MODULATION OF DOPAMINERGIC NEUROTRANSMISSION BY MORPHINE IN THE RAT

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PETER MOLEMAN

GEBOREN TE EINDHOVEN

PROMOTOR: DR. J. BRUINVELS

CO-REFERENTEN: PROF. DR. H.G.J.M. KUYPERS

PROF. DR. E.L. NOACH

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Wisdom is found on the desolate hillside, El-ahrairah, where none comes to feed, and the stony bank where the rabbit scratches a hole in vain.

R. Adams, Watership Down, MacMillan Publishing Company Inc., Avon Books, p. 285 (1975).

Several sections of this thesis are presented as papers:

I Rapid postmortem Changes in 3,4-dihydroxyphenylacetic acid (DOPAC), a Dopamine Metabolite, in Rat Striatum
J. Neurochem., in press
(With J. Bruinvels and B.H.C. Westerink)

II Differential Effect of Morphine on Dopaminergic Neurons in Frontal Cortex and Striatum of the Rat
Life Sci. 19, 1277 (1976)
(With J. Bruinvels)

III Morphine : a Modulator of Dopaminergic Neurotransmission in the Rat. I Frontal Cortical and Striatal Effects
Submitted for Publication
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IV Morphine : a Modulator of Dopaminergic Neurotransmission in the Rat. II Comparison with the Effect of Haloperidol in the Striatum and N. Accumbens after Manipulation of the Impulse Flow
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V Morphine Catalepsy in Relation to Striatal and Extrastriatal Dopamine
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(With J. Bruinvels)

VI Is Morphine-induced Catalepsy related to Activation of Dopaminergic Neurons?
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CONTENTS

ABBREVIATIONS

A	ampere
Ach	acetylcholine
αMpT	α-methyl-para-tyrosine (-methylester.HCl)
C	curie
cAMP	3',5'-cyclic-adenosinemonophosphate
CNS	central nervous system
COMT	catechol-O-methyltransferase
cpm	counts per minute
CSF	cerebrospinal fluid
DA	dopamine
df	degrees of freedom
$DMPH_4$	2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxyphenylacetic acid
dpm	desintegrations per minute
EDTA	ethylene-diamine-tetra-acetic acid
GABA	γ-aminobutyric acid
GHB	γ-hydroxybutyric acid
h, hr	hour(s)
HVA	3-methoxy-4-hydroxyphenylacetic acid homovanillic acid
i.p.	intraperitoneal
i.v.	intravenous
k	rate constant of DA loss, fractional turnover rate
MAO	monoamineoxidase
3MT	3-methoxytyramine
n	nucleus
NA	noradrenaline
n.s.	not significant
p	probability
PG(E)	prostaglandin (E)
S.D.	standard deviation
S.E.(M.)	standard error (of the mean)
TH	tyrosine hydroxylase
T.O.	tuberculum olfactorium
TYR	tyrosine

INTRODUCTION

The pleasant effects of opium were already known 6000 years ago and opium has been used for medical purposes for at least 3500 years. Opium, and its main constituent morphine, evoke a feeling of well-being and always relieve pain of any origin, in other words, a perfect analgesic and euphoric drug. However, there are unpleasant repercussions. Thus, the pleasant effects are followed by a period of dysphoria. With the first, moderate dose of opiate this rebound is not important. But euphoric and analgesic effects disappear with repeated administration unless the dose is steadily increased. When the opiate is withdrawn after repeated administration of high doses, the dysphoric rebound will gain dangerous proportions and this is one of the reasons for continued (addicted) use of the opiate. Thus, the perfect analgesic and euphoric drug produces a perfect dependence. One of the most important stimuli to the promotion of research into the mechanism of action of opiates is the desire to control opiate-dependence. Such control would help two groups of users: those who need a strong analgesic would not necessarily become dependent and those who become dependent would not necessarily always need opiates. The acute effects of morphine are highly interrelated phenomena such as analgesia and euphoria, while dependence is ascribed to an adaptation of the cells of the body. In order to relate or dissociate these effects of opiates it is necessary to analyse the cellular and molecular events related to the acute effects of the opiate. If these cellular and molecular events are the same as those underlying opiate-dependence, it will not be possible to dissociate the analgesic and euphoric effects of opiates from their ability to produce dependence. However, if it appears that differences do exist between the mechanism of these two opiate-induced phenomena then control of opiate-dependence may be possible.

In the experiments described here, the effects of acute morphine administration on dopaminergic neurons in the rat were investigated. Dopamine is an important neurotransmitter in limbic and extrapyramidal brain areas and plays an important role in the integration of emotional responses. Furthermore, the analgesic and rewarding (euphoric?) properties of morphine have been related *inter alea* to interactions with dopaminergic systems. An attempt has been made to analyse molecular mechanisms involved in the action of morphine on dopaminergic neurons in different brain areas and to analyse the relation of both these actions and behavioural responses to acute morphine administration.

PART I

ACUTE MORPHINE ADMINISTRATION

When morphine is administered for the first time to man or animal, a vast variety of responses can be evoked. Peripheral effects of morphine include constipation, reduced secretion of a number of endocrine glands, bronchoconstriction, dilation of capillaries and stimulation of the uterus (1). The appearance of these and also of the central effects of morphine is mainly dependent on the species and dose of morphine. The above-mentioned effects are of peripheral origin, but it is quite clear, that these effects are mediated through an action on the nervous system. This brings, therefore, the mechanism of peripheral and central effects closer together. Thus, besides research on central effects per se, the action of morphine has also been investigated using less complex peripheral models. The intestine provides an excellent preparation to study interactions of morphine with cholinergic and serotonergic neurotransmission (2, 3, 4).

Central effects of morphine include analgesia, respiratory depression, depression of spinal reflexes, depression or stimulation of locomotion, nausea, vomiting, pupillary constriction (pin point pupil), Straub tail (in mice), release of antidiuretic hormone, hyperglycaemia and hypo- or hyperthermia (1, 5). Again the effects depend on species and dose of morphine.

In spite of many attempts, no success has been obtained in ascribing the effects of morphine on these physiological systems to the interaction of this drug with one clearly defined type of neuron, whether the classification be morphological, anatomical or chemical. The subcellular and molecular mechanisms accounted for in the following sections are therefore, mostly indirectly related to the effect of morphine on the physiological systems described above.

BIPHASIC EFFECTS

Some responses to morphine show a biphasic character in relation to time and dose. In rats a high dose of morphine first induces depression and later stimulation of locomotor activity (6-8). Low doses of morphine only produce the stimulatory response (6, 8-10). Moreover, upon repeated treatment with high doses of morphine, the depression of locomotor activity diminishes, while the stimulation increases and is observable at an earlier period of time after the administration of morphine (6). Other biphasic responses (8), like changes in temperature (11), self-stimulation rate (12) and electrophysiologically recorded neuronal firing (13), show similar characteristics. Specific molecular or cellular events have been called in to account for these biphasic effects (7, 8). The most important observation in all these biphasic responses, however, is that no tolerance appears for the stimulant action in contrast to the depressant effect. Moreover, the stimulant effects are not antagonized by naloxone, a pure opiate antagonist (13, 14). This latter observation has led to the opinion that the stimulatory effects are non-specific. It may very well be, however, that the observed biphasic responses provide an important clue to the understanding of the dependence

producing properties of morphine (8).

METABOLISM OF MORPHINE

The metabolism of morphine and related compounds has been studied extensively in various species (15, 16). It was suggested on the basis of such studies, that heroin (diacetylmorphine) may be potent, because it enters the brain very rapidly. The active component would not be heroin itself, but either 6-monoacetylmorphine (17) or morphine itself (18).

Only recently, however, the metabolism of morphine has been studied in relation to its analgesic activity (19, 20). Dahlström et al. (19) provided a computer-designed model, which related the time-course of morphine distribution in restricted brain regions to different analgesic responses.

Another recent report showed that one of the principal biotransformations of morphine, N-demethylation, is localized in sites with high opiate receptor concentrations (21). This observation may have an important impact on the understanding of the conformation of the opiate receptor and the pharmacokinetics of opiates.

BRAIN SITES OF MORPHINE ACTION

The localization of morphine effects to restricted brain areas has been explored extensively in relation to analgesia and abstinence signs. Evidence is accumulating that the mesencephalic periaqueductal gray area is an important site for the analgesic action of morphine (22, 23). This localization in a rather restricted area was explained by an interaction of morphine with specific pain pathways. The sensitive sites in the periaqueductal gray are closely related to the midbrain raphé nuclei, the major cell body areas of central serotonergic neurons. Lesions (24, 25) and stimulation (26) of these nuclei have marked effects on analgesic responses to morphine. These data suggest that specific pathways from opiate-sensitive sites in the periaqueductal gray to the raphé nuclei are of major importance in morphine's analgesic action. In an extensive account of these data it was also emphasized, however, that direct spinal effects of morphine may contribute considerably to its analgesic action after systemic administration (23).

The specific brain sites thought to be involved in morphine abstinence signs have been studied by means of lesions (27) and application of a morphine antagonist in dependent rats (28, 29). The picture emerging from these studies is not quite clear for several reasons. In an early report the medial thalamus was advanced as a major site. However, the evidence was inconclusive since mainly "wet dog shakes" (one of the abstinence signs in rats) were quantified (28). From other reports it appears that a variety of signs may be elicited more easily from several different brain regions (27, 30). Moreover, "wet dog shakes" may also be antagonized in other brain regions by morphine (31). It thus appears that the thalamus, periaqueductal gray, preoptic area, locus coeruleus and hypo-

thalamus are involved in induction or mediation of various abstinence signs.

NEUROTRANSMITTERS

The question of whether morphine interacts with a specific neurotransmitter or not, has been studied extensively. A number of recent reviews deal with the results of such studies (32-40). Actually, morphine seems to interact with all the neurotransmitters investigated. In most cases the effect of morphine on the levels of the transmitters were studied. Since this does not permit firm conclusions concerning the real effect of morphine, it was appreciated that investigations of the turnover of transmitters would yield more information.

Acetylcholine (Ach)

A decrease in Ach turnover rate has been reported in the rat and cat cortex (8, 41), and in the rat n. accumbens (41), but not in the rat striatum (8, 41). Reduced release of Ach from the cortex of anaesthesized rats and cats has been observed by several investigators (8). Increased cortical release of Ach, however, has been reported to occur in unanaesthesized cats (41). Whether or not contradictory results can be explained by biphasic effects on Ach release, as suggested by Domino et al. (8), remains to be established. A clear involvement of Ach in certain physiological effects of acute opiate administration can not be concluded from the present state of knowledge (40).

Serotonin

As mentioned before, serotonin may play a major role in the mediation of analgesic effects of morphine. Studies of serotonin turnover have shown effects of morphine, but several contradictory results and theoretical objections to the results reported hamper a clear interpretation. There is no doubt that morphine interferes with serotonergic neurotransmission, but the implications of the reported results are quite obscure, as discussed by Eidelberg (40).

Catecholamines

An account of the effects on dopaminergic neurotransmission will be given in some of the following sections. Since profound effects on DA turnover were found, the effects on noradrenergic neurotransmission seem to be neglected to a certain extent. This may be due to the fact that measurement of NA turnover goes hand in hand with measurement of DA turnover. The turnover of NA seems to be increased by morphine in mice (43), while the same appears to apply to the rat, but only in the medulla/pons and not in whole rat brain (44). This latter effect may link up with the observed effect of morphine on the locus coeruleus, a major site of NA cell bodies in the medulla (45) and also a major site of opiate receptors (vide infra).

Other Neurotransmitters

Few reports are available on the role of other putative transmitters in the actions of morphine. The reasons for this lack of attention are obvious. The transmitters described above are reasonably well defined and

more or less accepted as such. Investigations on putative neurotransmitters leave the question open as to whether the observed effects really are involved with changes in neurotransmission. Thus, Sherman and Gebhart (46) investigated the effects of morphine, in relation to painful stimuli, on the levels of aspartate, GABA and glutamate, three putative amino-acid neurotransmitters (47). They observed decreased glutamate levels in the periaqueductal gray after application of a painful stimulus, an effect which is apparently not related to stress in general, since it was not observed after immobilization stress. Morphine, however, induced a rise in glutamate in the same brain area and also abolished the effect of the painful stimulus on glutamate levels. This suggests a role for glutamate in the analgesic action of morphine. As discussed by the authors, however, the role of glutamate as a neurotransmitter is not clear and, therefore, the relevance of these findings is not clear either (46).

GABA has been implicated in the spinal effects of morphine (48). Manipulation of GABA levels antagonized the effects of morphine on DA metabolism (49). Direct effects on GABA-ergic neurotransmission are extremely difficult to assess and information in this field is lacking.

ADENYLATE CYCLASE

Collier and Roy (50, 51) observed that morphine blocks the stimulation of cAMP formation by prostaglandin E(PGE) in brain tissue homogenates. They suggested that this may be the prime site of opiate action. In reaching this hypothesis an important consideration was, that morphine affects many neurotransmitters. A mechanism involving all of them might account for its universal effects. cAMP functions as a second messenger in many hormone-receptor interactions (52), probably also in the interaction of catecholamines in the CNS with their putative receptors (53, 54, 55). The findings of Collier and Roy were confirmed with clonal cells of central nervous origin, which permit more controlled experiments (56, 57). A mechanism of opiate tolerance and dependence based on the effects on cAMP and adenylate cyclase was proposed (57). Morphine does not seem to interact, however, at the same site on the adenylate cyclase as PGE (58). Moreover, the role of PGE-stimulated adenylate cyclase in central neurotransmission is not clear, although PG's have been reported to modulate neurotransmitter release (59). Thus, the PGE-stimulated adenylate cyclase may be a molecular substrate for the central effects of morphine, but the implications for physiological effects of morphine have yet to be assessed, perhaps in relation to the opiate receptors and endorphines described below.

OPIATE RECEPTORS

Specific opiate receptors in nervous tissue were postulated many years ago, but their direct demonstration was only achieved recently, due to technical difficulties (60, 61, 62). Since that time, however, an enormous number of reports have been published on the properties of these receptors. The information available has been cited in some recent

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DOPAMINERGIC NEURON SYSTEMS

Application of fluorescence histochemical techniques as developed by Falck et al. (1-3) resulted in a stereotaxic mapping of monoamine pathways in the rat brain (review: 4). At that time three main dopaminergic systems were discerned:

- the nigrostriatal DA system, with cell bodies in the substantia nigra (A9) and nerve terminals mainly in the caudate-putamen complex, but also in the central nucleus of the amygdala;
- the mesolimbic DA system, with cell bodies in the ventral tegmental area (A10) adjacent to the substantia nigra, and nerve terminals in limbic forebrain structures like the n. accumbens (septi), the n. interstitialis striae terminalis and the tuberculum olfactorium;
- the tubero-infundibular DA system, with cell bodies in the n. arcuatus (A12) of the hypothalamus and nerve terminals in the median eminence.

A modification of the fluorescence histochemical technique, using glyoxylic acid, allowed for the discrimination of DA pathways, which were undetectable before (5).Recently, two other dopaminergic systems were described utilizing this technique:

- the mesocortical DA system (6,7) with cell bodies in the substantia nigra and ventral tegmentum (A9, A10) and nerve terminals in various neocortical areas, like frontal cortex, anterior cingulate cortex, pyriform cortex and entorhinal cortex;
- the incerto-hypothalamic DA system (8), with cell bodies in the caudal thalamus, the posterior and rostral periventricular hypothalamus and the medial zona incerta (A11, 13, 14) and with nerve terminals in hypothalamic, preoptic and possibly septal nuclei.

Moreover, the mesolimbic DA system has been analyzed in more detail (5). A projection from the ventral tegmentum (A10) to the lateral septal nucleus was described (9), which also seems to contain nerve terminals to the incerto-hypothalamic system. In addition, the nigrostriatal and mesolimbic projections seem to be less strictly separated than previously considered. This is also indicated by the fact that mesocortical projections originate from the cell body areas of both these systems.

It is evident from these more recent observations that dopaminergic projections in the telencephalon are more widespread than realized before. Notably the cortical projections introduce new aspects in the role of DA in the brain, since direct influence on neocortical function implicate direct influence at another level of information processing.

The histochemical definition of different DA systems has been paralleled by their functional definition. The nigrostriatal DA system belongs to the basal ganglia, a part of the extrapyramidal motor system. The basal ganglia are regarded as the highest sensory-motor integrating system at the subcortical level. Thus the nigrostriatal DA system is implicated in extrapyramidal dysfunctions like Parkinson's disease in humans and catalepsy in laboratory animals like rats (review: 10). Classicaly, the extrapyramidal motor system was regarded as an independent system converging on the spinal motor apparatus, while the separate pyramidal motor system was assumed to descend directly from the motor cortex to the spinal cord. It is, however, clear that this simple picture can not be regarded as correct any more. The anatomical con-

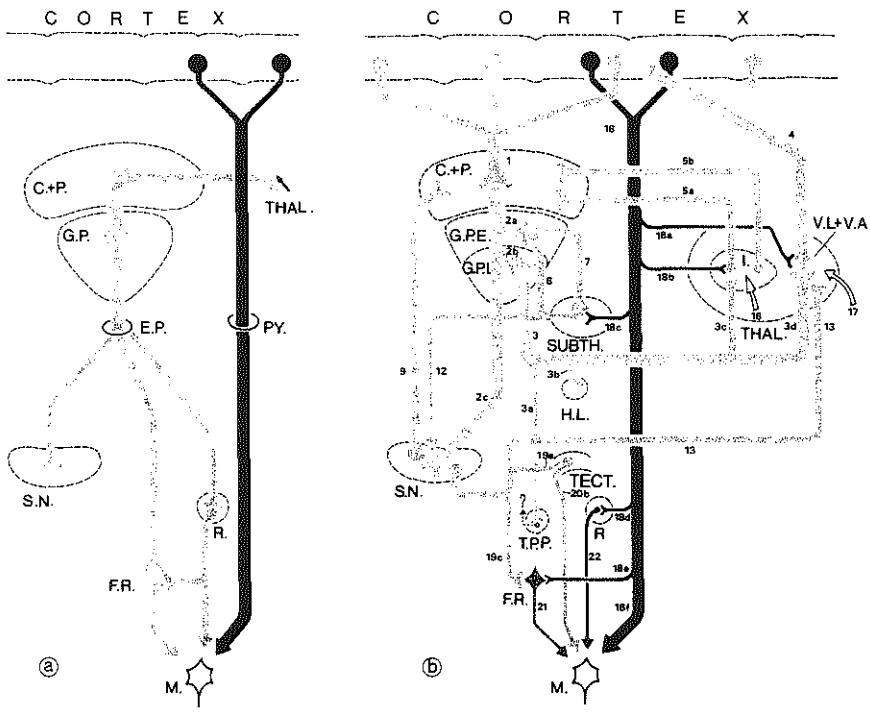

Fig. 1. Diagrams summarizing (a) the classical concept of the pyramidal (PY.) and extrapyramidal (E.P.) motor systems, and (b) our present-day knowledge of the fiber connections of the motor cortex and the striatum. Abbreviations: C., nucleus caudatus; F.R., formatio reticularis; G.P., globus pallidus; G.P.E., globus pallidus, pars externa; G.P.I., globus pallidus, pars interna; H.L., lateral habenular nucleus; I., intralamina nuclei; M., motor neuron; P., putamen; R., nucleus ruber; S.N., substantia nigra; SUBTH., corpus subthalamicum; TECT., tectum mesencephali; THAL., thalamus; T.P.P., nucleus tegmenti pedunculopontinus, pars compacta; V.A., nucleus ventralis anterior; V.L., nucleus ventralis lateralis.
(From ref. 11, fig. 6).
For a detailed description of fiber connections see ref. 11.

nections and interrelations have recently been reviewed (11). It appears that the pyramidal and extrapyramidal systems are closely connected: both contain fibers descending from the motor cortex and other connections exist in thalamic and subthalamic nuclei and in the reticular formation. Fig. 1 (from 11) gives an impression of these connections and also of a number of circuits existing in these systems.

