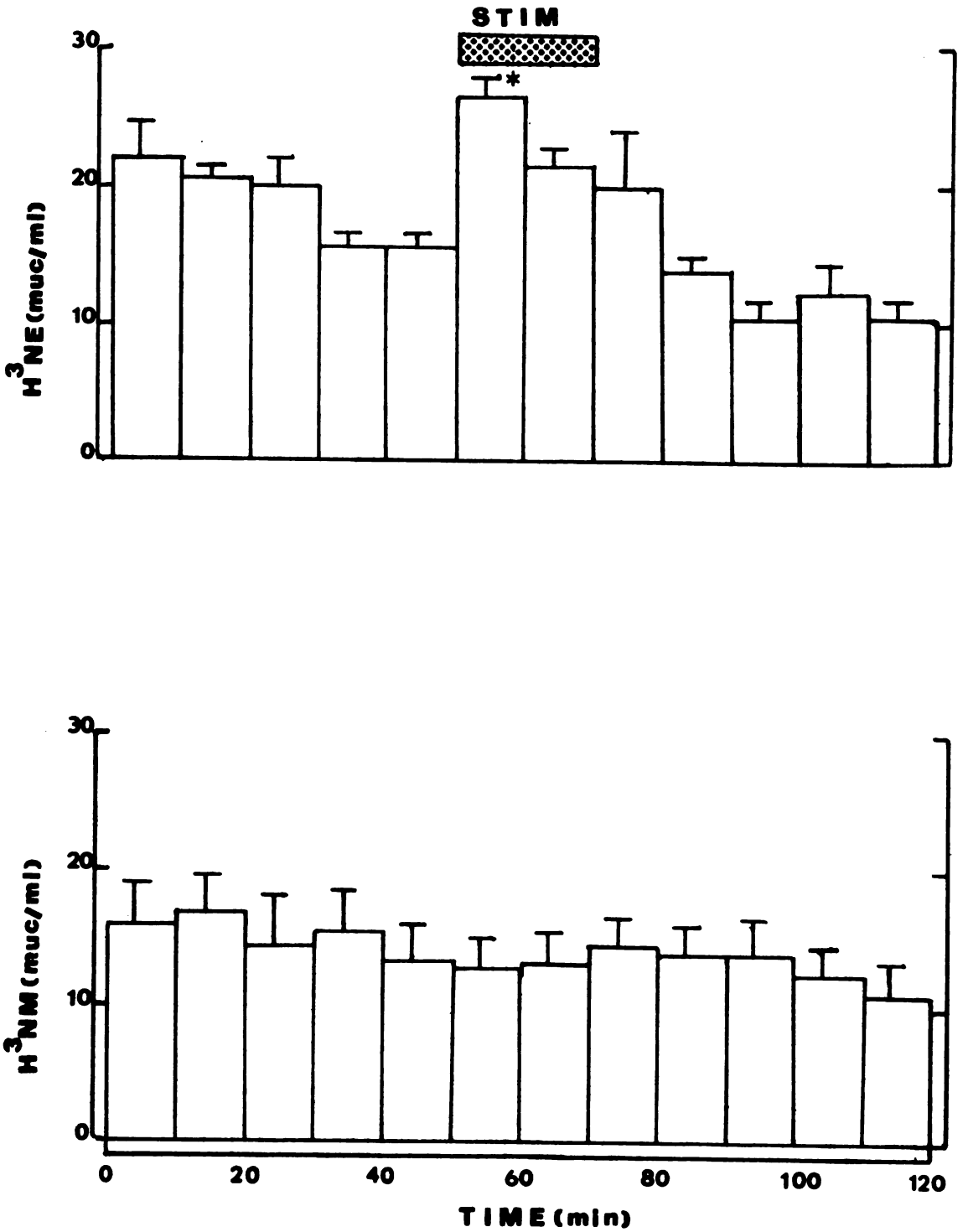
1. Electrical stimulation of caudate nucleus

The effects of a 20 minute period of direct electrical stimulation of the caudate nucleus upon the release of H^3-norepinephrine and H^3-normetanephrine into the ventricular perfusates of spinal cats are summarized in Figure 1. The efflux of H^3-norepinephrine but not of H^3-normetanephrine was significantly increased. Analysis of the total radioactivity in the perfusates revealed that approximately 50% was present as catechols. Further analysis of this catechol fraction with ion-exchange chromatography revealed that H^3-norepinephrine comprised more than 80% of this radioactivity, the remainder was presumably deaminated catechol metabolites. The concentration of these metabolites did not increase during or following stimulation. In subsequent experiments no attempt was made to separate the deaminated catechols from the catecholamines so that the radioactivity in the alumina eluate is reported as the amines. The remaining 50% of the total perfusate radioactivity, the alumina supernatant, was likewise separated into its amine and deaminated fractions by ion-exchange chromatography. This procedure demonstrated that about 70% of the non-catechols

Figure 1. Effects of 20 minutes of electrical stimulation of the caudate nucleus on the cerebroventricular effluent concentrations of H^3-norepinephrine (H^3NE) and H^3-normetanephrine (H^3NM).

The height of each bar represents the mean concentration (vertical lines denote 1 standard error) of H^3NE or H^3NM in the cerebroventricular effluent collected over 10 minute periods from 4 cats.

* The effluent concentration of H^3NE during the 20 minutes of stimulation (100 hz, 60 uA, 1 msec) was significantly greater than it was during the 20 minute prestimulation period ($P < .05$).


STIM
*
30
20
10
0
H^3NE (muc/ml)
H^3NM (muc/ml)
0
20
40
60
80
100
120
TIME (min)

present were H^3-normetanephrine; the remainder was probably deaminated-O-methylated metabolites. The concentration of the later fraction did not increase during or after stimulation.

In the initial experiments the cerebroventricular system was perfused at a rate of 0.1 ml per minute. At this rate a 10 minute period was required to collect enough perfusate (1 ml) to permit accurate chemical analysis. A faster rate of perfusion, 0.5 ml per minute, was utilized in subsequent experiments so that an analyzable amount of perfusate could be collected in 2 minutes. In this manner a shorter period of electrical stimulation could be employed, thereby lessening the possibility of electrolytic damage to the cerebral tissues. As illustrated in Figure 2, the increased rate of perfusion was accompanied by a general reduction in perfusate concentrations of H^3-norepinephrine and H^3-normetanephrine; nevertheless, a 2 minute period of electrical stimulation of the caudate nucleus still caused a significant increase in the efflux of H^3-norepinephrine. As with the longer period of stimulation, the efflux of H^3-normetanephrine and H^3-deaminated-O-methylated metabolites was not affected.