While the extrapyramidal DA system may be involved in Parkinsonism, the mesolimbic DA system has been implicated in schizophrenia. The involvement of DA systems in schizophrenia was gathered from the observation that some drugs which changed dopaminergic neurotransmission, also marked effects on the symptoms of schizophrenia. Thus neuroleptic drugs, regarded as DA receptor blockers (12), alleviate such symptoms. Besides this action they often induce Parkinson-like symptoms. It was suggested that these side effects may be related to DA receptor blockade in the striatum (the extrapyramidal system), while the beneficial effect on schizophrenic symptoms was ascribed to DA receptor-blockade in the mesolimbic DA system (13, 14).

At present the value of this hypothesis can not be fully appreciated. A number of reports indicate quantitative differences in effects of neuroleptic drugs on mesolimbic and nigrostriatal DA systems, which parallel their quantitative differences in antipsychotic and extrapyramidal effects (14-17). This supports the validity of functional distinction between mesolimbic and nigrostriatal DA systems. On the other hand, it has been suggested that the relation between mesolimbic DA and antipsychotic activity may not be as intimate as suggested above (16, 18, 19). Evidence has been reviewed that balances between various neurotransmitter systems, including DA, may be of more importance than the state of activity of a single system (20). Restricting the DA systems to a functional division into mesolimbic and nigrostriatal DA may also be too simple. Thus, the typical extrapyramidal symptom of catalepsy may be related to mesolimbic structures (21). Moreover, the various structures of the mesolimbic system show different reactions to various neuroleptics in behavioural as well as biochemical parameters (16, 22). From the anatomical point of view the distinction between mesolimbic and extrapyramidal structures is also not very strict. Powell and Leman (22) concluded from an analysis of connections of the (mesolimbic) n. accumbens, that it may serve as a bridge between limbic and extrapyramidal motor systems. Besides the described functions, DA systems have been reported to be directly involved in the mediation of reward (23-25) and in memory consolidation (26).

It seems, therefore, that the knowledge of DA systems has increased in recent years to the effect that they are far more widespread in anatomical and functional sense than appreciated before. At the same time knowledge about specific functions of DA systems in extrapyramidal motor regulation and psychotic behaviour may not have made as much progress as was anticipated, perhaps because these functions are less specifically mediated by these systems than presumed.

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DOPAMINE TURNOVER AND THE DYNAMICS OF DOPAMINERGIC NEUROTRANSMISSION: A CRITICAL SURVEY OF METHODS

SUMMARY

Several *in vivo* methods to study dopaminergic neurotransmission are reviewed, with the aim of defining the processes at the molecular level which can be studied using each method. This may provide a basis for the evaluation of effects of drugs and other manipulations. Also, the limitations of each method and their implications for dopamine turnover and functional activity are discussed. It is concluded that drug-effects, observed with any of the present methods, can not be interpreted in terms of changes in dopamine turnover or functional activity of dopaminergic neurons without additional information obtained using other methods.

INTRODUCTION

Many experimental approaches have been introduced to study dopaminergic neurotransmission. Over the course of several years more than 20 parameters have been put forward that might provide information about the functional activity of dopaminergic neurotransmission under normal and drug altered states. Many of these parameters are claimed to be measures of DA turnover, on the basis that increased/decreased DA turnover is an indication of increased/decreased functional activity of DA neurons and vice versa.

Turnover in an open system refers to the velocity at which a substance is formed and broken down while the system is in a steady state. Thus, DA turnover refers to the velocity at which DA is synthesized and degraded while its concentration does not change. It has to be assumed then, that the relevant transformations take place in one compartment. The essential property of a neurotransmitter like DA is, however, that it can be released from the neuron. The assumption that only one compartment is involved, is therefore not valid. It might be assumed, however, that DA is only degraded when released and likewise only synthesized to replenish the released DA.

The relation between synthesis, release and catabolism would be such, that a change in any one process might be associated with a similar change in the other processes. It would be sufficient then to measure one of these processes to permit a conclusion about the overall activity of the system, i.e. the functional activity (1). This approach may be valid in studying the turnover in the physiological situation, since it seems justified to assume that the physiological processes are indeed in balance. When studying the effect of drugs or other exogenous stimuli, however, the aim is mostly to detect a disturbance of the physiological system. It can not be assumed *a priori* therefore, that the balance between the various processes is not disturbed. Thus, observed drug-induced changes in DA turnover often do not represent a reliable index of changes in functional activity. Apart from this, most of the methods

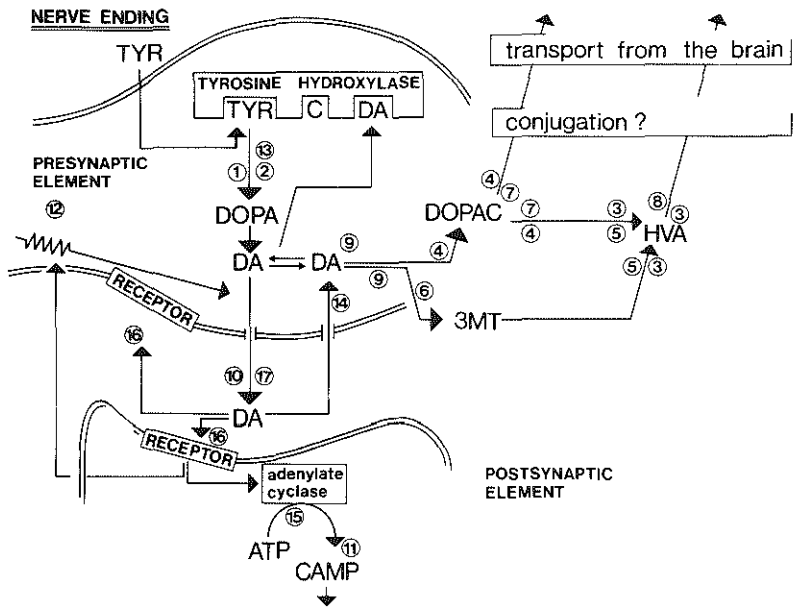

Fig. 1. Dopaminergic nerve ending with synaptic cleft and postsynaptic element. The cellular and molecular events assumed to be of significance for dopamine turnover and for the activity of DA as a transmitter are indicated. Tyrosine hydroxylase is shown separately, because it is assumed to be the rate-limiting enzyme of DA biosynthesis. The activity of the enzyme is regulated by the cofactor (C) and product (DA) (modified from ref. 33). Whether the presynaptic DA really exists as separate pools for release and reuptake is not certain (see text). The numbers associated with the arrows refer to the processes which are directly measured using the methods summarized in table 1.
Abbreviations: TYR: tyrosine; C: cofactor(a pterin); DA: dopamine; DOPA: L-dihydroxyphenylalanine; ATP: adenosinetriphosphate; cAMP: cyclic-adenosinemonophosphate; 3MT: 3-methoxy-tyramine; DOPAC: dihydroxyphenyl-acetic acid; HVA: 3-methoxy-4-hydroxyphenylacetic acid, homovanillic acid.

employed are often not a reliable index of the turnover of DA under the conditions to be studied.

Nevertheless, all the methods used provide a considerable amount of information on the changes occuring in and around dopaminergic neurons when the impact of the results is restricted to the actual process which is studied. The purpose of the present review is to survey the molecular events which are actually measured using several common in vivo methods and to indicate the other parameters on which their reliability depends. This will serve then to evaluate some of the limitations of these methods for assessing effects of drugs or of other manipulations on dopaminergic neurons.

In addition,a short account of in vitro techniques with a direct bearing on in vivo studies is included. Since most studies are performed in rats, the data apply mostly to this species with some reference to mice and cats.

SUMMARY OF METHODS

Fig. 1 shows a dopaminergic nerve ending with synaptic cleft and postsynaptic element. The cellular and molecular events assumed to be of significance for neurotransmission are represented schematically. The conversion of tyrosine, via dopamine, to its major catabolites is shown with special attention being paid to the enzyme tyrosine hydroxylase (E.C. 1.14.3. a), the rate-limiting enzyme in DA biosynthesis (2-4). The presynaptic DA is presented as separate pools for release and reuptake, because it seems that newly synthesized DA may be preferentially released (5.6). The problem of presynaptic DA compartmentation, however, is the subject of considerable debate (6-8). As a result, very little significance should be attached to the break-down of DA being related to a single pool in the present scheme, or to other compartmental divisions. The cellular location of the catabolites DOPAC, 3MT and HVA is not detailed for similar reasons (9,10).

The numbers on the subsequent list of currently available methods refer to the primary molecular event(s) with which they are connected, as shown in fig. 1. An attempt is made to discern which processes are actually measured and which processes are often presumed to be measured.

Table 1.

IN VIVO

	METHOD	PROCESSES ACTUALLY MEASURED	PROCESSES SUPPOSED TO BE MEASURED
1	conversion of labelled tyrosine to DA "labelled precursor method"	DA biosynthesis (TH activity)	DA turnover

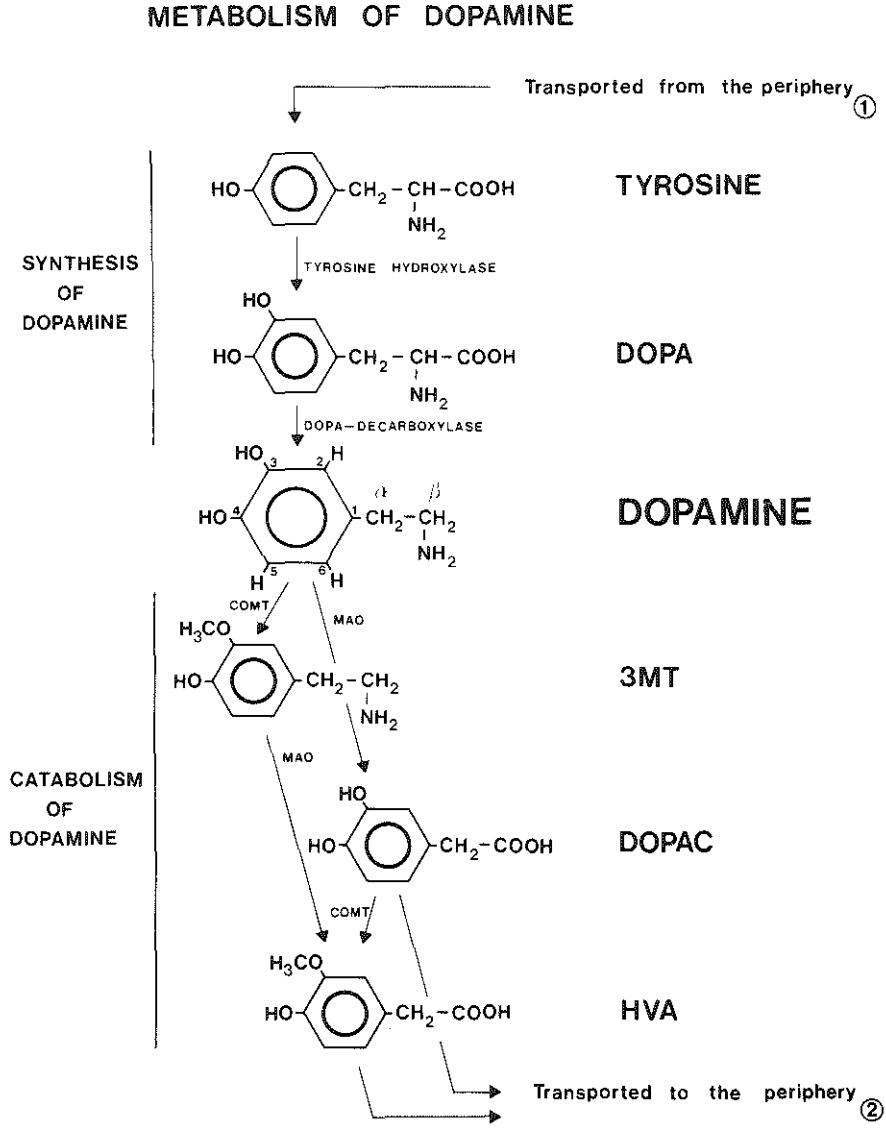

Fig. 2. Metabolism of Dopamine. ① Tyrosine can be transported from the periphery to dopaminergic neurons, but tyrosine can also be formed from phenylalanine in the CNS. ② HVA is transported to the periphery. DOPAC may be converted to HVA, but also transported to the periphery (10). Both these metabolites are possibly conjugated before transport (53). Abbreviations: DOPA: L-dihydroxyphenylalanine; 3MT: 3-methoxytyramine; DOPAC: dihydroxyphenylacetic acid; HVA: 3-methoxy-4-hydroxy-phenylacetic acid, homovanillic acid; MAO: monoamineoxidase; COMT: catechol-O-methyltransferase.

Table 1 (continued)

	METHOD	PROCESSES ACTUALLY MEASURED	PROCESSES SUPPOSED TO BE MEASURED
2	accumulation of DOPA after inhibition of DOPA-decarboxylase "DOPA-accumulation method"	Tyrosine hydroxylation	DA biosynthesis DA turnover
3,4	concentration of HVA and/or DOPAC	net result of formation, break-down, conjugation and transport of HVA and/or DOPAC	DA catabolism DA metabolism DA turnover
5	accumulation of HVA after inhibition of transport "Probenecid method"	formation (and conjugation?) of HVA	DA catabolism DA metabolism DA turnover
6	accumulation of 3MT after MAO-inhibition	formation of 3MT	DA release
7	disappearance of DOPAC after MAO-inhibition	net result of conversion to HVA, conjugation and transport	DA catabolism DA metabolism DA turnover
8	disappearance of HVA after inhibition of MAO and COMT	conjugation and transport of HVA	DA catabolism DA metabolism DA turnover
9	disappearance of DA after inhibition of TH	catabolism of DA	DA turnover, impulse-flow-induced DA release
10	accumulation of DA in fluid superfusing brain tissue	net result of release and catabolism of DA	neuronal release of DA
11	concentration of cAMP	net result of formation and break-down of cAMP	DA-receptor stimulation
		IN VITRO	
13	conversion of labelled tyrosine to DOPA or DA in tissue preparations	TH activity and kinetic parameters	
14	uptake of labelled DA into slices or synaptosomes	net result of uptake and efflux of DA	reuptake of released DA

Table 1 (continued)

	METHOD	PROCESSES ACTUALLY MEASURED	PROCESSES SUPPOSED TO BE MEASURED
15	conversion of ATP to cAMP, stimulation by DA	DA-sensitive adenylate cyclase	DA-receptor stimulation
16	"specific" binding of labelled DA to membrane preparations	affinity for putative DA-receptor	
17	accumulation of labelled DA from tissue preparations into bathing fluid	release of DA	

IN VIVO CONVERSION OF LABELLED TYROSINE TO DA (LABELLED PRECURSOR METHOD)

(^{14}C)- or (^{3}H)- Tyrosine is administered intravenous and the accumulation of labelled DA measured (11, 12, 13). This parameter, therefore, is an indication of the rate of DA biosynthesis. Since the method is based on steady state kinetics (12), several conditions have to be met in the first instance to obtain meaningful results:

-the steady state levels of tyrosine in plasma and brain tissue must not be disturbed;

-the steady state level of DA has to be maintained;

-the specific activity of tyrosine has to be measured, as the incorporation of the label into DA is related to the amount of labelled precursor available.

These conditions can be met and the method seems to provide then a stable measure of relative DA biosynthesis in vivo in untreated experimental animals (12, 13). No absolute value, however, is obtainable, since some of the labelled DA is degraded even after a short pulse label of 10-15 min (14). Moreover, it is assumed that the specific activity of tyrosine represents the precursor pool of DA. Since tyrosine is only metabolized to DA to a minor extent, this assumption is not necessarily valid. The observation that the specific activity of tyrosine and catecholamine formation from labelled tyrosine depends on the time of day at which the experiment is performed, emphasizes the importance of this factor (15).

The method, therefore, provides a measure of DA biosynthesis in vivo, and its reliability depends on whether i) the transport of tyrosine into the relevant metabolic compartment, ii) the degradation of DA and iii) the steady state of tyrosine and DA levels are not changed.