A stimulation frequency of 100 hz was used in the preliminary experiments shown in Figures 1 and 2. An examination of the frequency-response relationship, however, revealed that this rate of stimulation was not optimal. The data summarized in Figure 3 demonstrate that increased

Figure 2. Effects of 2 minutes of electrical stimulation of the caudate nucleus on the cerebroventricular effluent concentrations of H^3-norepinephrine (H^3NE) and H^3-normetanephrine (H^3NM).

The height of each bar represents the mean concentration (vertical lines denote 1 standard error) of H^3NE or H^3NM in the cerebroventricular effluent collected over 2 minute periods from 4 cats.

* The effluent concentration of H^3NE was significantly greater during and following stimulation as compared to the 2 periods previous to stimulation. ($P < .05$).

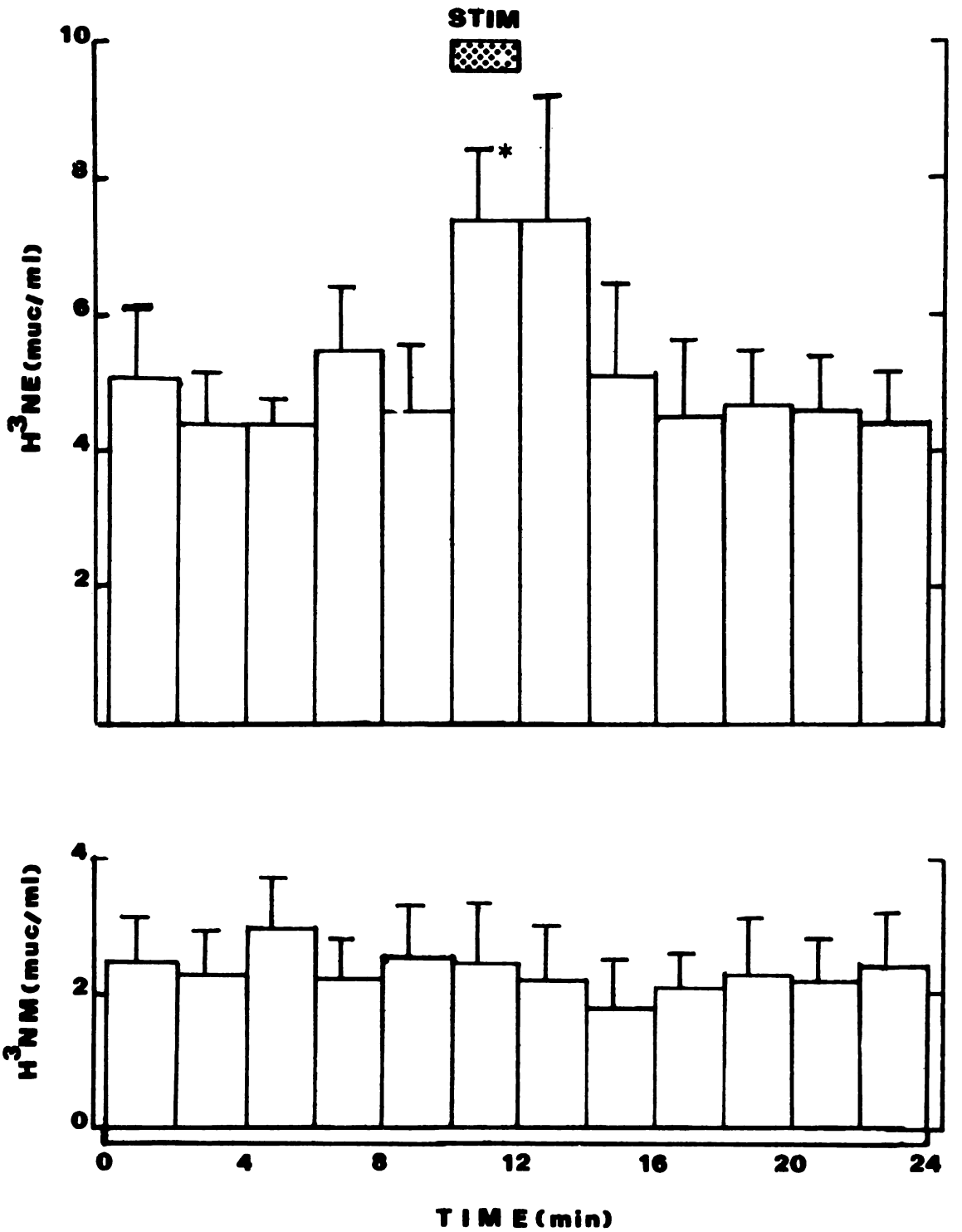
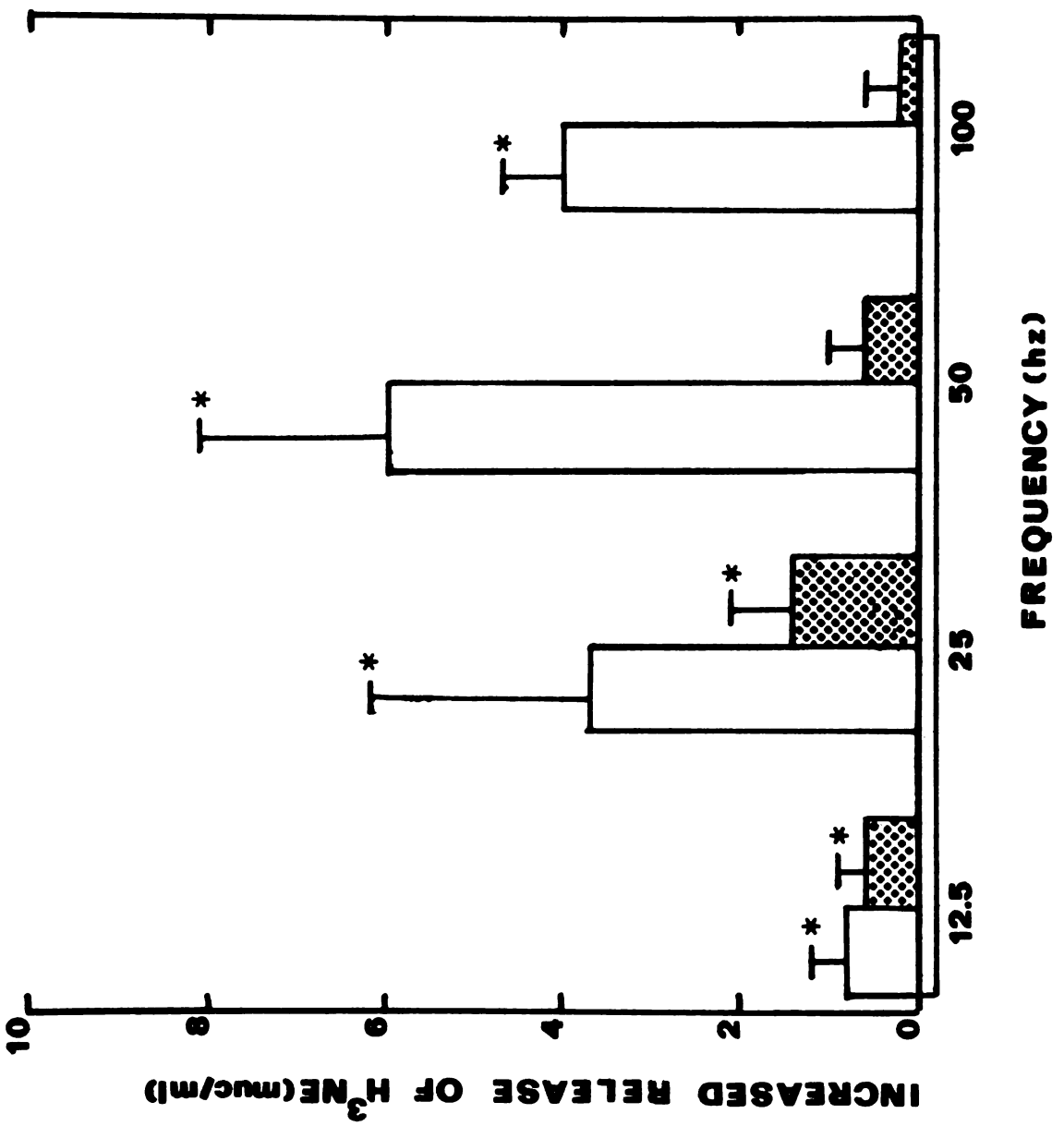

STIM
10
8
6
4
2
H^3NE (muc/ml)
*
4
2
0
H^3NM (muc/ml)
0
4
8
12
16
20
24
TIME (min)

Figure 3. Increase in H^3-norepinephrine (H^3NE) release into the ventricular perfusate upon caudate nucleus stimulation at various frequencies (350 uA, 1 msec).

The height of the open bars represents the increased concentration of H^3NE upon initial stimulation; the stippled bars represent the increase upon a second stimulation 8 minutes later (vertical lines denote 1 standard error). Increases in H^3NE concentration are calculated as the differences between the concentration during and after stimulation as compared to the concentration during the 4 minute period, immediately before stimulation in a total of 4 experiments.

* Increase in H^3NE concentration is statistically significant ($P < .05$).


INCREASED RELEASE OF H^3NE (muc/ml)
0
2
4
6
8
10
12.5
25
50
100
FREQUENCY (hz)
*

H^3-norepinephrine release occurred coincident with the initial periods of electrical stimulation at various frequencies. With 12.5-50 hz the efflux of H^3-norepinephrine increased progressively; however, 100 hz stimulation produced an efflux of lesser magnitude than 50 hz. The latter frequency would, therefore, appear to be closest to optimal for the release of amines from the caudate nucleus. The results in Figure 3 also indicate that the response to stimulation decreased in repeatability as the frequency was increased. Significant increases in H^3-norepinephrine release were obtained during a second period of stimulation at 12.5 and 25 hz but not at 50 and 100 hz.

Because the chemical analysis for H^3-norepinephrine are simpler and more reproducible than they are for H^3-dopamine, the former was used in the preliminary experiments. However, since dopamine is the predominant catecholamine in the caudate nucleus, it seemed appropriate to extend these studies to include the effects of electrical stimulation on the release of H^3-dopamine. Since H^3-dopamine can be hydroxylated *in vivo* to H^3-norepinephrine, ion-exchange chromatography was utilized to eliminate H^3-norepinephrine from the alumina eluate. Since H^3-norepinephrine was found to be present only in minute amounts, this separation procedure was deleted in subsequent experiments using H^3-dopamine. Since H^3-norepinephrine was essentially absent in these perfusates, the O-methylated-

amine metabolite was assumed to be that of H^3-dopamine, namely, H^3-3-methoxytyramine. The effects of direct caudate stimulation upon the efflux of H^3-dopamine and H^3-3-methoxytyramine are summarized in Figure 4. The perfusate concentration of the O-methylated-amine did not increase with stimulation. Because of variability the apparent increased release of H^3-dopamine is not statistically significant. This variability may be due in part to the additional steps involved in the H^3-dopamine - H^3-norepinephrine separation procedure.

In the foregoing studies it was tacitly assumed that radioactive catecholamines are selectively taken up by catecholamine-containing nerve endings and it is from this store that they are subsequently released when depolarizing electrical stimulation is applied. It is possible, however, that electrical stimulation could release amines from non-neuronal stores. To test the specificity of the stimulation-induced release of catecholamines from the caudate nucleus, the efflux of urea, a substance not generally considered to be a transmitter, was examined. The data summarized in Figure 5 indicate that electrical stimulation of the caudate nucleus failed to alter the efflux of C^{14}-urea into cerebroventricular perfusates.

2. Electrical stimulation of the substantia nigra pars compacta

Since the previous experiments had indicated that

Figure 4. Effects of 2 minutes of electrical stimulation (100 hz, 350 uA, 1 msec) of the caudate nucleus on the cerebroventricular effluent concentrations of H^3-dopamine (H^3D) and H^3-3-methoxytyramine (H^3 3-MT).

The height of each bar represents the mean concentration (vertical lines denote 1 standard error) of H^3D or H^3 3-MT in the cerebroventricular effluent collected over 2 minute periods from 4 cats.