The method is used then to assess drug effects. Neuroleptics, for example, increase the conversion of labelled tyrosine to DA (11, 16, 17). Since tyrosine and DA steady state levels are not disturbed (11), the conclusion that DA turnover is increased seems to be correct. This does not mean, however, that the functional activity of dopaminergic systems is increased. Most probably the increase in DA biosynthesis is a consequence of DA receptor blockade (18, 19). Thus the inference that the observed synthesis increase indicates increased DA turnover may be correct, whereas the conclusion that functional activity is increased is

incorrect.

Lesions of the dopaminergic cell bodies in the substantia nigra also cause a tremendous increase in the accumulation of labelled DA (20-23). In this case, however, the observed synthesis increase can not be explained as an increased DA turnover, since DA concentrations also increase rapidly (21, 22, 24) and the degradation of DA seems to be interrupted for a short time (25, 26). Thus, neither increased DA turnover nor increased functional activity can be concluded from the observed increase in the incorporation of labelled tyrosine into DA.

Several modifications of the described method are known. The labelled tyrosine may be infused instead of administered as a pulse (11, 27). Since the aim of steady states in the labelled tyrosine and catecholamine pools is not even achieved within 60 min (11), this method does not seem to be superior. Moreover, the procedure is more complicated. The absence of steady states after 60 min shows, that mixing of the tyrosine pools in blood, extracellular fluids and neurons is slow. It may be expected therefore, that the specific activity of tyrosine in brain tissue, obtained in pulse label experiments, does not represent the precursor of DA (vide supra).

3,5(^{3}H)-tyrosine may also be introduced locally into the CNS to form (^{3}H)-H_2O upon hydroxylation (28). It is difficult to be sure then that steady state concentrations of tyrosine are maintained and that the labelled precursor is reproducibly distributed. The method does not provide an absolute measure of TH *in vivo* because of diffusion of (^{3}H)-H_2O (7, 28). It also provides data for TH activity in both dopaminergic and noradrenergic neurons, though this might not be a serious drawback when used in the striatum, since in this structure relatively little noradrenaline is present. An advantage of this method is the large amount of radioactivity that can reach the CNS.

More modifications are available, but the characteristics do not differ widely from the ones described.

In conclusion the labelled precursor method provides under conditions that can be reasonably well controlled, a relative measure of *in vivo* DA biosynthesis. When the effects of drugs or other manipulations are studied, the results obtained may be biased by changes in tyrosine transport and degradation of DA. The implications for DA turnover and functional activity can only be evaluated in conjunction with other methods.

IN VIVO ACCUMULATION OF DOPA AFTER DOPA-DECARBOXYLASE INHIBITION (BY NSD-1015 OR RO 4-4602)

Another method to measure TH activity *in vivo* was introduced by Carlsson et al. (29). The accumulation of DOPA after inhibition of its conversion to DA is measured and assumed to reflect the conversion of tyrosine to DOPA. Several conditions have to be met in the first instance to obtain meaningful results:

-inhibition of DOPA-decarboxylase has to be rapid and complete;

-disruption of the steady state levels of DOPA, DA and its metabolites must not affect TH activity;

-steady state levels of tyrosine must not be disturbed.

The first point is not entirely clear. The enzyme inhibition, in fact, seems to be rapid, since DOPA accumulates immediately (23). Whether the inhibition is complete or not, can not easily be evaluated from the data available. The levels of DA do not decline for up to 30 min after

NSD-1015 administration, although this would be expected when the synthesis of DA is inhibited. The lack of DA loss may be due then to an additional inhibitory effect of NSD-1015 on DA breakdown by MAO (29), which might subsequently result in decreased TH activity (14, 29, 30). On the other hand, an incomplete inhibition of DOPA-decarboxylase could account for the lack of DA loss.

Linear accumulation of DOPA for 30 min is taken as an indication that DOPA itself does not affect TH activity. In view of the variability of DOPA levels obtained, very severe deviations from linearity would be necessary to be detectable in an experiment. Moreover, other data suggest that DOPA-decarboxylase-inhibition does indeed affect tyrosine hydroxylation in vivo (28, 31). Whether or not the observed increase in endogenous tyrosine is of significance for the present method is not clarified (29).

Thus, a number of theoretical objections to the present method have not yet been disposed of. No absolute indication of DA biosynthesis can be obtained, therefore. This is also illustrated by the lower rate of DA synthesis which is obtained, when compared with other methods (29, 31). Moreover, some practical points merit attention. In order to measure TH activity in DA neurons, the method is only applicable to structures which are relatively abundant in these neurons, in comparison to noradrenergic neurons, since the contribution of noradrenergic neurons can then be neglected. Furthermore, the brain tissue of several rats has to be combined because of the low levels of DOPA (32).

Thus, the method may provide a measure of TH activity in vivo and its reliability depends mainly on possible interfering changes in tyrosine hydroxylation induced by DOPA itself, DA or altered precursor availability.

The method has been used to assess the effect of transsection of ascending DA fibers on TH activity (23) and leads to qualitatively similar results as the labelled precursor method (vide supra). It has also been used to evaluate the effect of γ-butyrolacton (GBL) or γ-hydroxybutyrate (GHB), drugs which seem to have a similar effect on DA biosynthesis as cerebral hemisection (21, 24, 33).

In evaluating such unequivocal effects on DA biosynthesis the labelled precursor method and DOPA accumulation method provide similar results. Javoy et al. (34), however, obtained different results with these two methods when evaluating atropine-induced effects on DA biosynthesis. Atropine blocks acetylcholine receptors and since an intimate interaction between cholinergic and dopaminergic systems exists in the striatum, atropine also changes dopaminergic neurotransmission. Thus 5 mg/kg atropine increased the accumulation of labelled DA,but was without effect on DOPA-accumulation. Whatever the interpretation of the mechanism of action of atropine may be, some observations merit attention. Utilizing the labelled precursor method, atropine was also observed to reduce endogenous tyrosine and labelled tyrosine levels. Although the specific activity of tyrosine was unchanged, these observations raise the suspicion that the real tyrosine precursor pool could be changed by atropine treatment (vide supra). The endogenous tyrosine concentration, on the other hand, was not changed by atropine, when the DOPA-accumulation method was utilized. But this implies a relative reduction in endogenous tyrosine levels, since tyrosine concentrations are normally increased after inhibition of DOPA-decarboxylase (29, 34). With the latter method no DA concentrations were reported (34). Thus, atropine treatment may bias measurement of DA biosynthesis with either one or both methods utilized, because of changes in precursor availability. Since both methods

are used to measure DA biosynthesis and application of the results of either method to the interpretation of DA turnover has to be carried out with caution, it might be dangerous to draw conclusions from differences in results obtained with both methods (34).

In conclusion, the DOPA-accumulation method provides a measure of in vivo DA biosynthesis. In the evaluation of drug effects it should be observed that inhibition of DOPA-decarboxylase by NSD-1015 already results in changes of TH activity and that this may interfere with the drug effect. Results can not be generalized to DA turnover or to the functional activity of the dopaminergic neurons.

IN VIVO CONCENTRATION, ACCUMULATION OR DISAPPEARANCE OF DA CATABOLITES HVA, DOPAC AND 3MT

A number of different methods have been developed concentrating on the measurement of one or more DA catabolites. The frequent use of such methods was triggered by the observations of increased levels of all DA catabolites induced by chlorpromazine (18, 35). Only recently, however, kinetic analyses of changes in catabolite levels have been reported to provide a theoretical foundation for the use of these methods (10, 36, 37). The methods employed can be divided into those analysing concentrations without interference with synthesis, breakdown or transport of the substance studied and those assessing accumulation or disappearance of the substance after interruption of synthesis, breakdown, transport or a combination of these. The former approach is most often applied in drug studies (vide infra). Since measurement of basic catabolite levels, without other parameters, however, does not provide information about their turnover, the latter approach was utilized to perform kinetic analyses.

IN VIVO CONCENTRATION OF HVA AND DOPAC

HVA and DOPAC are present in normal brain (38, 39) while 3MT is almost undetectable (40). Positive values for 3MT in normal brain may be due to postmortem accumulation (41). Increased HVA and DOPAC levels, such as those obtained after chlorpromazine treatment, are observed with most neuroleptics like haloperidol, thioridazine, clozapine, loxapine, pimozine and many others (e.g. 42-51).

This provides a pragmatic basis for the evaluation of drug effects similar to neuroleptics. It is, however, not clear why increased catabolism should result in increased concentrations of catabolites. A tempting suggestion is that the active transport out of the brain of acid catabolites is easily saturable (52). The transport of HVA would then be unable to keep up with its enhanced formation induced by neuroleptics. HVA, however, also seems to be conjugated in brain tissue to a significant extent (53). The same considerations apply to DOPAC, since it may also be transported out of the brain (10) and conjugated (53). Moreover, DOPAC is converted to HVA. Thus, increased concentrations of HVA and DOPAC seem to provide a measure of increased catabolism in vivo, and its reliability for measuring drug effects seems to depend on whether i) the transport out of the brain, ii) conjugation and iii) degradation (i.e. of DOPAC to HVA) are not changed.

Most often increased catabolism of DA is taken as an indication of

increased metabolism of DA, thus suggesting that the synthesis is also increased. In the case of neuroleptics, which probably only interfere with presynaptic mechanisms indirectly, i.e. via physiological feedback mechanisms (54, 55), this may hold true. Although assessing changes in HVA (and DOPAC) levels may provide, therefore, important information about the effects of various neuroleptics. It may be hazardous, however, to relate the potency of different neuroleptics to increase HVA and DOPAC concentrations to their behavioural potency (e.g. 48) and also to relate the potency to increase HVA and DOPAC concentrations in different brain structures to their potency to block DA receptors in these structures (42, 51). These relations may not be correct,because the often subtle differences between various neuroleptics in increasing HVA and DOPAC concentrations may very well be related to effects on e.g. transport or conjugation of the catabolites. These mechanisms may differ in various brain structures (10, 49).

Roth and coworkers, on the other hand, correlated changes in the levels of DOPAC with changes in the impulse flow of dopaminergic neurons (25, 32, 56). This correlation seems to apply in many cases. Nevertheless, changes in one of these parameters may not always be associated with changes in the other parameter. Apomorphine, for example, reduces DOPAC levels in the striatum even after interruption of the impulse flow by cerebral hemisection (26). Under these conditions DA biosynthesis is decreased by apomorphine (22), which may account for the decrease in DOPAC levels. The decrease in DOPAC levels, however, is not associated with decreased impulse flow.

In conclusion, measurement of drug-induced changes in catabolite concentrations provides a rough measure of DA catabolism. An advantage of the method is that no other intervention than drug administration is necessary. Special attention should be paid to possible interferences of drugs with the transport of the catabolites from the brain and with the conjugation of the catabolites (see also paragraph on disappearance of catabolites). However, information concerning these mechanisms is very scarce. To evaluate effects on DA turnover or functional activity of dopaminergic neurons,additional information is necessary.

IN VIVO ACCUMULATION OF HVA AFTER INHIBITION OF ITS TRANSPORT BY PROBENECID (PROBENECID METHOD)

The active transport of acids from the brain is inhibited by probenecid (57), which results in accumulation of HVA (9, 58) but not of DOPAC (9). The accumulation of HVA may be a better measure of HVA formation than the assay of mere HVA concentrations. Several conditions have to be met to obtain meaningful results:

-inhibition of transport has to be complete;

-formation of HVA must not be influenced by the increase of its levels.

Whether these conditions are met is not entirely clear. Westerink and Korf (10) observed an accumulation of HVA of only 2 nmol/g/h in rat striatum with the probenecid method, while other methods result in values of about 20 nmol/g/h. This may indicate that,at least in the rat, one or both of the conditions are not met. The probenecid method thus provides a measure of HVA formation. It is mostly used in studies of DA catabolism in human CSF. For animal studies it may be of less value,

because DOPAC cannot be studied in the same way, although it has been suggested that the transport of conjugated DOPAC is also very sensitive to probenecid (53). Moreover, the transport inhibition of HVA may differ in various brain regions (49, 59).

IN VIVO ACCUMULATION OF 3MT AFTER MAO INHIBITION (WITH PARGYLINE)

Recently Kehr (60) introduced a method, based on the assay of 3MT, which is utilized after inhibition of MAO to block all other catabolic pathways of DA. 3MT is almost absent in normal brain tissue, but rapidly accumulates after pargyline administration (40, 60). The method provides a measure of DA catabolism, i.e. methylation. Since it is obvious that not all DA is catabolized to 3MT but also to DOPAC under normal conditions, the method does not provide a measure of normal DA catabolism. In fact, it was not introduced as such, but it was suggested that the accumulation of 3MT is an indication of impulse-flow-induced DA release in rat brain in vivo (60), linking up with the suggestions of Roffler-Tarlov et al. (9) on compartmentation of DA catabolism. Several experimental data (60), however, seem to be incompatible with this concept:

-3MT accumulation was increased after cerebral hemisection which interrupts DA release;

-DA synthesis inhibition prevented almost entirely 3MT-accumulation during inhibition of DA release by cerebral hemisection;

-haloperidol-induced increase in 3MT was largely reduced by DA synthesis inhibition, while DA levels decreased in the same experiment indicating DA release.

These data seem to indicate that 3MT accumulation may be taken as much as an indication of DA biosynthesis as of DA release. Moreover, pargyline may not entirely inhibit MAO (30, 40), but does reduce DA biosynthesis within 10 min (23, 30).

In conclusion, unless the latter objections are dealt with, the method does not provide an absolute measure of one of the metabolic pathways of DA. Generalised conclusions on DA turnover or functional activity based on this method thus seem premature.

IN VIVO DISAPPEARANCE OF DOPAC AND/OR HVA AFTER INHIBITION OF MAO AND/OR COMT

In this experimental approach the formation of catabolites is prevented by inhibition of the enzymes involved in their formation and the disappearance of the catabolite is measured. The method provides, therefore, a measure of degradation and/or transport of the studied catabolite. Conditions for obtaining meaningful results are:

-the enzyme has to be entirely inhibited;

-disturbance of the steady state levels must not influence the first order kinetics of disappearance of catabolites.

As mentioned before, pargyline, the commonest MAO-inhibitor, may not entirely block MAO (30, 40). Furthermore, the disturbance of steady state levels of catabolites may not change the kinetics of their disappearance (10, 37). The influence of pargyline on DA biosynthesis (23, 30) is of less importance in this method, because changes in DA biosynthesis do not influence the catabolite pools after inhibition of catabolite formation

by MAO.

Furthermore, the decline of HVA after MAO inhibition will be delayed by de novo formation of HVA from DOPAC, but one can correct for this, either by also inhibiting COMT (10) or by analysing HVA disappearance when formation from DOPAC has declined to negligible amounts (10, 37). Consequently, the only draw-back seems to be the general toxicity of the inhibitors used. Thus, the method provides a measure of disappearance of catabolites in vivo, and its reliability seems to depend on whether the synthesis inhibition is complete or not.

The rate of disappearance of DOPAC is calculated to be about 20-24 nmol/g/h for rat striatum (10, 37 and calculated from data of 25), probably reflecting the total rate of disappearance of DA catabolites (10). Assuming steady state kinetics, under control conditions this may reflect total DA turnover and is close to values obtained with the labelled precursor method (13, 61) and αMpT method (13) (vide infra). Nevertheless, this observation is rather surprising, since, by this method it is actually the rate of disappearance of catabolites which is measured, while when using the labelled precursor method, the rate of the DA synthesis is measured and when using the αMpT method the rate of disappearance of DA is measured.

The latter two methods may yield similar values, since the concentration of DA is maintained within narrow limits by feedback mechanisms on DA biosynthesis, thus equilibrating loss of DA and its biosynthesis. When the disappearance of catabolites is measured, however, MAO (or MAO and COMT) are inhibited. This implies that changes in DA biosynthesis, the presumed rate-limiting step in DA turnover, have no influence on the disappearance of catabolites. Therefore, another rate-limiting step, entirely independent of the regulation of synthesis and release of DA, is involved in the disappearance of catabolites. This may also be deduced from the observation that DOPAC and HVA concentrations are considerably higher than zero, since these would have been almost zero if DA release would have been the only rate-limiting step in the chain of events leading up to removal of catabolites from the brain. Since DOPAC and HVA, but not 3MT concentrations are considerable, it may be speculated that transport or conjugation of acid metabolites is the rate-limiting step involved. In any case, this rate-limiting process will include a step involving low substrate affinity resulting in accumulation of catabolites up to levels at which a balance exists between the turnover of DA and of catabolites, and thus producing similar turnover rates, as discussed above.

Some important conclusions can be drawn from this concept. Evaluation of effects of drugs on the disappearance of DOPAC and HVA after MAO (or MAO and COMT) inhibition has, for this reason, no bearing on the effect of the drug on DA turnover. On the other hand, it would be of value as additional information for studies of drug effects on HVA and/or DOPAC levels, since changes may be induced by interference with (the rate-limiting step of) catabolite removal instead of, or in addition to, interference with DA turnover. Furthermore, it may be speculated that the profound differences in steady state levels of catabolites relative to DA and to each other in different species (62) point to differences in catabolic pathways, possibly caused by differences in the rate-limiting step of catabolite disappearance.

In conclusion, measurement of the disappearance of DA catabolites in vivo, when their formation is inhibited, provides information on the kinetics and pathways of DA catabolism. Assessment of drug effects on these parameters, however, can not provide any information about DA

turnover or functional activity.

IN VIVO DISAPPEARANCE OF DA AFTER INHIBITION OF ITS SYNTHESIS BY αMpT (SYNTHESIS INHIBITION METHOD)

The decline in DA levels can be measured after administration of a potent blocker, αMpT of TH, the rate-limiting enzyme of DA biosynthesis. In a strict sense, this decline is a measure of DA catabolism, since DA disappears as a result of catabolism. The method was introduced to obtain a generalized measurement of both DA synthesis rate and DA turnover, assuming steady state kinetics (1), i.e. assuming a balance between synthesis, release and catabolism (see Introduction). Although results have been obtained which were in reasonable agreement with other methods (labelled precursor method, disappearance of catabolites, vide supra), several conditions have to be met to validate the method:

-the enzyme inhibition must be instantaneous and complete in vivo;

-αMpT must not interfere with DA decline;

-the disturbance of DA steady state levels must not influence the kinetics of its decline.