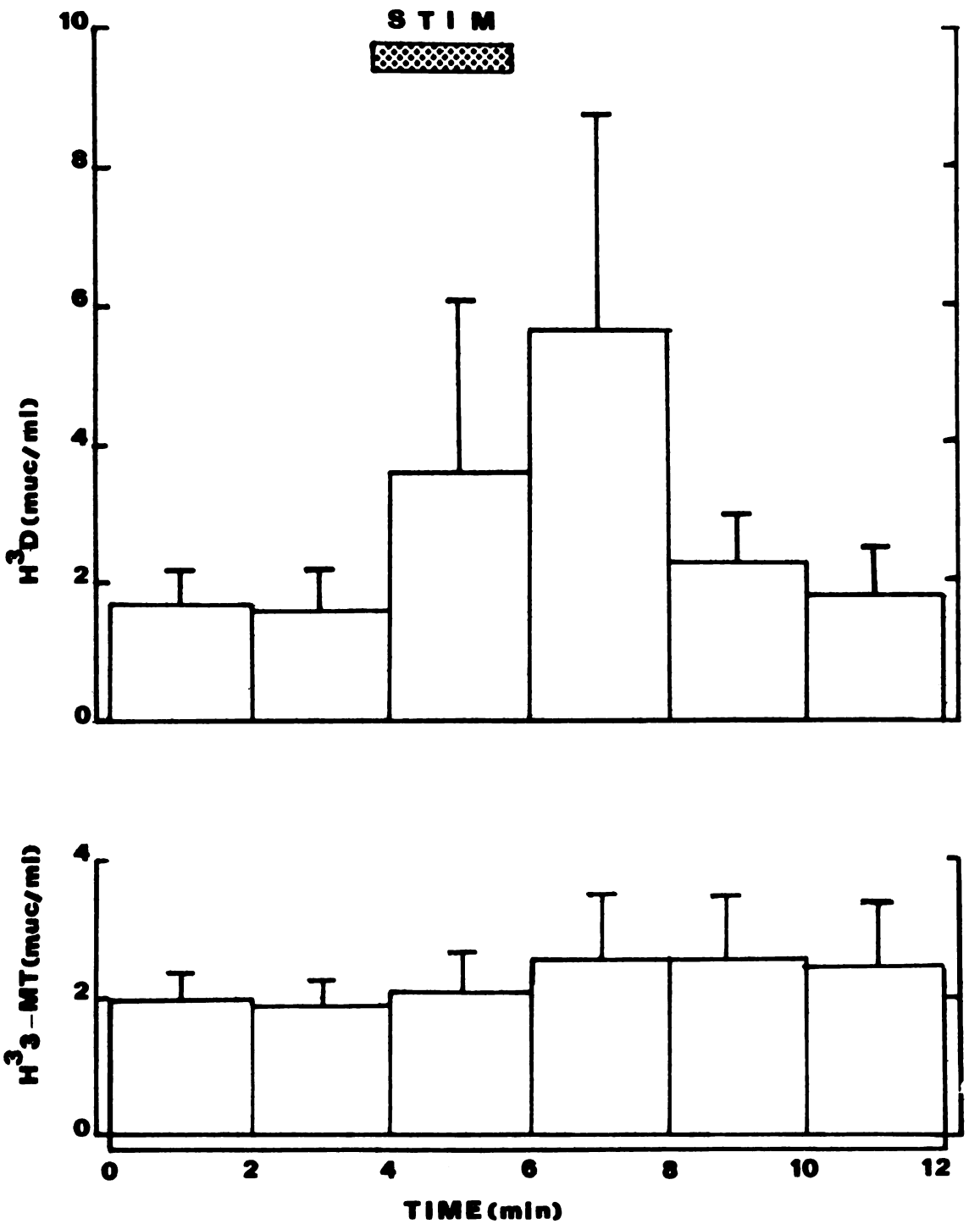
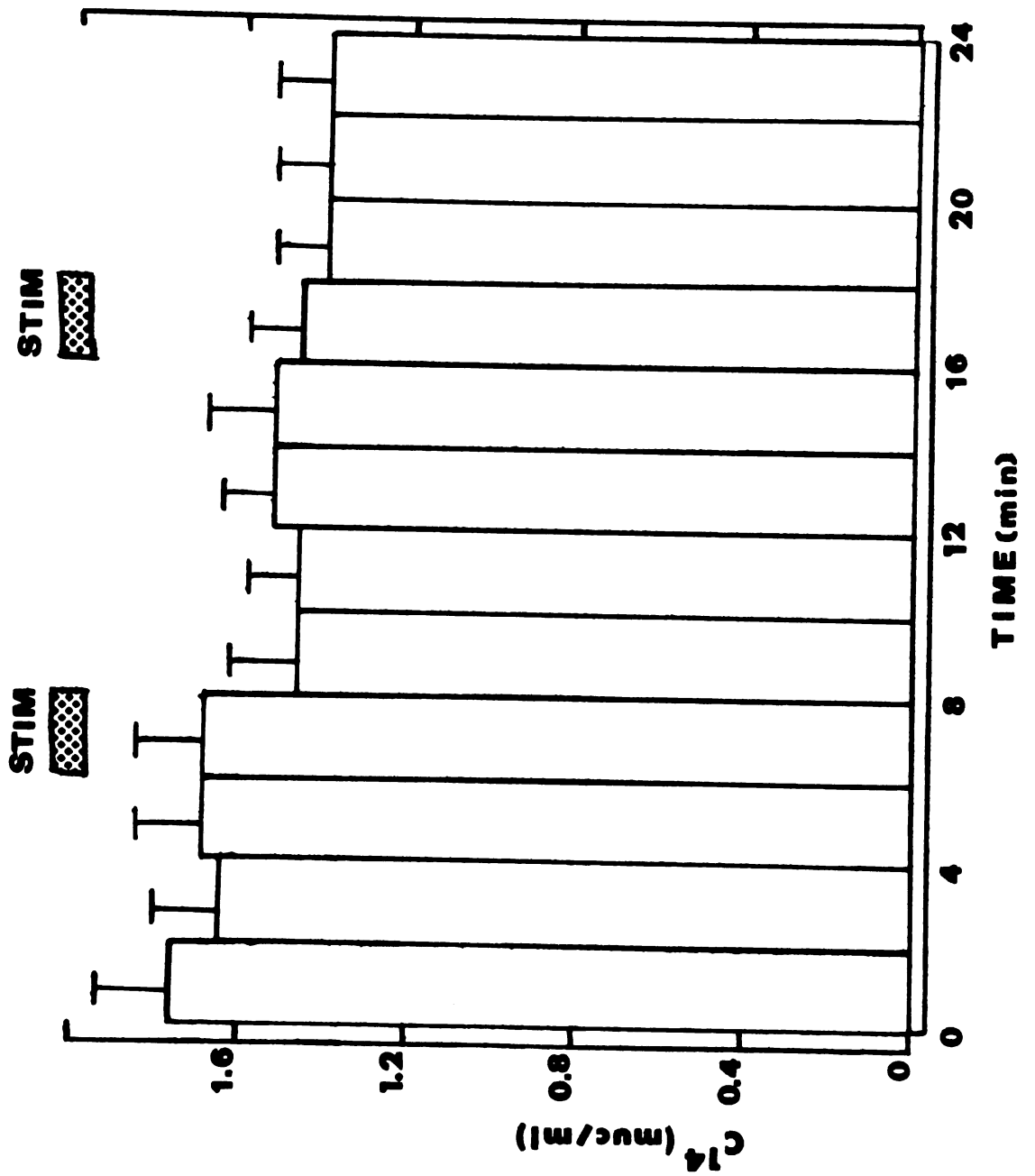

STIM
10
8
6
4
2
0
H^3D (muc/ml)
4
2
0
H^3 3-MT (muc/ml)
0
2
4
6
8
10
12
TIME (min)

Figure 5. Effect of electrical stimulation of the caudate nucleus (100 hz, 350 uA, 1 msec) upon the C^{14}-urea concentration in ventricular perfusates.

The height of the bars represents the concentration of C^{14}; the vertical lines denote standard errors for a total of 4 experiments.


STIM
STIM
1.6
1.2
0.8
0.4
0
C^{14} (muc/ml)
0
4
8
12
16
20
24
TIME (min)

direct electrical stimulation of an area of dopaminergic terminals, the caudate nucleus, increased the concentrations of H^3-catecholamines in the ventricular perfusates, it was of interest to investigate the effects of stimulating the cell bodies of these terminals. Accordingly, the substantia nigra pars compacta, a region containing cell bodies of dopaminergic neurons which terminate in the caudate nucleus, was electrically stimulated. The results of the initial study on the effects of substantia nigra pars compacta stimulation on the release of H^3-dopamine into the perfusing fluid are summarized in Table 1. The perfusate concentration of both H^3-dopamine and H^3-3-methoxytyramine were increased upon stimulation.

The release of H^3-dopamine evoked by the stimulation of the substantia nigra pars compacta appears to be frequency dependent. In the frequency-response studies illustrated in Figure 6, the substantia nigra pars compacta was stimulated at 3, 10, 30 and 100 hz in random order. Increasing the frequency up to 30 hz caused a progressive increase in the efflux of H^3-dopamine; however, stimulation at 100 hz was ineffective in causing a consistent release of H^3-dopamine. This point is further supported by a separate series of experiments summarized in Table 2; repeated stimulation at 100 hz did not cause a significant increase in the release of H^3-dopamine.

In order to establish an intensity-response curve for substantia nigra pars compacta stimulation, various

Table 1. Release of H^3-dopamine and H^3-3-methoxytyramine upon electrical stimulation of the substantia nigra pars compacta.

	Experiment Number	Control (muc/ml)	Stimulation (muc/ml)	Change
H^3D	1	1.06	1.18	.12
	2	1.85	1.99	.14
	3	0.98	1.18	.20
	4	0.85	1.00	.15
	$\bar{x} \pm$ SE	1.19 $\pm$ .23	1.34 $\pm$ .22	.15 $\pm$ .01*
H^3-3-MT	1	1.60	1.70	.10
	2	1.29	1.50	.21
	3	1.08	1.25	.17
	4	0.92	0.98	.06
	$\bar{x} \pm$ SE	1.22 $\pm$ .15	1.36 $\pm$ .16	.14 $\pm$ .03*

The control period represents the 2 minute perfusion period immediately before stimulation. During the stimulation period 30 hz, 200 uA, 1 msec duration electrical stimulation was applied.

* Increase in release statistically significant ($P < .05$).

Figure 6. The increase in H^3-dopamine (H^3D) released into the ventricular perfusate upon substantia nigra pars compacta stimulation at various frequencies (100 uA, 1 msec).

The height of each bar represents the mean (vertical lines denote 1 standard error) increase in H^3D release upon stimulation for a total of 8 experiments. Increase in release is calculated as the difference between the perfusate concentration of H^3D during the 2 minute prestimulation period and the 2 minute period of stimulation.

* Increase is statistically significant ($P < .05$).