It has been shown that in vivo the enzyme inhibition is complete (63), but not instantaneous, since 15 min after αMpT administration the inhibition is only about 60% (13, 63, 64). Moreover, αMpT, or its metabolites, seem to interfere with DA decline during the first 20-40 min after its administration (13). The impact of these observations is not entirely clear, since they provoked discussion on the fundamental problem of compartmentalization of DA (5, 6, 8, 13), which is beyond the scope of this review. However, it is clear that a biphasic decline in DA levels is obtained, during which the first rapid phase is important only up to 20 min after αMpT treatment (5, 13).

The synthesis inhibition method as introduced by Brodie et al. (1), was then applied to drug studies. The underlying concept was to administer the drug to be studied, which would then induce a new steady state of DA synthesis, release and catabolism, and then to administer αMpT. Assuming steady state kinetics, one could apply the drug effect on DA decline to DA biosynthesis and turnover (e.g. 42, 65-68). However, the disappearance of DA is measured while its synthesis is impaired. The value of this indirect assessment of drug effects on DA biosynthesis and turnover is thus seriously questioned by the observations described above. Moreover, it does not seem warranted to assume that the drug induces a new steady state. It does not seem justified, therefore, to apply the results to a discussion of DA biosynthesis and turnover.

A modified synthesis inhibition method was introduced to avoid these pitfalls. Thus the drug to be studied was administered at least 30 min after αMpT, when complete inhibition of DA synthesis was achieved (66, 69, 70, 71). This precludes any interpretation in terms of DA biosynthesis and DA turnover, since the drug can not change DA biosynthesis. Andén et al. (72) emphasized the importance of impulse flow for the disappearance of DA as a result of αMpT administration. Studying drug effects, however, it may be more accurate to state that the method provides a measure of DA disappearance, consisting of release and catabolism and possibly reuptake, and that its reliability depends on whether i) disturbance of DA steady state levels has an influence on the kinetics of its own decline and whether ii) newly synthesized DA is of particular importance. The first point is a serious draw-back of the method. DA

levels decline exponentially, because the available DA diminishes, and thus less DA will be released into the synaptic cleft. Since rapid feedback mechanisms are also in operation, which are regulated by the amount of DA reaching synaptic receptors (66, 73), it may very well be possible that the normal release of DA is changed after αMpT treatment. As to the second point, a possible separate pool of newly synthesized DA with high turnover will be entirely depleted 30 min after treatment with αMpT (5, 6, 7, 70), since its turnover time is less than 10 min (5). The drug effects studied are, therefore, concerned with the storage pool (70, 74), or, alternatively, when not taking recourse to a particular theory, they are concerned with effects on DA release (and catabolism) which are entirely unrelated to DA biosynthesis.

It has been observed that morphine does not affect the decline in DA levels in the rat striatum, when applying the modified synthesis inhibition method (63, 69), whereas it increases DA decline when utilizing the conventional method (67, 69, 75). This illustrates the fact that different results may be obtained with the two methods. The difference in this instance may be due to the fact that morphine markedly increases DA biosynthesis (76-78), which effect is eliminated utilizing the modified synthesis inhibition method but not utilizing the conventional method.

In conclusion, the conventional synthesis inhibition method seems to be of value only in control conditions. The modified method does provide a measure of DA catabolism which is probably related to release of DA. This distinction, however, has to be evaluated with the aid of other methods. Effects of drugs on these parameters can be studied when special attention is paid to the interference with receptor-mediated feedback because of declining DA levels. The effects obtained are not related to newly synthesized DA and those effects which depend on the presence of newly synthesized DA will be missed, therefore. The implications for DA turnover and functional activity have to be evaluated in conjunction with other data.

IN VIVO RELEASE OF DA

Many attempts have been made to measure DA release directly, since this is a crucial event in neurotransmission. It has often been claimed that methods such as those described above, provide an indirect index of DA release. The only way to avoid the pitfalls involved in interpreting the results obtained by these methods, is to measure DA release directly. For that purpose extracellular DA has to be trapped in one way or another when it is released from the neuron. *in vivo* methods aiming at this goal involve superfusion of brain tissue and analysis of DA in the perfusion fluid. Since all variations of these methods are technically difficult and time consuming, they are not in general use. Moreover, the specificity of results obtained is sometimes difficult to assess, since many artefacts are possible (79). Nevertheless, it seems to be the only approach available to obtain direct information about DA release *in vivo*.

Superfusion of DA rich brain tissue can be achieved by perfusion of the lateral ventricles bordering parts of the caudate nucleus (80-82), by superfusion of the caudate nucleus using a cup technique after removal of cortical tissue (83-85) or by the use of a push-pull canula placed into the brain tissue (86-88). All these techniques involve anaesthesia and fairly extensive surgery in the cats, which are utilized because of their size. With the first method the DA has to be released into the

ventricle and it is uncertain whether this reflects neuronal release.

The DA in the perfusion fluid is most commonly measured by radioisotopic techniques. Introduction of the label by application of radioactive DA itself may not label the appropriate transmitter pool. Therefore, it is preferable to introduce the label by addition of radioactive tyrosine to the perfusion fluid. Systemic application does not yield measurable amounts of DA. Local introduction of label as well as drugs will result in a gradient declining from the place of perfusion. It is not known whether this uneven distribution affects the results obtained. Similarly it is not known whether the results obtained locally can be applied to the whole structure studied. It has been observed with superfusion of striatal tissue that transsection of the nigrostriatal bundle not only inhibited DA release, but also DA biosynthesis (83). This latter observation is in conflict with many other reports (20-23), and may indicate that the results of the superfusion study can not be applied to the whole striatum.

The recently developed highly sensitive radioenzymatic assay of DA (89) made possible the measurement of authentic DA in the perfusion fluid (88). This may be a major advantage, since the pitfalls of radioactive labelling techniques are avoided.

Changes in released DA induced by various stimuli, are only detectable when they occur relatively slow. This may hamper the interpretation of results, especially when shortlasting or biphasic responses are induced.

In conclusion, superfusion of brain tissue seems to be the only technique available to measure DA release in vivo. Results obtained, however, are hazardous to interpret. Special attention has to be paid to the specificity of the release measured and the time course of responses (79).

IN VIVO cAMP CONCENTRATIONS

Evidence has been presented by in vitro experiments that the striatum may contain a DA-sensitive adenylate cyclase which may function as a DA receptor (90, 91). Adopting this concept Costa et al. showed that some DA agonists increased considerably the in vivo levels of cAMP in DA rich brain tissue (8, 54, 92, 61). The response was blocked by haloperidol, a receptor blocker (61, 92). Therefore, the method may be an important tool for the evaluation of DA receptor activity in vivo. On the other hand, the method has not yet been characterized extensively with respect to

-specificity for DA-receptor interaction;
-localization (pre- or postsynaptic or neither);
-time course of changes in cAMP levels.

The fact that in vitro DA-sensitive adenylate cyclase seems to be reasonably specific for DA does not imply that the in vivo observed changes of cAMP levels induced by DA agonists and antagonists are DA-specific phenomena. The relative sensitivity to other drugs has to be assessed (93). In striatal slices the increase in cAMP elicited by DA, is distinguishable from the increase induced by NA. This latter increase, however, is predominant (94) and the existence of a DA-specific adenylate cyclase in the striatum has even been doubted (95).

The implications of results for the functional activity of dopaminergic neurons depend on the localization of the presumed receptor. The DA-sensitive adenylate cyclase (96) which is assayed in vitro seems to be localized postsynaptically. However, this can not be taken as evidence

that the in vivo changes of cAMP levels are related to postsynaptic DA receptors. Because cAMP turnover in vivo is very rapid, an effect on cAMP levels can easily be missed (93).

In conclusion, changes in cAMP levels may present a measure of in vivo DA receptor stimulation, but the specificity and reliability of this method has to be investigated more extensively.

IN VITRO METHODS

The aim of the present review is to survey methods which provide direct information about in vivo processes. Information about specific processes like (re)uptake, receptor affinity, enzyme kinetics etc., however, can only be obtained in vitro. These data are indispensable for the evaluation of in vivo processes and effects of drugs. The in vitro methods are discussed here only in relation to the direct information they offer for in vivo studies.

IN VITRO TYROSINE HYDROXYLASE (TH) ACTIVITY

The in vitro method which has probably the most direct bearing on the turnover of DA in vivo, is the assay and kinetic analysis of TH activity. To a (striatal) tissue preparation (slices, synaptosomes, membrane-free supernatant) radioactively labelled tyrosine is added and and the conversion to DA during in vitro incubation is measured. When the label is recovered in (^3H)-H_2O, which is formed during the conversion of tyrosine to L-DOPA, the activity of TH is directly measured (7, 97). Often, however, (^{14}C)-CO_2 is measured, which is formed during the subsequent conversion of DOPA to DA (98, 99). Utilizing these techniques, quite a lot of information about the regulation of DA biosynthesis has been obtained (31, 100-103 and references therein). Among other things it appeared that the activity of TH is under the regulatory influence of both the cofactor, an unconjugated pterin,and the product DA and other catechols.

Many studies were performed with the soluble enzyme, which is only one of several species normally present and which possess widely different kinetic characteristics (102). Moreover, the artificial cofactor $DMPH_4$ is most often used instead of the putative natural cofactor, which displays different kinetic characteristics (100). A number of other factors, like ionic composition of the assay medium, are kept constant, while they may vary in vivo and affect TH and possibly change drug effects (104, 105).

It has been observed, for example, that TH activity of the soluble enzyme preparation is increased by an acute lesion of the nigrostriatal pathway (104), or by cerebral hemisection (106), while the opposite effect was observed in a synaptosomal preparation (105). Similar lesions result in increased DA biosynthesis in vivo (20-23). Injection of amphetamine, on the other hand produced an increase in biosynthesis of DA in vivo about 30 min after injection (33, 54, 107, 108), but failed to change TH activity in the soluble enzyme preparation (54) and even decreased TH activity in synaptosomes (102, 109, 110). When added in vitro, however, TH activity in the synaptosomal preparation is increased by amphetamine

(109, 110). Similarly, morphine did not affect TH activity in the soluble enzyme preparation (54, 111), while DA synthesis is stimulated in vivo (54, 76, 78). Whatever these manipulations may affect in vivo, these examples show that in vitro TH activity does not always reflect changes of DA biosynthesis in vivo. In some of these cases a factor may be involved in vivo, which is lost in the in vitro assay. Some factors of significance for DA biosynthesis in vivo and which are lost in vitro, may be:

-accumulation or disappearance of DA which acts as (feedback) inhibitor of TH;

-changes in cofactor levels;

-changes in different forms of TH, such as shifts in the equilibrium of soluble and membrane bound TH;

-changes in ionic environment (e.g. calcium);

-changes in precursor availability;

-changes in O_2-pressure.

In conclusion, in vitro assay of TH activity and analysis of its kinetic characteristics may provide information about the molecular mechanisms of changes in DA biosynthesis in vivo. Since changes in vivo, however, may be lost or altered in vitro, application of the results obtained in vitro to the interpretation of effects in vivo is not always justified.

IN VITRO UPTAKE OF DA

It is assumed that much of the DA which is released, is inactivated by reuptake into the neuron. Drugs may affect DA turnover and functional activity by interaction with this mechanism. The uptake of radioactive DA into synaptosomes or slices of striatal tissue, may provide information about effects of drugs on this parameter. It is, however, well-known, that DA can also be taken up extraneuronally in the CNS, although the function of such a mechanism is not entirely clear (112). Moreover, most drugs exhibit effects on DA uptake at one concentration or another and the relative potency in vivo may not always be assessed easily (e.g. 113). Furthermore, uptake of DA is not easily distinguished from DA release by such methods (114).

IN VITRO DA-SENSITIVE ADENYLATE CYCLASE

DA-sensitive adenylate cyclase activity can be assayed in vitro and may reflect DA receptor activity (90, 91). Possibly the enzyme is located postsynaptically (96) and may, therefore, provide information about the activity of drugs on postsynaptic DA receptors.

IN VITRO DA RECEPTOR BINDING

Radioactively labelled DA or haloperidol can be bound to membrane material of synaptosomal tissue preparations (115). This may reflect binding to the (postsynaptic?) DA receptor, when a number of controls, such as exclusion of aspecific binding, have been obtained (116). The receptor binding reflects the affinity for the binding site of the receptor. Some reports suggest that the DA receptor may exist in two conformations, one binding DA agonists, and the other binding DA antagonists (115, 116). Although a correlation of binding characteristics of neuro-

leptics in vitro with their antipsychotic activity in vivo has been suggested (117), the implications of the method for in vivo activity has yet to be assessed. The method will not provide information about intrinsic activity, but only about receptor affinity.

IN VITRO DA RELEASE

Radioactively labelled DA can be released in vitro from tissue preparations by drugs or field stimulation (79, 118). A number of conditions for such release seem to be similar to those for physiological release (79). Recently a method was presented with which the release of newly synthesized DA and the synthesis rate of DA can be measured at the same time in striatal slices (7, 21). (3,5-^{3}H)-Tyrosine was superfused over the slices and (^{3}H)-H_2O and (^{3}H)-DA measured in the superfusate. (^{3}H)-H_2O is formed from the (3-^{3}H) during conversion of tyrosine to DOPA, while the (5-^{3}H) is left behind in the DA molecule. Therefore, analysis of (^{3}H)-H_2O in the incubation medium provides a measure of DA synthesis and (^{3}H)-DA of DA release. DA release is almost nil in this situation, but can be stimulated by increasing K^+ concentration (119), which causes depolarization of the membranes. There were, however, marked differences from results of other investigations (105) which ask for evaluation.

The in vitro studies of DA release complement the in vivo experiments and allow controlled studies to be made, which are impossible in vivo. Interpretation of results in relation to in vivo processes, however, is not always possible (79).

CONCLUDING REMARKS

A survey has been made of methods which can be used to study various in vivo processes involved in the functional activity of dopaminergic neurons. Important processes involved are DA biosynthesis, release and catabolism. Each of these processes may be regulated by a number of mechanisms. DA biosynthesis, for example, may be regulated by intraneuronal product (DA) inhibition (6), by presynaptic receptors or by postsynaptic receptors via transsynaptic mediators (32) or via impulse flow, i.e. neuronal activity (40, 55). It seems that all these mechanisms can operate in the normal brain. Which regulatory mechanism is most important may depend on the way in which the physiological balance is altered by drugs or other manipulations.

It has been observed, for example, that the increase of DA biosynthesis and change of TH kinetic characteristics is prevented by haloperidol, a DA receptor blocking agent (18), when the nigrostriatal pathway is severed (106). This seems to imply, in keeping with other results (40, 55, 120), that striatal TH activity is regulated via impulse flow in the nigrostriatal DA pathway. It certainly does not imply, however, that haloperidol-induced changes in TH activity and kinetic characteristics in unlesioned neurons can not be mediated locally in the striatum by presynaptic receptors or product inhibition. This is evident by the fact that the lesion itself brings about changes in TH activity similar to those induced by haloperidol (106). It can be expected therefore, that haloperidol-induced changes will no longer be observed when the nigrostriatal

pathway is lesioned. Evaluation of the changes at the molecular level, brought about by a manipulation is necessary and often may exclude direct conclusions about the physiological process involved. This also implies that, for instance, antagonism of a drug effect by impairment of the impulse flow does not necessarily mean that the drug effect is mediated by the impulse flow. The point to be stressed is, that most experiments involve manipulations of the normal function of the dopaminergic neurons. Such manipulations may result in changes in set points of regulatory mechanisms, and in one experiment one mechanism may be most important, while in another experiment, involving another manipulation, also another regulatory mechanism may be most important.

Thus, the presence of one regulatory mechanism does not indicate the absence of another, or citing Elkes (121):".....for I suspect, that while using the "Either/Or", the language of the brain really is the language of the "Also" and the "And"." Actually, the philosophical statement of Elkes was concerned with undue emphasis on the role of DA as such.....

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PART II

I

RAPID POSTMORTEM CHANGES IN 3,4- DIHYDROXYPHENYLACETIC ACID (DOPAC), A DOPAMINE METABOLITE, IN RAT STRIATUM

INTRODUCTION

Several experimental approaches have been introduced to study the in vivo effects of drugs on dopaminergic neurons in the central nervous system. These include conversion of labelled precursors into dopamine, rate of disappearance of dopamine after inhibition of its synthesis (1) and estimation of metabolite concentrations (2,3). These procedures all include decapitation of the test animal, removal of the brain tissue, which often includes dissection, and extraction and assay of dopamine and/or one or more of its metabolites. To evaluate the reliability of such methods, short term postmortem changes in these substances should be studied. Carlsson et al. (4) reported postmortem changes in 3-methoxy-4-hydroxy-phenylethylamine (3-methoxytyramine, 3-MT) and dopamine 10 and 30 min after decapitation of rats, while other authors have only been concerned with considerably longer time intervals (5). In the present investigation postmortem changes in dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) have been studied from $2\frac{1}{2}$ to 20 min after decapitation.

MATERIALS AND METHODS

Male, albino Wistar rats (TNO, Zeist, The Netherlands) weighing 120-135 g were used. The animals were housed 5 to a cage, with standard food and water ad libitum. The lighting period was from 7.00 h to 21.00 h. Ambient temperature was approximately 18° C.

All chemicals were reagent grade. All water used was demineralized and subsequently redestilled in an all glass apparatus.

The rats were killed between 8.50 h and 9.35 h by means of a guillotine. The brain was removed and placed on an ice-cooled plate exactly 1 min after decapitation. The striata of one group of brains were dissected out, according to Gispen et al. (6), after exactly 0, $2\frac{1}{2}$, 5, 10 or 20 min on the ice-cooled plate (undissected "incubation") and frozen in liquid nitrogen exactly $1\frac{1}{2}$ min after starting the dissection. The striata of another group of brains were dissected out immediately and replaced on the ice-cooled plate exactly $1\frac{1}{2}$ min after starting the dissection. These striata were frozen in liquid nitrogen after exactly 0, $2\frac{1}{2}$, 5, 10 or 20 min on the ice-cooled plate (dissected "incubation"). Thus, the time interval between decapitation and freezing of the striata was $2\frac{1}{2}$ min plus the time during which the brain tissue was "incubated" on the ice-cooled plate, either undissected or dissected.