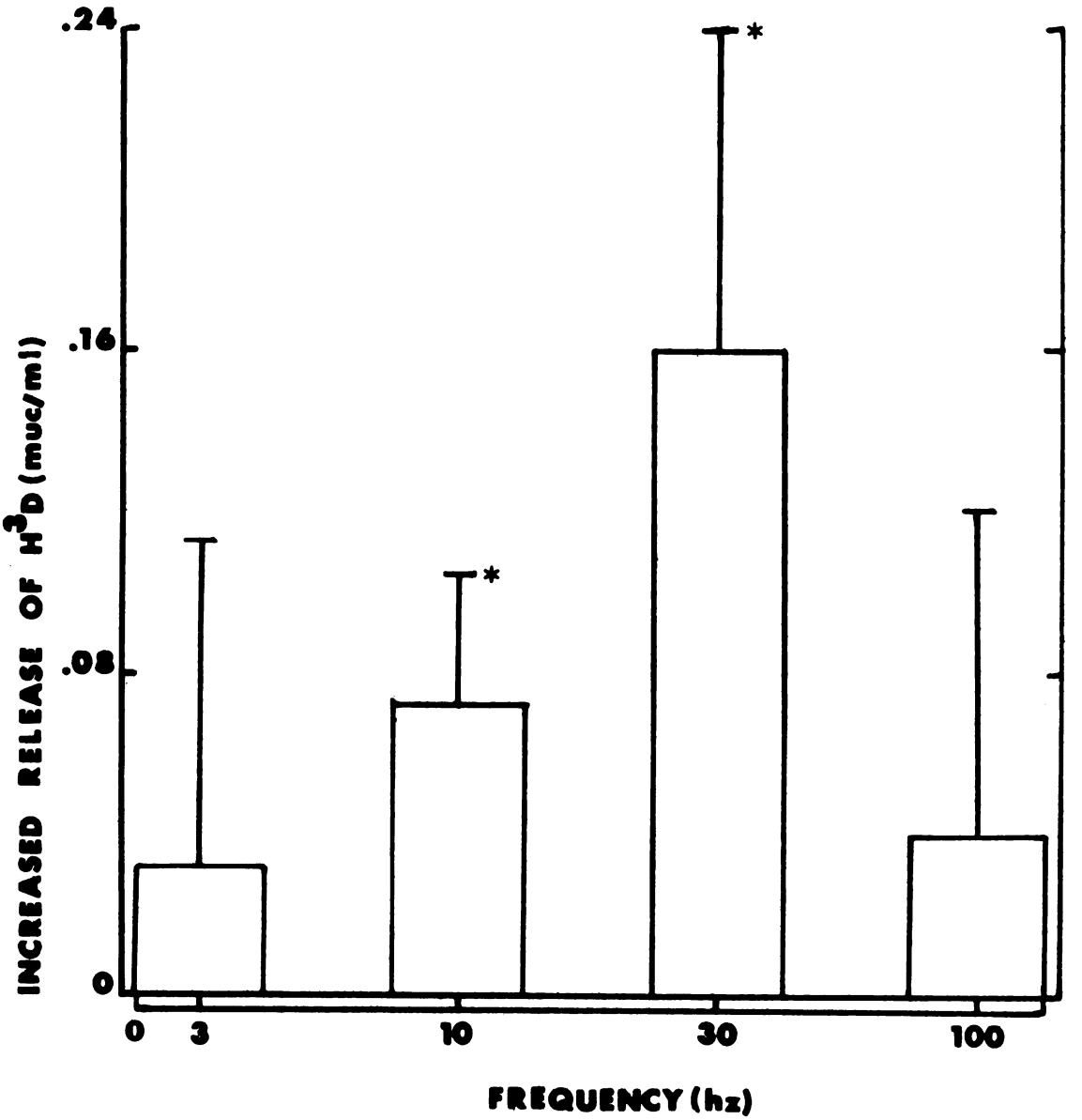

.24
.16
.08
0
INCREASED RELEASE OF H^3D (muc/ml)
*
*
0
3
10
30
100
FREQUENCY (hz)

Table 2. The effect of 100 hz stimulation of the substantia nigra pars compacta on the effluent concentrations of H^3-dopamine in ventricular perfusates.

	Experiment Number	Control H^3D (muc/ml)	Stimulation H^3D muc/ml	Change
(1st stimulation)	1	1.82	2.65	+ .83
	2	2.59	3.08	+ .49
	3	1.79	0.97	- .82
	4	1.90	1.94	+ .04
	5	2.70	2.41	- .29
	$\bar{x} \pm$ SE	2.16 ± .20	2.21 ± .36	.17 ± .29
(2nd stimulation)	1	1.89	2.14	+ .25
	2	1.93	2.00	+ .07
	3	0.76	0.93	+ .17
	4	1.95	1.69	- .26
	5	2.46	2.55	+ .09
	$\bar{x} \pm$ SE	1.80 ± .28	1.86 ± .27	.06 ± .09

The two periods of stimulation (100 hz, 100 uA, 1 msec duration) were separated by 10 minutes; in neither case did stimulation significantly increase H^3D release.

strengths of current were applied in a random fashion. The results of the initial experiments involving currents of 12.5 to 100 uA are illustrated in Figure 7a. A significantly increased release of H^3-dopamine was observed only at 100 uA. In a second series of experiments summarized in Figure 7b, higher intensities were utilized. Stimulation of the substantia nigra pars compacta at 100, 200 and 400 uA caused progressively greater increases in the release of H^3-dopamine.

After having found an optimal frequency of stimulation and an apparent intensity threshold of stimulation for the substantia nigra pars compacta mediated release of H^3-dopamine, it was of interest to establish the reproducibility of the response at these parameters. Table 3 demonstrates that repeated substantia nigra pars compacta stimulation at 100 uA and 30 hz is capable of eliciting two responses of similar magnitude.

In all the experiments reported herein the systemic blood pressure did not change significantly during the period of stimulation.

Figure 7a and 7b. The relationship of substantia nigra pars compacta stimulation intensity and the increased cerebroventricular perfusate concentration of H^3-dopamine (H^3D).

Each point represents the mean increase in H^3D upon stimulation at the indicated intensity as compared to the prestimulation H^3D perfusate concentrations for a total of 8 experiments. The vertical line denote 1 standard error.

* Increased concentration of H^3D is significant ($P < .05$).