In one series of experiments the striata were weighed and homogenized in 1 ml 0.4 N perchloric acid. The homogenate was centrifuged at 25,000 g

for 10 min at 2° C and the supernatant stored at -80° C. HVA and DOPAC were isolated and assayed as previously described (7). The procedure consisted of isolation of the metabolites on small Sephadex G 10 columns followed by an automatic fluorimetric assay. The recoveries of 50 ng HVA and 50 ng DOPAC, added to 50-60 mg cerebellar tissue and run in parallel throughout the isolation and assay procedure, were 81 ± 9% for DOPAC and 67 ± 7% for HVA (± S.D., n = 6). The mean weight of the striata was 59.0 ± 6.8 mg (±S.D., n = 36).

In another series of experiments dopamine assays were carried out on striata obtained in exactly the same way as in the previous experiment. The striata were weighed and homogenized in 10 ml 0.4 perchloric acid containing 0.5% EDTA and 0.05% $Na_2S_2O_5$. The homogenate was stored over night at -20° C and then centrifuged at 25,000 g for 10 min at 2° C. The supernatant was filtered through Whatman no. 40 filter paper and transferred to 50 ml glass stoppered tubes containing 0.35 g Al_2O_3, prepared according to Weil-Malherbe (8). 0.5 Ml 1 M Tris pH=8.4 was added and after adjustment of the pH to 8.4-8.5 with 5 N and 1 N NaOH, the tubes were shaken for 10 min. The supernatant was discarded and the Al_2O_3 washed three times with at least 10 ml water. Dopamine was then eluted with 2.5 ml 1 N HCl (containing 10 mg/l EDTA and 1 mg/l ascorbic acid) and assayed, in duplicate, in 1 ml aliquots, according to Atack (9).

The recovery of 0.5 g dopamine, added to brain tissue and run in parallel throughout the isolation and assay procedure was 67%. The striata weighed 52.2 ± 6.3 mg ±S.D., n = 50).

All values were corrected for recovery and expressed as µg/g wet tissue weight. Differences between values for 0 min "incubation" and values obtained after different periods of "incubation" were statistically evaluated by means of the test of Dunett (10) for comparison of several treatments with one control.

RESULTS

DOPAC concentrations changed considerably 7½ min or more after decapitation (fig. 1). An increase of 30-45% was observed in striata "incubated" for 5, 10 or 20 min after dissection (dissected "incubation"). On the other hand, a decrease of 20-30% was observed in striata removed from whole brains "incubated" for 10 or 20 min (undissected"incubation"). HVA levels showed no significant changes. They tended to rise for the first 5 min of "incubation" and then to decrease after "incubation" of dissected striata for 10 and 20 min and of undissected brain for 10 min.

The concentration of dopamine did not change substantially during the period of observation except that, after 20 min "incubation" of dissected striata, a 15% decrease in dopamine levels was observed, although this effect was not significant.

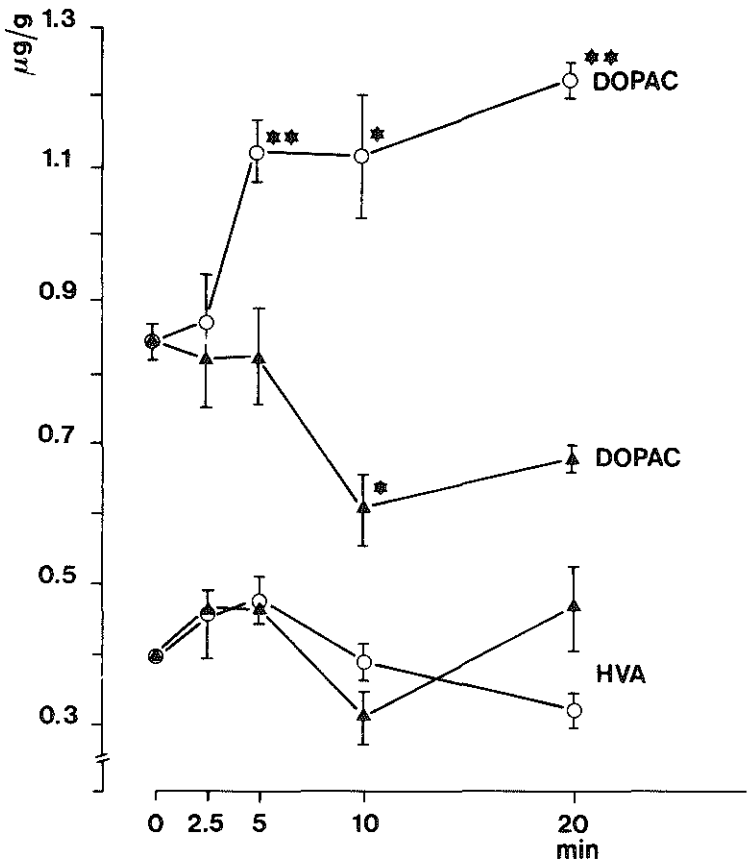

Fig. 1. Postmortem changes in HVA and DOPAC in rat striatum. Rats were decapitated, the brains removed and placed on a ice-cooled plate exactly 1 min after decapitation. Striata were frozen in liquid nitrogen exactly 1½ min after the start of the dissection. This represents the 0 min value. Values for the other time intervals indicated were obtained from striata "incubated" on the ice-cooled plate either before (undissected "incubation") or after dissection (dissected "incubation"). Values represent mean ± S.E. of 4 determinations, except for the 0 min value for HVA (n = 2). * $p < 0.05$, ** $p < 0.01$ vs 0 min value.
○ dissected, ▲ undissected.

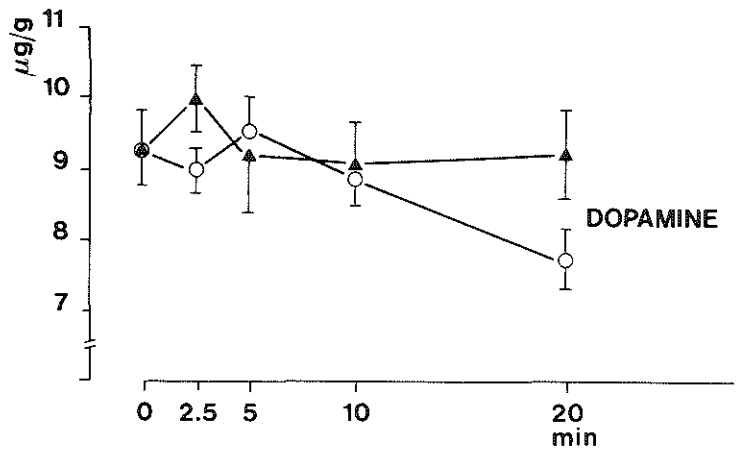

Fig. 2. Postmortem changes in dopamine in rat striatum. For explanation see fig. 1. Values represent mean ± S.E. of 6 determinations.
○ dissected, ▲ undissected.

DISCUSSION

The results presented show that no gross changes in dopamine, HVA and DOPAC concentrations occur from 2½ to 5 min after decapitation. Since no values can be obtained for the true concentrations at the moment of decapitation, levels of dopamine, HVA and DOPAC obtained from striata frozen within 5 min after decapitation, provide a reasonable alternative. However, when the period between decapitation and freezing of the brain tissue was extended beyond 5 min, considerable postmortem effects were observed for DOPAC. Remarkably, striatal DOPAC levels sharply increased when the striata were "incubated" at 0° C for more than 5 min (dissected "incubation"), while they decreased when the brain was "incubated" as a whole (undissected "incubation"). Oxidation of dopamine only occurred when the striatum was dissected and this may, therefore, reflect a difference in oxygen concentration. This is in agreement with the results and conclusion of Wiesel and Sedvall (5), who observed increases in HVA in striata when dissected and stored at 4° C for 6 h or more, but not when the brain was left *in situ* at 4° C for 6 h or more. Whether the observed DOPAC accumulation actually results from oxidation of dopamine or not, can not be concluded from the time course of dopamine levels obtained (fig. 2), although the decrease after 20 min "incubation" of dissected striata points in that direction. It should be noted, that dopamine concentrations are more than tenfold greater than the DOPAC concentrations and therefore, a change in DOPAC levels of 50% can be accounted for by a change of as little as 4% in the levels of dopamine. The decrease in dopamine after 20 min "incubation"

probably coincides with the onset of the disappearance reported by Wiesel and Sedvall (5) at 6 h after decapitation under similar experimental conditions.

While DOPAC levels decreased in whole brain after 10 min, no increase in HVA concentration was observed. This seems to indicate, that the disappearing DOPAC is not O-methylated to HVA. A possible reason for the disappearance of the DOPAC may be the formation of a conjugate, as recently indicated by Gordon et al. (11), although it is not known whether conjugation can take place postmortem. Another observation also suggests that O-methylation did not take place to an appreciable extend in our experiments:namely, the massive increase in DOPAC levels in dissected striata was not paralleled or followed by an increase in HVA concentration. Although Carlsson et al. (4) observed considerable postmortem changes in 3-MT and dopamine in whole brain 10 or 30 min after decapitation, this does not contradict our results, since these effects occurred when the brain was left in situ at 37°C. However, when the brain was stored at 23°C for 10 or 30 min no effects on 3-MT or dopamine concentrations were observed (4).

In conclusion, there seem to be no gross postmortem changes in the levels of dopamine and its acid metabolites between 2½ and 5 min after decapitation, under the conditions of the present experiments. However, at longer time intervals, dramatic changes in DOPAC levels were observed. These changes seem to result from oxidation and possibly conjugation, while O-methylation does not play an important role.

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II

DIFFERENTIAL EFFECT OF MORPHINE ON DOPAMINERGIC NEURONS IN FRONTAL CORTEX AND STRIATUM OF THE RAT

SUMMARY

Inhibition of dopamine synthesis by a single injection of α-methyl-para-tyrosine (200 mg/kg, i.p.) was complete from 30 to at least 300 min. after administration.

When morphine (20 mg/kg) was given intraperitoneally 30 min. after αMpT treatment, an enhanced decline of dopamine was observed in frontal parts of the cortex but not in the striatum. These results indicate that morphine affects dopaminergic neurons in frontal parts of the cortex in a way differently from those in the striatum of the rat.

This may be caused either by a difference in the properties of dopaminergic nerve endings in both structures or by an effect of morphine on the input to the cortical system which is lacking in the striatum.

INTRODUCTION

Several laboratories reported that morphine enhances the synthesis of dopamine in the corpus striatum (1, 2, 3) and in other brain structures of the rat (4). Many effects of drugs on the synthesis of dopamine seem to be initiated by compensatory mechanisms after disturbance of the dopaminergic system. It is postulated, for example, that neuroleptic drugs block dopaminergic receptors (5) and as a consequence of this blockade activate the synthesis of dopamine by some feedback mechanism. Kehr et al. (6) suggested a local feedback,while Andén et al. (7) proposed a feedback via modulation of the impulse flow at the level of the substantia nigra. Likewise, changes in release and catabolism of dopamine or changes in impulse flow can affect dopamine synthesis (8, 9). After inhibition of the biosynthesis of dopamine these compensatory mechanisms are blocked and effects on release, catabolism or impulse flow can be uncovered by measuring the changes in dopamine concentration.

The present experiments evaluate the effect of morphine on dopaminergic systems in the corpus striatum and in frontal parts of the cortex after inhibition of the dopamine synthesis by α-methyl-para-tyrosine (αMpT). A first series of experiments was performed to determine the time intervals at which αMpT exhibits maximal inhibition of the synthesis. In a second series of experiments the effect of morphine during this time interval was investigated.

MATERIALS AND METHODS

Animals

Male albino Wistar rats weighing 80-110 g were used. The animals were housed 5 in a cage with ad libitum water and standard food. Lights were on from 7 a.m. to 9 p.m.

Drugs

Drug solutions were freshly prepared before each experiment in 0.9% NaCl. Drugs used were: α-methyl-para-tyrosine-methylester. HCl (αMpT, AB Labkemi), morphine. HCl and L-^{14}C-tyrosine (uniformly labelled 483 mC/mmol, Radiochemical Centre, Amersham). αMpT (200 mg/kg as base) and morphine. HCl (20 mg/kg) were injected intraperitoneally (5 ml/kg). L-^{14}C-tyrosine was freeze-dried, dissolved in 0.9% NaCl and injected into the tail vein (300-500 μC/kg, 2 ml/kg).

Experiment 1

At graded intervals after administration of αMpT (15, 30, 60, 120 or 240 min) or saline (15, 60 or 240 min) L-^{14}C-tyrosine was injected. The rats were decapitated 60 min after L-^{14}C-tyrosine administration and the brain without bulbi olfactorii was rapidly removed. The cerebellum was discarded. The rest of the brain was homogenized in 5 ml 0.4N perchloric acid containing 0.5% EDTA and centrifuged at 25,000 g for 15 min at 2°C. Dopamine and tyrosine were isolated from the supernatant by chromatography on Dowex 50 WX 4 columns as described by Atack and Magnusson (10) and Atack (11). The chromatography was performed at 4°C. Aliquots of the eluates containing dopamine and tyrosine were added to 16 ml Instagel (Packard). Radioactivity was measured by liquid scintillation spectometry and corrected for quenching with an external standard. L-^{14}C-dopamine and L-^{14}C-tyrosine were added to brain tissue samples of untreated rats and run in parallel through the extraction and isolation procedure. Experimental values were corrected for recovery of L-^{14}C-dopamine and contamination of L-^{14}C-tyrosine in the dopamine fraction.

The percentage inhibition of dopamine synthesis was calculated from the following equation: $\% \text{ inhibition} = \frac{C - E}{E} \times 100$, where C and E are values for radioactivity in the eluates containing dopamine from saline and αMpT treated rats respectively. C equals the average value from the three saline treated groups (15, 60 and 240 min before L-^{14}C-tyrosine). The mean radioactivity in the dopamine fraction of these controls was 7168 ± 404 dpm (± S.E. n=9). The results were statistically evaluated by analysis of variance according to Snedecor and Cochran (12).

Experiment 2

Morphine or saline was administered 30 min after αMpT treatment. Rats were killed by decapitation 2½ hours after injection of αMpT. After decapitation the brain was rapidly removed without bulbi olfactorii, dissected, weighed and frozen in liquid nitrogen within 4 min. after decapitation.

Frontal cortex was dissected from the tissue frontal to a vertical section through the optic chiasm and anterior commissure as follows: tuberculi olfactorii were removed and all tissue medial to a vertical cut through the anterior commissure and dorsal to the corpus callosum

(containing septum, nucleus preopticus and nucleus accumbens) was discarded. The frontal parts of the caudate nuclei were removed and added to the caudal parts of striatal tissue (13). The remaining cortical tissue is specified as frontal cortex. The mean tissue weight (± S.D., n=90) per brain was: striatum 120.8 ± 10.1 mg and frontal cortex 203.1 ± 22.0 mg.

Pooled frontal cortices and pooled striata of 4 rats were homogenized in 10 ml perchloric acid containing 0.5% EDTA. Dopamine was isolated and assayed fluorimetrically as described by Atack (11). The recovery of 0.5 µg dopamine added to brain tissue samples and run in parallel through the procedure was 72.0 ± 5.7% (mean ± S.D., n=7). The values were not corrected for this recovery. Differences in dopamine levels were statistically evaluated by Student's t-test.

RESULTS

The results as shown in Table I demonstrate that a single injection of αMpT inhibited the synthesis of dopamine for more than 95% from 30 min after administration until the longest period of observation (4 hr). However, a lower percentage of inhibition (87.9%) was achieved when the precursor L-^{14}C-tyrosine was injected 15 min after αMpT administration. Statistical evaluation of the data by an analysis of variance (Table II) showed a significant difference between treatments ($p < 0.005$).

TABLE I

Time Course of Inhibition of Dopamine Synthesis by αMpT

Treatment Group	Time Schedule (min after αMpT)		% Inhibition of Dopamine Synthesis
	Injection of L-^{14}C-Tyrosine	Decapitation	
1	15	75	87.9 ± 3.3
2	30	90	97.3 ± 2.4
3	60	120	95.2 ± 2.2
4	120	180	95.8 ± 1.9
5	240	300	96.2 ± 3.1

200 mg/kg αMpT was administered i.p. and at indicated time intervals thereafter L-^{14}C-tyrosine i.v.. % Inhibition of dopamine synthesis was calculated as described in "Materials and Methods". Values represent mean ± S.E., n = 3.

TABLE II

Analysis of Variance of the Data of Table I

Source of Variation	Sum of Squares	df	Mean Sum of Squares	F	p
between treatments[a]	168.21	4	42.05	33.91	<0.005
1st vs other groups[a]	161.05	1	161.05	129.88	<0.005
among other groups (2-5)[a]	7.16	3	2.39	1.92	>0.1
within treatments (error)	9.91	8	1.24		

a. Treatment groups as indicated in Table I.

Inhibition of the synthesis of dopamine in the first treatment group (L-^{14}C-tyrosine 15 min after αMpT) significantly differed from the other groups ($p < 0.005$), while no significant effect was observed within these other groups ($p > 0.1$). The average value for the other groups amounted to 96.1%, which represents the mean inhibition from 30 min until 5 hr after administration of αMpT. The value obtained for the first treatment group (87.9%) represents the mean inhibition between 15 and 75 min after αMpT treatment.

TABLE III

Effect of Morphine on the αMpT induced Decline of Dopamine

	Dopamine	
Treatment	Frontal Cortex (ng/g)	Striatum (µg/g)
None	415 ± 26 (9)	4.30 ± 0.21 (10)
αMpT + Saline	143 ± 7 (6)	1.82 ± 0.13 (4)
αMpT + Morphine	111 ± 7 (4)^{x}	1.81 ± 0.19 (5)

Rats were decapitated at T=0 (no treatment) or injected with αMpT (200 mg/kg,i.p.). αMpT treated rats received saline or morphine (20 mg/kg,i.p.) at T=½hr and were decapitated at T=2½hr. Values represent mean levels of dopamine ± S.E. and number of observations in parenthesis.
^{x} Differs from saline control ($p < 0.01$).

Assuming that the inhibition in this treatment group from 30 min until decapitation (75 min) is the same as for the other groups from 30 min on (96.1%), the inhibition between 15 and 30 min can be calculated to be about 63%.