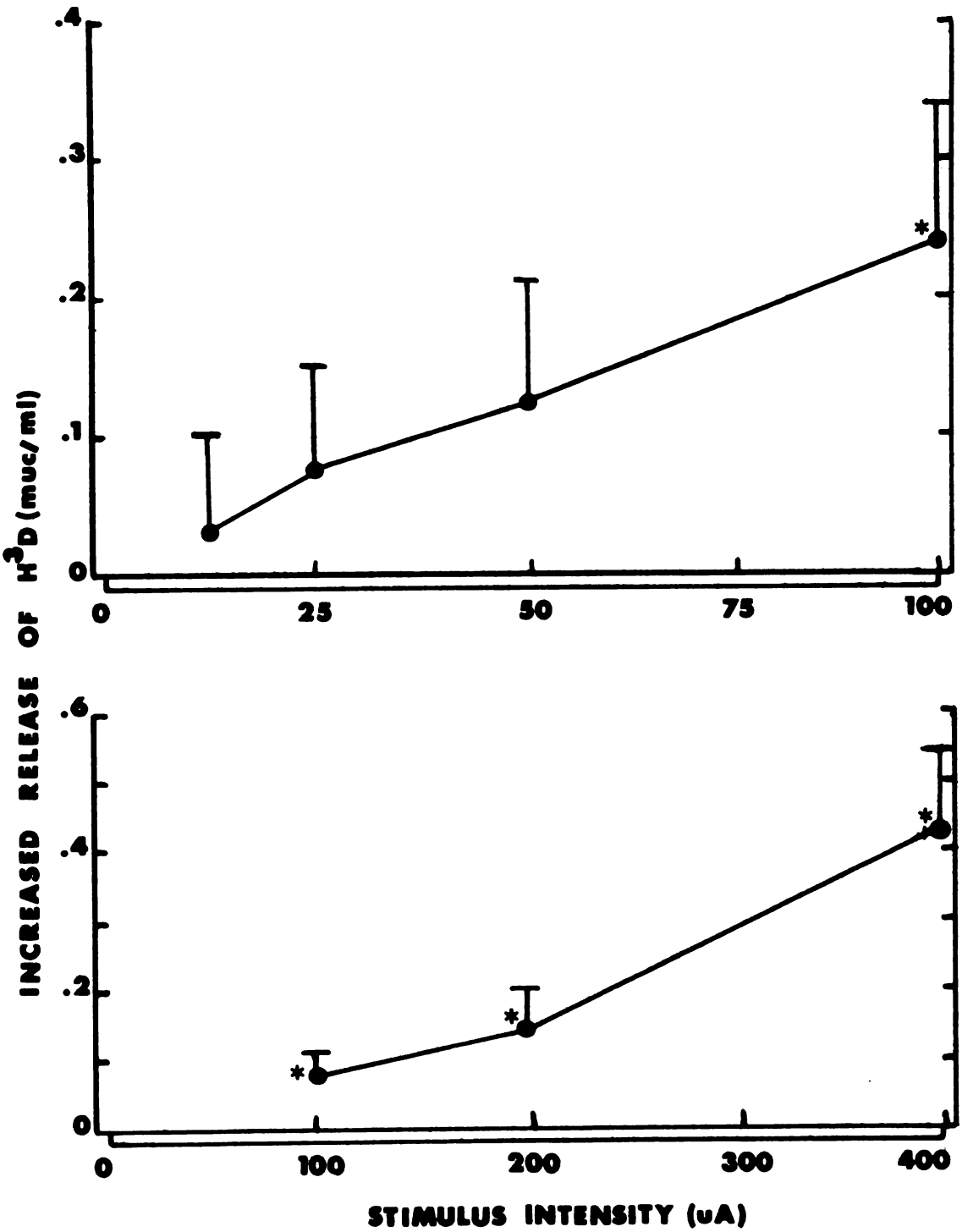

.4
.3
.2
.1
0
0
25
50
75
100
.6
.4
.2
0
0
100
200
300
400
INCREASED RELEASE OF H3D (muc/ml)
STIMULUS INTENSITY (uA)

Table 3. Effect of repeated 100 uA stimulation of the substantia nigra pars compacta upon the perfusate effluent concentration of H^3-dopamine (H^3D).

	Experiment Number	Control H^3D (muc/ml)	Stimulation H^3D (muc/ml)	Change
(1st stimulation)	1	2.25	3.25	+ 1.00
	2	1.12	1.86	+ 0.74
	3	1.03	0.98	- 0.05
	4	0.68	0.71	+ 0.03
	5	2.18	2.52	+ 0.34
	6	1.88	2.08	+ 0.20
	$\bar{x} \pm$ SE	1.52 ± .27	1.90 ± .39	.38 ± .17*
(2nd stimulation)	1	2.58	2.84	+ 0.26
	2	1.03	1.79	+ 0.76
	3	0.87	0.80	- 0.07
	4	0.63	0.97	+ 0.34
	5	1.87	1.96	+ 0.09
	6	1.95	2.36	+ 0.41
	$\bar{x} \pm$ SE	1.49 ± .31	1.79 ± .32	.30 ± .12*

The control is expressed as the effluent concentration of H^3D during the two minute prestimulation period. The 2 two minute stimulations (30 hz, 100 uA, 1 msec) were separated by 10 minutes.

* Increase from the control to stimulation is significant ($P < .05$).

DISCUSSION

This study clearly indicates that the efflux of brain catecholamines into the cerebroventricular system may be increased by electrical stimulation of the caudate nucleus or substantia nigra pars compacta. The significance of these results depends upon a thorough understanding of the mechanisms involved in this response. Indeed, the evoked release demonstrated here has little significance if it is not causally related to the physiological release of neurotransmitter from the presynaptic terminal.

The regional and subcellular distribution of injected H^3-catecholamines is of major importance in interpreting these results. Carr and Moore (1969[a]) have demonstrated, using an identical intraventricular injection technique, that H^3-catecholamines are distributed primarily to the ipsilateral caudate nucleus and hypothalamus. Essentially none are distributed to areas adjacent to the substantia nigra. It, therefore, seems unlikely that evoked release by substantia nigra pars compacta stimulation is a local effect. Likewise, autoradiographical studies (Schubert and Labisich, 1969) indicate that intracisternally administered H^3-norepinephrine penetrates only 0.5 mm into monoaminergic areas. Exogenously administered H^3-norepinephrine

has the same subcellular distribution as endogenous norepinephrine (Green *et al.*, 1969); therefore, conditions which cause the release of the endogenous compound should have a similar effect on the H^3-norepinephrine. It does bear mention, however, that exogenous dopamine may displace endogenous 5-hydroxytryptamine (Everett, 1970), implying that H^3-catecholamines may also be distributed to and possibly released from serotonergic neurons.

The direct caudate nucleus stimulation studies presented here are similar to the slice stimulation studies (Baldessarini and Kopin, 1966) in that terminals are directly stimulated. However, in the *in vivo* studies reported here, the disruption of normal cellular and tissue barriers was avoided. Nevertheless, direct stimulation of the caudate nucleus *in vivo* was effective in increasing the release of H^3-catecholamines.

Once the effect of direct stimulation upon the release of H^3-catecholamines was verified, it was of interest to determine if the release was from dopaminergic terminals. For this purpose the substantia nigra pars compacta, the site of the cell bodies of these terminals, was stimulated. This stimulation effectively released H^3-dopamine, suggesting that a specific neuronal effect was involved. H^3-dopamine would not appear to be released from the substantia nigra pars compacta *per se* because this region is some distance from the ventricular system and the site of H^3-dopamine distribution. Similarly, the evoked

release of H^3-dopamine localized in non-dopaminergic terminals may be eliminated by stimulating specifically the dopaminergic neurons of the substantia nigra pars compacta. Therefore, while direct caudate nucleus stimulation may release H^3-catecholamines from serotonergic as well as dopaminergic terminals, stimulation of the exclusively dopaminergic neurons of the substantia nigra pars compacta should lead to release of H^3-dopamine from primarily the dopaminergic terminals. This technique of stimulating the axons and cell bodies distant from the terminals is one which would be difficult to utilize in an *in vitro* system. Philippu *et al.*(1970) failed to utilize such a technique to verify the specificity of their observed release of C^{14}-norepinephrine upon *in vivo* electrical stimulation of the hypothalamus.