Complete inhibition of dopamine synthesis, therefore, was achieved 30 min but not 15 min after administration of αMpT. For this reason morphine was administered 30 min after αMpT and this resulted in an enhanced decline of dopamine in the frontal cortex (Table III). The level of dopamine obtained 2½ hours after injection of αMpT was significantly lower in morphine treated rats as compared to saline treated rats ($p < 0.01$).

However, in the striata of the same rats no difference between the levels of dopamine in morphine and saline treated rats was observed ($p > 0.95$).

DISCUSSION

From 30 min until 5 hr after a single intraperitoneal injection of αMpT (200 mg/kg) the inhibition of dopamine synthesis was complete (96.1%), while only a partial inhibition was obtained from 15-30 min (63%). This is in agreement with the results of Doteuchi et al. (15) who reported a 53% inhibition of dopamine synthesis between 5 and 15 min after administration of the same dose of αMpT.

Morphine, when administered to rats in which dopamine biosynthesis was completely inhibited, had no effect on the decline of dopamine in the corpus striatum. A number of factors such as release generated by impulse flow, release generated directly at the nerve ending (tyramine- or reserpine-like effects) and catabolism of dopamine determine the decline of dopamine when its synthesis has been inhibited. The influence of morphine on these processes can be ruled out,as the drug had no effect on the decline of dopamine in the striatum.

A single injection of morphine was shown to increase dopamine synthesis in the rat striatum (1, 2, 3). Although this effect of morphine has been attributed to a direct influence on the biosynthesis itself (3,16), another possibility should be taken into consideration. It has been suggested that morphine may block dopamine receptors in a way similar to neuroleptics (17, 18). Such a blockade would enhance the synthesis of this amine via a local feedback or via an enhanced impulse flow, as proposed for neuroleptics (6, 7). The latter possibility is apparently not supported by the present results. However, a feedback at the level of the striatum is still plausible, but cannot be detected in our experiments due to prior αMpT treatment.

The augmented decline of dopamine by morphine in the frontal cortex after αMpT treatment indicates that the drug either enhances the impulse flow in dopaminergic neurons in this structure or that it increases release or breakdown of dopamine at the nerve endings. In the case of an enhanced impulse flow, morphine may affect the input to the dopaminergic neurons projecting to the frontal cortex,but not to those projecting to the striatum. However, a direct effect of morphine on dopamine release or catabolism at the nerve endings in the frontal cortex can not be excluded from the present experiments. Therefore, a second explanation

for the discrepancy between frontal cortex and striatum might be a difference in properties of the dopaminergic nerve endings in both structures.

In conclusion, the data presented reveal a differential effect of morphine on striatal and frontal cortical dopaminergic systems. Morphine may selectively modulate the input to the dopaminergic systems in the frontal cortex. Alternatively, a direct effect on dopaminergic nerve endings in the frontal cortex is possible.

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III

MORPHINE: A MODULATOR OF DOPAMINERGIC NEUROTRANSMISSION IN THE RAT
1. FRONTAL CORTICAL AND STRIATAL EFFECTS

ABSTRACT

Morphine (20 mg/kg i.p.) increased the rate of dopamine (DA) loss by ca 40% in the frontal cortex of rats when the drug was administered 30 min after treatment with the synthesis inhibitor α-methyl-para-tyrosine methylester (αMpT). This effect was specific as it was antagonized by naloxone. Under the same injection schedule the rate of DA decline in the striatum was unaltered in one experiment and increased slightly in another experiment. Moreover, striatal levels of homovanillic acid (HVA) and 3,4-dihydroxy-phenylacetic acid (DOPAC) were unaffected by morphine under exactly the same experimental design. It is suggested, therefore, that, under conditions whereby the synthesis of DA is entirely inhibited, morphine increases DA release in the frontal cortex in contrast to the striatum. This differential effect could not be ascribed to a delayed inhibition of DA synthesis in the frontal cortex when compared with the striatum. Subdissection of the frontal cortex showed that most of the DA (80%) was present in basal parts of the forebrain.

When morphine, on the other hand, was administered before inhibition of DA biosynthesis (30 min before αMpT) an increase in the rate of DA loss was also observed in the striatum and a more marked increase was seen in the frontal cortex.

The results suggest that morphine exerts two different actions on dopaminergic neurons: an increase of DA biosynthesis in most or all dopaminergic structures, presumably by a direct action, and an increase in DA release in the frontal cortex. It is suggested that extrastriatal, including frontal cortical, dopaminergic structures, are of more importance than striatal structures for morphine-induced effects related to the functional activity of dopaminergic systems.

INTRODUCTION

The effect of morphine administration on the activity of dopaminergic neurons in the central nervous system of the rat has been extensively studied in recent years. The enhanced turnover of dopamine (DA) induced by morphine in the striatum and other DA-rich brain areas has been shown, using several different experimental techniques. These have included enhanced conversion of radioactive tyrosine into DA in the corpus striatum (1, 2, 3) and in other brain areas (4, 5), increases in the DA metabolite homovanillic acid (HVA) in the striatum (6, 7, 8), nucleus accumbens and tuberculum olfactorium (7), increases in 3,4-dihydroxy-phenylacetic acid (DOPAC) in these brain structures (9) and accelerated decline of DA in the striatum (10, 11) and in whole rat brain (12) after

treatment with the synthesis inhibitor α-methyl-para-tyrosine (αMpT).

Since the turnover of DA is dependent on several closely linked processes like release, reuptake, synthesis and catabolism (13), the reports cited above do not indicate which of these components is primarily affected by morphine. We recently reported that morphine did not affect the decline in striatal DA after αMpT treatment, when the drug was administered 30 min after αMpT, while it increased DA decline in the frontal cortex under these conditions (14). The major feature of this experimental approach is a strict exclusion of drug effects contingent on DA biosynthesis, which is not achieved when morphine is administered before αMpT (10, 11) or by other techniques as described above. The present experiments are concerned with the localization and specificity of the reported enhanced DA decline in the frontal cortex and the absence of this effect in the striatum.

MATERIALS AND METHODS

Male, albino Wistar rats were purchased from T.N.O. Zeist, The Netherlands. The animals were housed 5 in a cage with free access to food and water. The period of light was from 7.00 h to 21.00 h. Ambient temperature was ca 18° C. All animals weighed 100-135 g on the day before the experiment, when they were placed in a separate housing room where the experiments were performed. The first treatment (injection or decapitation) of all animals was between 9.00 h and 10.30 h and treatments were randomized to avoid influences of diurnal fluctuations in DA, HVA and DOPAC.

Drug solutions were freshly prepared before each experiment in 0.9% NaCl. Drugs used were: α-methyl-para-tyrosine methylester.HCl (αMpT, AB Labkemi) 200 mg/kg as base, Morphine.HCl 20 mg/kg, Naloxone.HCl 1 mg/kg (Endo Lab.), L-(3,5-^{3}H)-tyrosine (42 C/mmol) and S-adenosyl-L-(methyl-^{3}H)-methionine (10 C/mmol), both from Radiochemical Centre Amersham. (^{3}H)-tyrosine was freeze-dried and dissolved in 0.9% NaCl. All chemicals were reagent grade. All water used was demineralized and subsequently distilled in an all glass apparatus.

EXPERIMENTAL SCHEDULES

Assessment of DA Synthesis Inhibition

About 1 mC of (^{3}H)-tyrosine was injected into the tail vein in a volume of 0.2 ml. αMpT or saline was administered 30 or 60 min before (^{3}H)-tyrosine and rats were decapitated 30 min after (^{3}H)-tyrosine injection.

Morphine Experiments

Rats were injected at t_o with αMpT and decapitated at t=2½ h. Controls were decapitated at t_o (0 HR values). Morphine or saline was administered 30 min before αMpT (Sal + αMpT and Mor + αMpT) or 30 min after αMpT (αMpT + Sal and αMpT + Mor). The 0 HR control for the Mor + αMpT group received morphine 30 min before decapitation. Naloxone was given together with morphine or alone 30 min after αMpT (αMpT + Mor + Nal and αMpT + Nal). These rats received a second injection of naloxone 60 min later, while groups not treated with naloxone in the same experiment received saline.

All drugs were injected intraperitoneally in a volume of 5 ml/kg. The injection schedule is summarized in table 1.

Table 1.

Time Schedule of Treatments (min).

-30	0	+30	+90	+ 150	Treatment Group (Code)
Saline	Decapitation				0 HR Sal
Morphine	Decapitation				0 HR Mor
Saline	α MpT	--	--	Decapitation	Sal+αMpT
Morphine	α MpT	--	--	Decapitation	Mor+αMpT
--	α MpT	Saline	--/Saline	Decapitation	αMpT+Sal
--	α MpT	Morphine	--/Saline	Decapitation	αMpT+Mor
--	α MpT	Naloxone	Naloxone	Decapitation	αMpT+Nal
--	α MpT	Morphine + Naloxone	Naloxone	Decapitation	αMpT+Mor+ Nal

All injections were i.p. in a volume of 5 ml/kg
Morphine: 20 mg/kg as HCl salt
αMpT: α-methyl-para-tyrosine-methylester.HCl, 200 mg/kg as base
Naloxone: 1 mg/kg as HCl salt

DISSECTION

Dissection of the frontal cortex

After decapitation the brain of the rat was rapidly removed from the skull and placed on an ice-cooled plate for dissection. The olfactory tubercula and bulbi were removed by blunt dissection. Subsequently the frontal pole of the forebrain was separated from the rest of the brain by a transverse cut through the optic chiasm and the anterior commissure (cut A, fig. 1). From the dissected frontal pole of the forebrain the "frontal" cortex was dissected in two steps. First, the septal and preoptic areas were removed. For this purpose two parasagittal cuts were made passing through the floor of the lateral ventricle and the lateral olfactory tract, which made it possible to remove the midline tissue by peeling it loose from the corpus callosum. Subsequently the striatum on either side was removed by means of blunt dissection sparing the basal parts of the forebrain. The two striata were kept and further analysed together with the striatal tissue obtained from the caudal half of the forebrain, according to the method described by Gispen et al. (15). The remaining tissue of the rostral pole of the forebrain will be referred to

as frontal cortex, which includes, however, part of the corpus callosum and the basal parts of the forebrain. The dissected frontal cortex and striata were put into liquid nitrogen within 4 min after decapitation.

Subdissection of the frontal cortex

In one experiment the "frontal" cortex was subdivided in four parts. First the frontal pole of the forebrain was separated from the rest of the brain by the transverse cut through the anterior commissure and the optic chiasm as described above. The frontal pole of the forebrain was then divided into two parts by a transverse cut through the level of the genu of the corpus callosum (cut B, fig. 1). The cortical tissue rostral to this cut will be referred to as area 1.

From the brain slice between the transverse cuts A and B the septal and preoptic area as well as both striata were removed as described above. The remaining cortical tissue was further subdivided into five parts by means of four longitudinal cuts, i.e. two parasagittal cuts about 1 mm from the midline, which dissected the corpus callosum and the medial aspects of the hemisheres (area 2, fig. 1) and two other parasagittal cuts which separated the basal parts of the forebrain (area 4, fig. 1) from the lateral convexity of the hemishere (area 3, fig. 1).

ASSAYS AND STATISTICS

HVA and DOPAC

Single striata were weighed and homogenized in 1 ml 0,4 N perchloric acid for HVA and DOPAC assay. Homogenates were centrifuged at 25,000 g for 10 min at 2° C. HVA and DOPAC were isolated and assayed according to Westerink and Korf (9). The recoveries of 50 ng HVA and 50 ng DOPAC added to 50-60 mg cerebellar tissue and run in parallel through the total procedure were 67 $\pm$ 7% for HVA and 81 $\pm$ 9% for DOPAC ($\pm$ S.D., n=6). Values were expressed as µg/g wet tissue weight without correction for recovery.

Fluorimetric DA Assay

For the assay of DA the striata and frontal cortex of one rat were weighed. Striata of one rat and pooled frontal cortices of 4 rats were homogenized in 10 ml 0.4 N perchloric acid containing 0.5% EDTA and 0.05% $Na_2S_2O_5$. Homogenates were centrifuged at 25.000 g for 10 min at 2° C. The supernatant was filtered through Whatman no. 40 filter paper into 50 ml glass stoppered tubes containing 0.35 g Al_2O_3 which was prepared according to Weil-Malherbe (16). 0.5 Ml 1 M Tris pH = 8.4 was added and the pH adjusted to 8.4-8.5 with 5 N and 1 N NaOH and the tubes shaken for 10 min. The supernatant was discarded and the Al_2O_3 washed three times with at least 10 ml water. DA was eluted with 2.5 or 3 ml 1 N HCl containing 10 mg/l EDTA and 1 mg/l ascorbic acid and assayed fluorimetrically in duplo in 1 ml aliquots according to Atack (17). The recovery of 0.5 µg DA added to brain tissue extracts and run in parallel through the procedure was 70.8 $\pm$ 3.0% ($\pm$ S.D., n = 13). Values were expressed as µg/g wet tissue weight without correction for recovery. Rate constants of amine loss were calculated according to Brodie et al. (18).

Radiochemical Assay of DA and NA

DA and NA in dissected brain areas of the frontal cortex (see Dissection) were assayed by a radiochemical method (19) as modified by Versteeg et al. (20). Briefly, the brain tissue was homogenized in 0.1 N

perchloric acid, centrifuged and the supernatant used for assay. DA and NA were converted to their (^{3}H)-methoxy derivatives by means of incubation with rat liver COMT in the presence of (^{3}H)SAM. The derivatives were separated by extraction and paper chromatography.

Assay of Radioactive DA and Tyrosine

(^{3}H)-tyrosine and (^{3}H)DA in striata and frontal cortex of one rat were isolated as described before (14). Briefly this consisted of perchloric acid extraction of the brain tissue, centrifugation and chromatography of the supernatant on Dowex 50 WX 4 columns. Radioactivity was measured by liquid scintillation spectrometry. The percentage inhibition of DA biosynthesis was calculated from the equation $\frac{C-E}{C} \times 100$, where C and E represent the ratios of radioactivity in the DA and tyrosine containing eluates from saline and αMpT treated rats respectively.

Statistics

Differences between DA levels and rate constants were tested with two-tailed Student's t-test. In the experiment involving determination of HVA and DOPAC (table 5) analysis of variance (21) was performed.

Values of p less than 0.05 were considered significant.

Table 2

Inhibition of Dopamine Biosynthesis by αMpT in Frontal Cortex and Striatum

Time-interval: ^{3}H-TYR injection to decapitation	% inhibition of DA synthesis [a]	
	Frontal Cortex [b]	Striatum [b]
30 - 60 min	85 ± 6	96;96
60 - 90 min	89 ± 5	95 ± 3

a. Values represent mean ± S.E. of 3 experiments.
b. Frontal cortices weighed 194 ± 21 mg and striata 108 ± 11 mg (mean ± S.D., n = 9).

RESULTS

INHIBITION OF DA BIOSYNTHESIS BY αMpT IN FRONTAL CORTEX AND STRIATUM

Table 2 shows that DA biosynthesis in both the frontal cortex and striatum was inhibited to a similar extend 30-60 and 60-90 min after αMpT administration. The calculated % inhibition in the frontal cortex, however, did not amount to the same value as in the striatum. The amount of radioactive DA formed in the frontal cortex of control rats (no αMpT) was considerably less than in the striatum (1410 ± 385 dpm vs 13,504 ± 1,830 dpm), while more radioactive tyrosine was recovered in the frontal cortex (93 ± 9 dpm.10^{+3} vs 61 ± 6 dpm.10^{+3}).

Table 3.

Effect of Morphine Administered before or after αMpT on the Decline of Dopamine in Frontal Cortex and Striatum[a]

	Frontal Cortex[b]			Striatum[b]		
Treatment[c] group	DA concentration (ng/g)	Rate constant[d] (h^{-1})	ΔRate constant (h^{-1})	DA concentration (μg/g)	Rate constant[d] (h^{-1})	ΔRate constant (h^{-1})
0 H Sal	292±23 (3)			4.38±0.13 (9)		
0 H Mor	334±26 (3)			4.82±0.23 (10)		
αMpT+Sal	148± 4 (3)	0.271±0.0334	0.102±0.0467	1.99±0.06 (14)	0.317±0.0181	-0.002±0.0247
αMpT+Mor	115± 4 (4)^{x}	0.373±0.0316^{x}		1.99±0.06 (13)	0.315±0.0168	
Sal+αMpT	160± 9 (3)	0.241±0.0399	0.167±0.0506	2.22±0.06 (13)	0.271±0.0157	0.099±0.0299^{f}
Mor+αMpT	120± 4 (4)^{x}	0.407±0.0322^{x}		1.91±0.09 (12)^{x}	0.371±0.0254^{xx}	

x $p < 0.01$ vs matching saline xx $p < 0.002$ vs matching saline f $p < 0.02$ vs inversed treatment

a. Values represent mean ± S.E. and number of observations are given in parenthesis. The number of experiments on separate days was 4.

b. Frontal cortices weighed 208 ± 18 mg and striata 115 ± 8 mg (± S.D., n = 96).

c. See table 1 for injection schedule and codes for treatment groups.

d. Rate constants for Sal + αMpT, αMpT + Sal and αMpT + Mor treatments were calculated utilizing the 0 H Sal value, while the 0 H Mor value was used for Mor + αMpT treatment.

Table 4

Effect of Morphine and Antagonism of Naloxone in Frontal Cortex and Striatum[a].

	Frontal Cortex[b]		Striatum[b]	
Treatment[c] group	DA level (ng/g)	Rate constant (h^{-1})	DA level (μg/g)	Rate constant (h^{-1})
0 H Sal	248±10(4)		5.28±0.26(11)	
αMpT+Sal	119±8 (5)	0.297±0.0356	2.74±0.10(17)	0.263±0.0237
αMpT+Mor	86±6 (5)^{x,f}	0.427±0.0378^{x,f}	2.36±0.10(24)^{x}	0.325±0.0268^{x}
αMpT+Nal	110±11(6)	0.339±0.0583	2.66±0.11(22)	0.277±0.0263
αMpT+Mor+Nal	122±9 (5)	0.289±0.0370	2.60±0.08(16)	0.281±0.0217

x. $p < 0.02$ vs αMpT+Sal f. $p < 0.01$ vs αMpT+Mor+Nal

a. Values represent mean ± S.E. and number of observations in parenthesis. The number of experiments on separate days was 5.

b. Frontal cortices weighed 197 ± 19 mg and striata 102 ± 11 mg (± S.D., n = 115).

c. See Table 1 for injection schedule and codes for treatment groups.

ADMINISTRATION OF MORPHINE 30 MIN AFTER αMpT: EFFECT ON DA AND ANTAGONISM BY NALOXONE

Frontal Cortex (table 3 & 4)

Morphine administration 30 min after αMpT resulted in lower DA levels in the frontal cortex when compared with saline treatment. The rate constants of DA loss calculated from these concentrations showed an increase of 38% (table 3) and 44% (table 4), after morphine treatment. Moreover, this effect was blocked by the specific morphine antagonist naloxone, which had no effect of its own (table 4).

Striatum (table 3, 4 & 5).

When morphine was administered 30 min after αMpT, no effect on DA decline was observed in the striatum, as shown in table 3. However, in another experiment (table 4) a decrease in the DA levels was obtained. Although the rate constant only increased by 24% when compared with 44% in the frontal cortex, the difference from saline treatment was significant. The difference between morphine and combined morphine and naloxone injection, however, was not significant in the striatum. Moreover, it was observed, that the rate constant of DA loss in the striatum of saline treated rats was smaller in the experiment reported in table 4 when compared with that shown in table 3, while they were similar in the frontal cortex.

The concentrations of the DA metabolites HVA and DOPAC were analysed under exactly the same experimental schedule. Although the morphine injection resulted in slightly lower levels of both metabolites when compared with saline treatment, an analysis of variance showed no significant effect of treatments (HVA: F=0.25, df=3/12 and DOPAC: F=1.50, df=3/13, both n.s.). The HVA and DOPAC levels in the frontal cortex were below the limit of detection 2½ h after αMpT administration, thus no values for this brain area are reported.

Table 5

Effect of Morphine and Naloxone on HVA and DOPAC in Striatum after Synthesis Inhibition [a].

Treatment group[b]	HVA (ng/g)[c]	DOPAC (ng/g)[c]
0 H Sal	375±41 (4)	729±39 (4)
αMpT+Sal	141± 3 (3)	325±31 (4)
αMpT+Mor	131±16 (4)	291±11 (4)
αMpT+Nal	129±21 (4)	271±36 (4)
αMpT+Mor+Nal	141±10 (4)	343±27 (4)

a. Values represent mean ± S.E. and number of experiments in parenthesis. Each individual value consists of the mean value of left and right striatum.
b. See Table 1 for injection schedule and code for treatment groups.
c. Single striata weighed 54.8 ± 7.9 mg (± S.D., n = 48).
No significant effect of treatments was detected by analysis of variance (see Results).

COMPARISON OF THE EFFECT OF MORPHINE ON DA DECLINE, WHEN ADMINISTERED 30 MIN BEFORE OR AFTER αMpT (TABLE 3)

DA concentrations in control rats were 292±23 ng/g and 4.38±0.13 µg/g in the frontal cortex and striatum respectively. After 30 min pretreatment with morphine a rise in DA levels of 14% in the frontal cortex and of 10% in the striatum was observed, but these values were not significantly different from controls. In the frontal cortex, significantly lower levels of DA were obtained when morphine was administered 30 min after, as well as 30 min before αMpT, when compared with saline treatment. In the striatum, however, no difference was observed when morphine was administered 30 min after αMpT while injection of morphine 30 min before αMpT resulted in a significantly lower DA concentration than in saline treated rats.

A better quantitative insight into these effects of morphine is provided by comparison of rate constants of DA loss, as calculated from the DA concentrations. These rate constants were of the same order of magnitude in frontal cortex and striatum under control conditions. Administration of morphine 30 min after αMpT treatment increased the rate constant by 38% in the frontal cortex, while after the reversed treatment, viz. morphine 30 min before αMpT, a larger increase of 69% was observed. In the striatum, on the other hand, no difference in rate constants was revealed when morphine was administered after αMpT, while an increase of 37% was observed after treatment with morphine 30 min before αMpT. Therefore, administration of morphine after αMpT induced an increased rate of DA loss in the frontal cortex and had no effect in the striatum, while inversion of the treatment with morphine and αMpT induced an additional increase in the rate constant of 31% in the frontal cortex and an increase of 37% in the striatum. This latter increase was statistically significant, as can be seen by comparing the ΔRate constants (table 3), while the increase in rate constant in the frontal cortex of 31% was not statistically significant.

DISTRIBUTION OF DA AND NA IN THE FRONTAL CORTEX

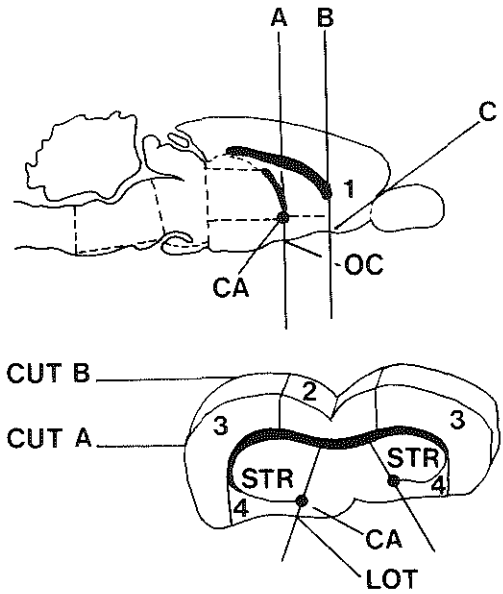

Fig. 1. Subdissection of the frontal cortex. See Materials and Methods "Subdissection of the frontal cortex" for detailed description. Abbreviations: C.A.: commissura anteriora; O.C.: optic chiasm ; STR: striatum; L.O.T.: lateral olfactory tract. Numbers of the dissected areas correspond with table 6. Figures modified from Gispen et al. (15).

Fig. 1 shows the dissection of the frontal cortex. The areas 1 and 2 contain the dopamine terminals of frontal cortex and anterior cingulate cortex respectively, while area 3 probably contains dopamine terminals in the transition zone between neocortex and piriform cortex as described by Lindvall et al. (22). Table 6 shows, that most of the DA (ca 80%) in the total frontal cortex originates from basal parts of the forebrain (area 4), which contains much less NA. The other areas contribute only minor amounts to the DA of the total frontal cortex, while they contain about 3-4 times as much NA. The total frontal cortex contains roughly equivalent amounts of DA and NA; the DA concentration (37 ng in 171 mg) of ca 216 ng/g corresponds well with other experiments (see table 3 & 4).

Table 6

Distribution of DA and NA in Dissected Areas of the Frontal Cortex.

		DA		NA		DA/NA
area[a]	tissue weight (mg)	ng	%	ng	%	
1	82 ±7	1.4±0.1	4.2±0.6	7.9±0.2	23.1±0.3	0.18
2	28.4±1.7	1.8±0.3	5.2±1.1	6.4±0.3	18.6±0.7	0.28
3	50 ±2	3.8±0.6	10.9±1.7	17.2±0.4	50.4±1.2	0.22
4	10.9±0.9	30 ±6	80 ±3	2.7±0.3	7.9±0.8	3.80
Total	171±10	37±6	100	34.2±0.6	100	1.08

a. Numbers as indicated in fig. 1.
All values represent mean ± S.E. of the absolute amount of amine, n = 5.

DISCUSSION

The present experiments show that morphine, administered during complete inhibition of DA synthesis, enhances the decline in DA levels in the frontal cortex but not in the striatum (table 3). However, in the experiment shown in table 4 an enhanced disappearance of DA was also observed in the striatum.

A possible interpretation of these contradictory observations could be that morphine causes a marginal increase in DA loss in the striatum, which only occasionally exceeds the experimental error. To test this possibility the levels of the DA metabolites HVA and DOPAC were analysed under exactly the same experimental conditions, to obtain another indication of possible

increased DA release. Morphine treatment, however, failed to increase the concentration of either metabolite in the striatum. Moreover, no effect of morphine was observed on the decline in DA in five similar series of experiments (table 3 (14, 23)) nor in an experiment utilizing a dose range of morphine from 2½ to 40 mg/kg (24). The enhanced DA decline in the frontal cortex, on the other hand, was clearcut and reproducible (table 3 & 4 (14)). Morphine, therefore, does not seem to affect DA release in the striatum, in contrast to the frontal cortex, when the biosynthesis of DA has been entirely inhibited. Nevertheless, it might be possible that the rats in the experiment reported in table 4 belong to a different population with respect to the action of morphine. In favour of this suggestion is the smaller rate constant of DA decline observed in striata of saline treated rats, when compared with the values from the experiment reported in table 3, presumably reflecting a lower basal activity of the striatal neurons. It has been reported elsewhere that morphine causes release of DA in the striatum when the impulse flow is inhibited (23). It is possible that a similar effect is exerted by morphine when the basal activity of the dopaminergic neurons is as low as in the experiment reported in table 4.

Inversed administration of morphine, i.e. 30 min before instead of 30 min after αMpT, on the other hand, resulted in an increased DA decline in the striatum, which is in agreement with the observations of Puri et al. (11) and Sugrue (10). This enhanced loss of DA in the striatum can not, in all probability, be ascribed to increased DA release (25), since increased DA release would also have been observed when morphine was administered after αMpT. Moreover, the enhanced loss of DA can not be attributed to increased catabolism of DA (6) for the same reason and also because no increase was observed in metabolite levels (table 5). It has been suggested that catabolism of newly synthesized DA in particular, is stimulated by morphine (6). If so, this would not have been observed in the present experiments because of the prior treatment with αMpT. The increase in DA concentration 30 min after morphine administration (2, 12, 26), however, seems to indicate that DA biosynthesis is stimulated before DA catabolism, rather than the reverse. This is in accord with direct observations of increased DA biosynthesis in rat striatum after morphine treatment (1, 2, 3). Increased levels of HVA and DOPAC after morphine administration (6, 7, 9) might then be secondary to this increased biosynthesis of DA. This would also be in line with the present observation that there are no increases in HVA and DOPAC concentrations when the synthesis of DA is inhibited by αMpT.

The stimulation of DA biosynthesis in the striatum by morphine, however, was not accompanied by changes in any indirect parameter of DA receptor stimulation (3, 27), a fact which is in accord with the present direct observation that DA is not released by morphine. We conclude, therefore, that morphine does not affect DA release and catabolism in the striatum, but directly stimulates DA biosynthesis.

As morphine does not alter tyrosine hydroxylase activity in striatal homogenates (3, 28), it is tempting to suggest that the increased DA synthesis *in vivo* is caused by a factor which is lost in these *in vitro* assays. This suggestion is also substantiated by the observation of increased tyrosine hydroxylation in striatal slices of mice (29). Such a factor which is lost *in vitro* could be a change in precursor availability (2) or a shift in the equilibrium of membrane-bound and soluble tyrosine hydroxylase (30), but other factors may be involved as well.

The increased biosynthesis of DA seems to be a common effect of morphine in all dopaminergic brain structures, since it has been observed

in the hypothalamus, midbrain (4) and n. accumbens (5). Moreover, increased levels of HVA and DOPAC, indicating increased biosynthesis of DA, have not only been observed in striatum and n. accumbens, but also in the tuberculum olfactorium (9) and in the frontal cortex (31).

The present results suggest that apart from the general enhancement of DA biosynthesis, morphine also exerts another effect on dopaminergic neurons in the frontal cortex, since an enhanced decline in DA levels was also observed in this structure when morphine was administered after αMpT. It might be argued, however, that this effect of morphine is due to an incomplete inhibition of DA synthesis at the moment of morphine injection. This might not have been detected in an earlier experiment in which it was shown that DA biosynthesis in whole rat brain is completely inhibited 30 min after administration of αMpT (14). The present results, however, show that the inhibition of DA synthesis in the frontal cortex and striatum 30 min after αMpT amounted to the same value as 60 min after αMpT. The calculated inhibition in the frontal cortex did not surpass 90%, as in the striatum, but this can be ascribed to the limitations of the techniques employed. The radioactive DA formed in the frontal cortex of control rats only amounted to about 1% of the radioactive tyrosine present, while in the striatum it amounted to more than 20%. Since, in αMpT treated rats, only about 100 cpm was counted in the DA fraction of the frontal cortex, most or all of this radioactivity could be accounted for by contamination from the tyrosine fraction (about 30,000 cpm). Thus, the synthesis inhibition of 85-90% observed in the frontal cortex is the maximal obtainable value. The results, therefore, do not necessarily indicate that there is a real difference in the inhibition of DA biosynthesis by αMpT between the two structures.

Since naloxone antagonizes the increase in DA loss in the frontal cortex, this effect seems to be specific for morphine. Most probably the increase in DA loss reflects an enhanced release of DA, but it is not possible to conclude from the present experiments whether this was due to an increased impulse flow or to a direct action of morphine on the nerve terminal. It is tempting to accept the latter explanation, since this would line up with morphine's releasing action in the striatum, as observed after inhibition of the impulse flow (23).

Morphine, therefore, seems to exert two separate effects on dopaminergic neurons under the conditions used in these studies: i) an increase in DA biosynthesis, common to most or all dopaminergic brain structures and ii) an increased release of DA in the frontal cortex (and tuberculum olfactorium unpublished observations), but not in the striatum and the n. accumbens (23). This dual effect of morphine might be an important clue to the interpretation of functional effects of this drug on dopaminergic neurons. Papeschi et al. (12) suggested, on the basis of similar experiments to those reported here, that it is unlikely that morphine alters the functional activity of dopaminergic neurons. These authors studied whole rat brain and because their results are in keeping with our observations in the striatum, which contains the bulk of whole brain DA, their suggestion seems to apply to this brain structure. Since morphine enhances DA release in the frontal cortex and tuberculum olfactorium under the same conditions, it in fact does alter the functional activity of dopaminergic neurons, but not those in the striatum and n. accumbens. The frontal cortex and tuberculum olfactorium and possibly other extrastriatal structures, which were not investigated, are probably of more importance therefore, for morphine-induced effects related to the functional activity of dopaminergic systems, such as catalepsy (6,8). This suggestion has also been made by others (32, 33).

In this context, it is of interest to know which dopaminergic nerve terminals were actually included in the frontal cortex, as defined in these experiments. As can be seen from fig. 1 and table 6, most of the DA (80%) orginates from basal parts of the forebrain (area 4). Therefore, the results obtained with the frontal cortex in the present experiments can probably be ascribed to processes in this dopaminergic area and not in the frontal (area 1) or anterior cingulate cortex (area 2), as described by Lindvall et al. (22). The origin of the DA in area 4 remains to be established. It should be stressed that the utmost care was taken not to contaminate the frontal cortex with tissue from the tuberculum olfactorium, n. accumbens or striatum. Moreover, the reproducibility of the amount of DA obtained from the frontal cortex or area 4, does not seem compatible with contamination by small pieces of tissue containing high concentrations of DA.

Equal amounts of DA and NA were found in the total frontal cortex. Since the amount of DA present as the percursor of NA in noradrenergic neurons has been calculated to be less than 4% of the NA present(34), more than 95% of the DA is presumed to originate from dopaminergic neurons in the frontal cortex. The results show that area 4 contained even considerably more DA than NA, indicating that we were dealing with dopaminergic neurons.

In conclusion, morphine seems to increase DA biosynthesis, regardless of the dopaminergic brain structure(1-4)and also causes release of DA from neurons in the frontal cortex and tuberculum olfactorium, but not from neurons in the striatum and n. accumbens.

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IV

MORPHINE: A MODULATOR OF DOPAMINERGIC NEUROTRANSMISSION IN THE RAT
2. COMPARISON WITH THE EFFECT OF HALOPERIDOL IN THE STRIATUM AND N. ACCUMBENS AFTER MANIPULATION OF THE IMPULSE FLOW

ABSTRACT

The present experiments show that morphine administration results in dopamine (DA) release in the striatum, but only when the impulse flow is interrupted by means of a lesion of the dopaminergic cell bodies or when the impulse flow is decreased by administration of apomorphine. Morphine had no effect on DA release when the impulse flow was normal nor when the impulse flow was increased by haloperidol treatment. Haloperidol increased DA release from the striatum at normal impulse flow, but this effect disappeared after a lesion of the dopaminergic cell bodies. In the n. accumbens, qualitatively similar drug effects were observed, although morphine seemed to have a stronger and haloperidol a weaker effect.

These results suggest that morphine releases DA by a direct effect on dopaminergic neurons. It is suggested that this releasing effect is overwhelmed by the impulse-flow-coupled release at normal or increased impulse flow. An underlying mechanism for this observation is proposed. It is also concluded from the present results that haloperidol-induced DA release involves a striatonigral feedback pathway rather than a local receptor-mediated feedback. Moreover, in our rats, DA release induced by morphine in the frontal cortex and tuberculum olfactorium seems to predominate over that in the striatum and n. accumbens under normal conditions. It is suggested, however, that this dominance is dependent on the state of activity of the dopaminergic systems involved.

The implications of these mechanisms for behavioural effects of morphine are discussed. It is concluded that morphine does not have an independent effect on dopaminergic neurons, but is a modulator of dopaminergic neurotransmission and dopamine-dependent behaviour.

INTRODUCTION

Morphine has been reported to considerably increase the turnover of DA in the rat striatum (1, 2, 3, 4, 5). Following the recent reports that morphine does not alter the rate of DA decline in the rat striatum, when the drug is administered 30 min after α-methyl-para-tyrosine methyl-ester (αMpT) (6, 7, 8), it was suggested that the increase in turnover reflects a direct stimulation of DA biosynthesis (8, 9). Since an increase in the rate of DA loss was induced by morphine in the frontal cortex as opposed to the striatum (6, 8), it was not clear whether this apparent DA-releasing action of the drug in the frontal cortex was absent in the striatum or whether it was also present in the striatum but was

counteracted in one way or another. The following considerations are of significance in this respect.