While the technique of substantia nigra pars compacta stimulation would seem to be free from problems associated with nonspecific release of H^3-catecholamines, the technique of direct caudate stimulation would seem more prone to these problems. Since the stimulating current is applied directly to the area of H^3-catecholamines distribution, one might imagine that gross ionic shifts, metabolic alterations, or even electrophoretic effects related to stimulation could introduce artifacts into this system. However, the studies reported here on the effect of caudate stimulation upon the efflux of C^{14}-urea suggest that such artifactual effects are not a serious problem. For example, C^{14}-urea

concentrations in perfusates could not be altered by electrical stimulation. Other workers (Philippu et al., 1970; Stein and Wise, 1969) who have studied the effects of stimulation upon catecholamine release have failed to check their systems for nonspecific release by the use of urea or other non-transmitter substances. In the experiments of Philippu et al. (1970) this may be an important oversight since they utilized large non-tracer injections of C^{14}-norepinephrine which must have been distributed, at least in part, extracellularly and thus, may have been readily available for and sensitive to non-specific stimulation effects. Correspondingly, Stein and Wise (1969) should have been more concerned with non-specific effects on apparent H^3-norepinephrine release. In a push-pull cannula system similar to theirs, Kopin and Chase (1968) demonstrated that urea and inulin as well as H^3-norepinephrine or its metabolites could be released.

The intensity and frequency-response studies reported here were undertaken primarily to determine the optimal parameters of stimulation for the release of H^3-catecholamines. These results also reveal something about the release mechanism. The release of H^3-catecholamines by stimulation of the caudate nucleus and the substantia nigra pars compacta was frequency dependent; the optimal frequency was similar for both structures. This suggests that the mechanisms by which these two types of stimulation cause release may be the same. It is also of interest that

the pool from which H^3-catecholamines are released is sensitive to frequency; that is, high frequency stimulation, although not as effective at releasing H^3-catecholamines, decreases the chance of release upon a second stimulation. The dose-response curve has been a classical method of relating a given effect to a drug. In the present study an intensity-response curve was utilized to relate the intensity of substantia nigra pars compacta stimulation to the increase in H^3-dopamine release. This study demonstrated that increases in stimulus intensity caused corresponding increases in H^3-dopamine release; thus, the effect was confirmed to be stimulus-dependent.

The release or lack of release of catecholamine metabolites coincident with induced release of H^3-catecholamines may also shed some light upon the mechanism of release. Since the deaminated catechols, O-methylated-metabolites, and deaminated O-methylated-metabolites are all present in the brain of cats after intraventricular injection of H^3-catecholamines (Carr and Moore, 1969[a]) and are also present in the ventricular perfusates (Carr and Moore, 1969[b]) as confirmed by this study, one would expect any non-neuronally mediated increase in efflux to affect the perfusate concentrations of all of these compounds. This was not the case. Direct caudate nucleus stimulation released only the catecholamines. Axelrod (1968) has suggested that the post-synaptic receptor in

adrenergic synapses may be adjacent to catechol-O-methyltransferase. If this is true, then some of the catecholamines released at adrenergic synapses should be metabolized to their O-methylated products and show up in that form in the ventricular perfusates during stimulation. Indeed, during stimulation of the substantia nigra pars compacta the perfusate concentrations of H^3-3-methoxytyramine, as well as those of H^3-dopamine, increased. This effect was not seen with direct caudate nucleus stimulation. The explanation for this result may be the specificity of the release following stimulation of the substantia nigra pars compacta as compared to that following direct caudate stimulation. The former is a specific dopaminergic synapse mediated effect; therefore, the H^3-3-methoxytyramine is produced and effluxes coincident with stimulation. In the latter case, however, H^3-dopamine may be released predominately from non-dopaminergic nerve terminals (possibly serotonergic sites) that lack catechol-O-methyltransferase. Hence, the increase in O-methylated-metabolites is not seen coincident with stimulation.

These proposals are reinforced by the findings of other investigators who are concerned with the functions of dopamine in the striatum and with the possibility of a dopaminergic nigro-striatal pathway. The fact that H^3-dopamine may be released <u>in vivo</u> from the striatum upon stimulation is further evidence for a neurotransmitter function for dopamine. That stimulation of the substantia

nigra pars compacta is capable of releasing H^3-dopamine, presumably from the caudate nucleus, is additional evidence for a dopaminergic nigro-striatal pathway. Although only theoretical at this time, the idea of an association of the adrenergic receptor with catechol-O-methyltransferase also finds support from these data.

A number of questions relating to these experiments remain to be answered. Perhaps, the specificity of direct caudate stimulation-induced release of H^3-catecholamines is one of the more pressing of these questions. Moore et al., (1970) recently reported the entire course of the nigro-striatal pathway in the cat and demonstrated that discrete lesions in this tract cause almost complete destruction of dopaminergic terminals in the caudate nucleus. By perfusing the ventricles of cats that have lesions in the nigro-striatal pathway, one should be able to determine the specificity of direct electrical stimulation on H^3-catecholamines release. That is, if the H^3-catecholamines originate in dopaminergic terminals, direct stimulation of the caudate in chronically lesioned animals should not evoke the release of catecholamines. The specificity of the observed release of H^3-catecholamines could also be tested by labeling amine stores with H^3-tyrosine rather than H^3-catecholamines. Since tyrosine hydroxylase is present only in adrenergic neurons, any H^3-catecholamines released would necessarily be of adrenergic neuronal origin.

L-Dopa has been used in the treatment of Parkinsonism in an attempt to replenish depleted dopamine stores (Hornykiewicz, 1966[a]). The experimental system developed in this study could also be used to determine if peripherally administered L-dopa can be taken up, metabolized to dopamine and subsequently released upon stimulation of the dopaminergic terminals in the striatum. Previous experiments (Everett, 1970) have demonstrated that L-dopa can increase whole brain concentrations of dopamine but no one has shown that the dopamine thus formed functions as a neurotransmitter.

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