An effect of morphine on DA decline was occasionally obtained in the striatum (8) and in these experiments the striatal dopaminergic neurons seemed to have a lower basal activity. A possible explanation for this might be that morphine releases DA only when the neurons involved are relatively inactive. Furthermore, it has been suggested that morphine stimulates an inhibitory input to the nigro-striatal dopaminergic neurons (10). Also Gessa et al. (11) have suggested that another narcotic analgesic, methadone, might have dopamine agonistic as well as antagonistic properties in the rat; and Kuschinsky and Hornykiewicz (12) concluded that morphine releases DA in the striatum of mice, but not rats. This led us to the hypothesis, that morphine induces DA release in the striatum of the rat as in the frontal cortex, but simultaneously inhibits the same neurons. These effects would cancel each other out in normal rats. We have, therefore, analysed the effect of morphine on DA decline in the rat striatum after manipulation of the nervous impulse flow.

It was anticipated that morphine would increase the loss of DA when the impulse flow was decreased, while it would inhibit DA decline during increased impulse flow.

MATERIALS AND METHODS

Male, albino Wistar rats were purchased from TNO, Zeist, The Netherlands. Animals used for lesion experiments weighed 195-205 g, for other experiments 100-135 g. The first treatment (αMpT injection or decapitation) of all animals was carried out between 9.00 h and 11.30 h and treatments were randomized.

α-Methyl-para-tyrosine methylester.HCl (αMpT, AB Labkemi), 200 mg/kg as base and Morphine.HCl 20 mg/kg, were dissolved in 0.9% NaCl, Haloperidol (Janssen Pharmaceutica, Beerse, Belgium), was dissolved in a few drops of acetic acid, diluted with 0.9% NaCl and the pH adjusted to 4-5 with NaOH. Apomorphine.HCl (Sandoz A.G., Basel, Switzerland) was dissolved in 0.9% NaCl containing 0.2 mg/ml ascorbic acid. In the experiment with apomorphine this solvent was utilized for saline injections.

All drugs were injected i.p. in a volume of 5 ml/kg.

All chemicals were reagent grade. All water used was demineralized and subsequently distilled in an all glass apparatus.

LESION EXPERIMENT

Unilateral electrolytic lesions of the right mesencephalic dopaminergic cell bodies were made stereotaxically by means of an iron electrode, 1 mm thick, coated with epoxy varnish except for 0,5 mm at the tip. A current of 2 mA was passed through the electrode for 3 min. The coordinates of the electrode were 6.6 mm caudal, 2.0 mm lateral and 7.7 mm ventral from the bregma. Midbrains were removed at the end of the experiment, fixed in formalin and examined histologically for electrode placements.

All rats were injected with αMpT 25 min before the lesion was made.

17 Min after lesioning, morphine (20 mg/kg), haloperidol (1 mg/kg) or saline was administered. Most animals at the moment of drug injection, had recovered from the light ether anaesthesia applied before lesioning. Rats were decapitated 2 h later. Striata and nuclei accumbens from each side of the brain were dissected out as described in "Dissection" and analysed separately.

OTHER EXPERIMENTS

After 30 min αMpT pretreatment morphine (20 mg/kg), apomorphine (4 mg/kg), haloperidol (3 mg/kg), saline or indicated combinations of these drugs were administered. Apomorphine (4 mg/kg) injection was repeated 60 min later. Rats were decapitated 2½ h after αMpT injection. Combined striata of one rat were analysed after dissection as described in "Dissection".

DISSECTION

After decapitation, the brain of the rat was rapidly removed, dissected and frozen in liquid nitrogen within 4 min of decapitation. Striata were dissected out according to Gispen et al. (13) and nuclei accumbens according to Horn et al. (14).

ASSAYS AND STATISTICS

Dissected brain areas were weighed before homogenization. Mean wet tissue weights are reported in the legends to the figures.

Assay of striatal DA

Striatal DA was isolated on Al_2O_3 and assayed fluorimetrically as reported earlier (8). Recovery of 0.5 µg DA added to brain tissue extracts was 66 ± 4.2% (± S.D., n = 8).

Assay of DA in n. accumbens

Since the fluorimetric method is not sufficiently sensitive, DA in n. accumbens was assayed by a radiochemical method (15) as modified by Versteeg et al. (16).

Statistics

DA concentrations are expressed as µg/g wet tissue weight without corrections for recovery. Rate constants were calculated according to Brodie et al. (17).

Differences between DA levels in the lesion experiments were analysed by means of Duncan's new multiple range test (18) and in the other experiments by means of two-tailed Student's t-test. Differences between rate constants were tested with two-tailed Student's t-test. Values of p less than 0.05 were considered significant.

RESULTS

EFFECT OF MORPHINE AND HALOPERIDOL ON DA DECLINE IN STRIATUM AND N. ACCUMBENS AFTER A LESION OF THE MESENCEPHALIC DOPAMINERGIC CELL BODIES

Lesion of the mesencephalic cell bodies, after inhibition of DA biosynthesis, reduced the decline in DA virtually to zero in both striatum

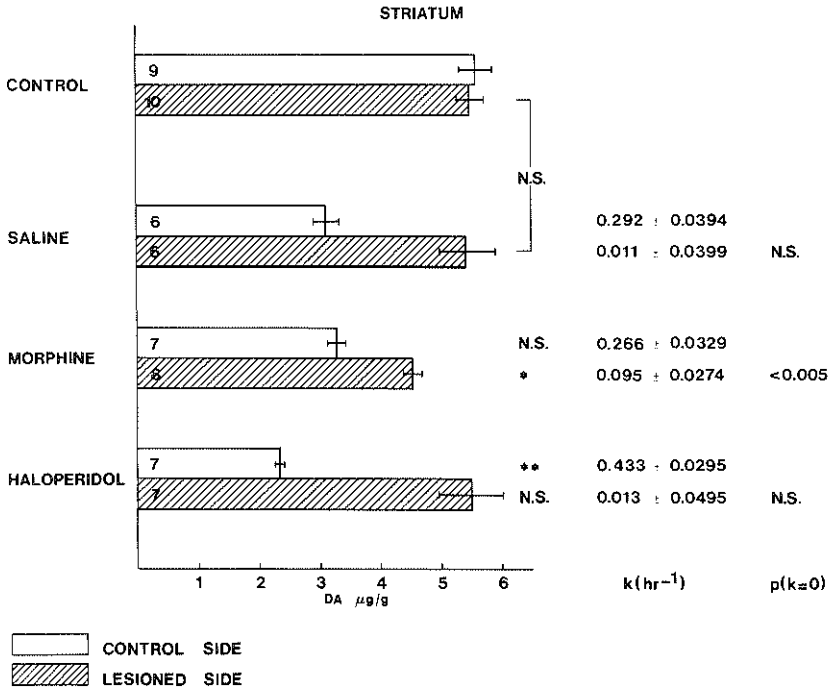

Fig. 1a. Effect of morphine and haloperidol on the αMpT-induced decline in DA in the striatum of rats with unilateral lesion of the mesencephalic dopaminergic cell bodies. Values represent mean ± S.E., number of observations are indicated at the base of the columns. DA concentrations in the lesioned side (hatched bars) and control side (open bars) are given at the moment of drug administration (CONTROL, 42' after αMpT) and 2 h later after saline, morphine (20 mg/kg) or haloperidol (1 mg/kg) treatment. See also Materials and Methods "Lesion Experiment".

Statistics: Duncan's new multiple range test and Student's t-test for p (k=0): n.s.: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$ when compared with the appropriate saline controls. All DA levels in control sides were significantly different from lesioned sides and from DA levels in CONTROL ($p < 0.01$).

Mean wet tissue weight was 46.1 ± 5.3 mg (± S.D., n = 60).

and n. accumbens, as shown in fig. 1. Morphine (20 mg/kg) then induced a loss of DA in both structures on the lesioned side, as evident from the fall in DA levels and from the rate constants, which were significantly different from zero, in contrast to saline treated rats. Haloperidol (1 mg/kg) was without effect on the lesioned side. This drug, however, enhanced the normal rate of DA loss in the control side in both structures, while at this side morphine was without effect.

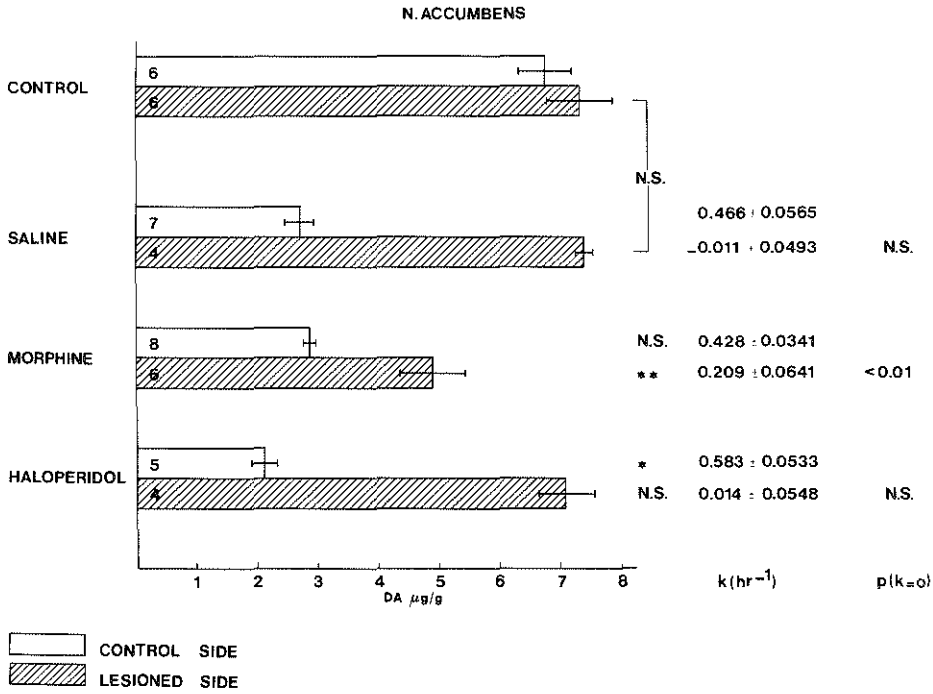

Fig. 1b. Effect of morphine and haloperidol on the αMpT-induced decline in DA in the n. accumbens of rats with unilateral lesion of the mesencephalic dopaminergic cell bodies. Values represent mean ± S.E., number of observations are indicated at the base of the columns. DA concentrations in the lesioned side (hatched bars) and control side (open bars) are given at the moment of drug administration (CONTROL, 42' after αMpT) and 2 h later, after saline, morphine (20 mg/kg) or haloperidol (1 mg/kg) treatment. See also Materials and Methods "Lesion Experiment".

Statistics: Duncan's new multiple range test and Student's t-test for p (k=0): n.s.: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$ when compared with the appropriate saline controls. All DA levels in control sides were significantly different from lesioned sides and from DA levels in CONTROL ($p < 0.01$).

Mean wet tissue weight was 8.4 ± 2.0 mg (± S.D., n = 48).

To compare the drug effects in both structures, the rate constants were calculated in proportion to the rate of DA decline at the control side after saline treatment. These control values of 0.292 h^{-1} in the striatum and 0.466 h^{-1} in the n. accumbens were set arbitrarily at 100%. Morphine resulted in a relative rate of DA loss on the lesioned side of 33% in the striatum as against 45% in the n. accumbens. Haloperidol, on the other hand, increased the rate constant on the unlesioned side to 148% and 125% in the striatum and n. accumbens, respectively.

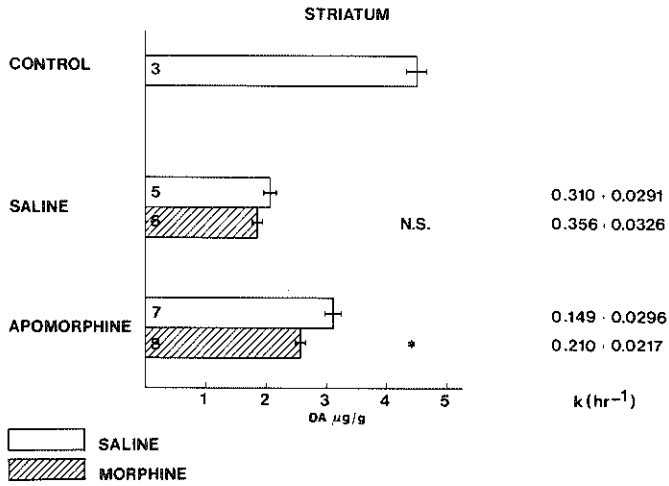

Fig. 2. Effect of morphine on the αMpT-induced decline of DA in the striatum of rats, simultaneously treated with apomorphine. Values represent mean S.E., number of observations are indicated at the base of the columns. CONTROL: DA level at the moment of αMpT administration. DA levels 2 h after saline (open bars) or morphine (20 mg/kg, hatched bars) administration are given for rats simultaneously treated with saline or apomorphine (2 x 4 mg/kg). These drugs were injected 30 min after αMpT. See also Materials and Methods "Other Experiments".

Statistics: Student's t-test, n.s.: $p > 0.05$; *: $p < 0.005$ when compared with appropriate saline. DA levels of apomorphine treated rats significantly differed from the appropriate saline controls ($p < 0.001$). Striata of one rat weighed 105 ± 8.3 mg (± S.D., n = 29).

EFFECT OF MORPHINE ON THE DECLINE OF DA IN STRIATUM OF RATS SIMULTANEOUSLY TREATED WITH APOMORPHINE OR HALOPERIDOL (FIG. 2 & 3)

The rate of DA decline in the striatum was reduced to about 50% of the control value by two injections of 4 mg/kg apomorphine. Morphine caused an increase in the rate of DA loss in apomorphine but not in saline treated rats (fig. 2).

Haloperidol (3 mg/kg), on the other hand, increased the rate of DA loss to 162% of the control value. Simultaneous treatment with morphine was without effect in haloperidol as well as saline treated rats.

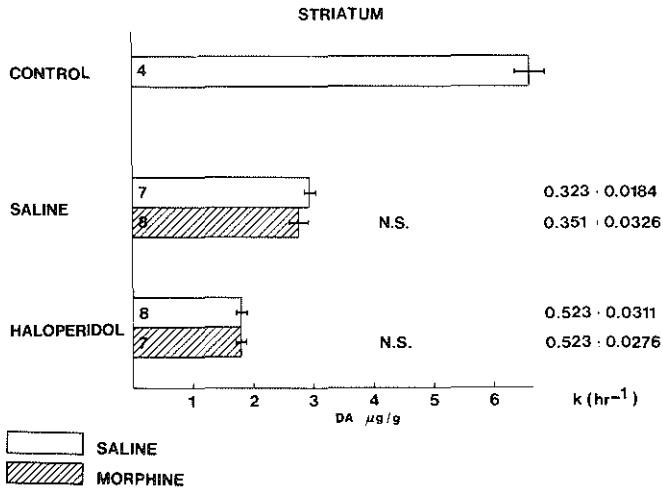

Fig. 3. Effect of morphine on the αMpT-induced decline of DA in the striatum of rats simultaneously treated with haloperidol. Values represent mean ± S.E., number of observations are indicated at the base of the columns. CONTROL: DA level at the moment of αMpT administration. DA levels 2 h after saline (open bars) or morphine (20 mg/kg, hatched bars) administration are given for rats simultaneously treated with saline or haloperidol (3 mg/kg). These drugs were injected 30 min after αMpT. See also Materials and Methods"Other Experiments".

Statistics: Student's t-test, n.s.: $p > 0.05$ when compared with the appropriate saline. DA levels of haloperidol treated rats significantly differed from the appropriate saline controls ($p < 0.001$). Striata of one rat weighed 101 ± 8.4 mg (± S.D., n = 34).

DISCUSSION

The present results show that morphine administration results in release of DA in the rat striatum, when the impulse flow of the nigro-striatal pathway is reduced. Interruption of the impulse flow, by means of a lesion of the cell bodies, resulted in complete inhibition of basal DA release. Under these conditions, morphine could not produce further inhibition of DA release and consequently, its releasing effect was uncovered. This was also the case when the basal DA release was reduced to about ca 50% by apomorphine. Since all communication with other neuronal systems is presumably eliminated by interruption of the impulse flow, the morphine-induced release probably resulted from a direct action on dopaminergic neurons. At normal impulse flow, however, no release of DA was induced by morphine.

These observations support the hypothesis that morphine simultaneously stimulates and inhibits release of DA by two independent mechanisms (see

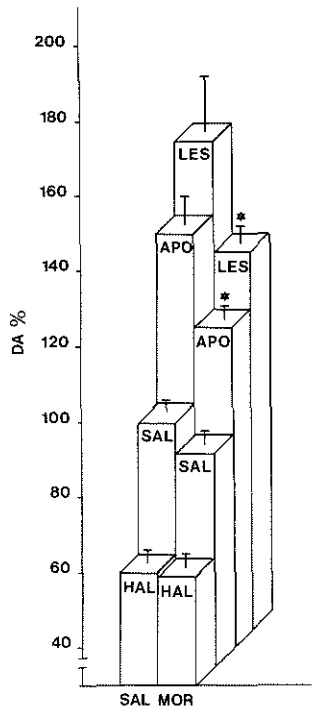

Fig. 4. Impulse flow dependent effect of morphine on DA release in the striatum. Compilation of data of figures 1 - 3 . The concentration of DA 2½ h after αMpT treatment was set to 100% for each experiment and the DA concentration obtained from rats, treated in addition with other drugs or lesioned in addition, were calculated relative to this value. Treatments: HAL: haloperidol; SAL: saline; APO: apomorphine; LES: lesion. All these treatments were either combined with SAL: saline or MOR: morphine treatment. *: $p < 0.05$ vs corresponding treatment without morphine. See also figures 1-3.

Introduction). If two independent effects really do exist, prior increase of impulse flow would be expected to result in the predominance of the inhibitory effect of morphine. The drug, however, had no effect when DA release was considerably increased with haloperidol. Therefore, there does not seem to be a balance between two independent effects of morphine. Since the drug, apparently, does induce release of DA during reduced impulse flow, the results probably indicate that this releasing effect is overwhelmed by the normal impulse-flow-coupled release itself as vizualized in fig. 4. As the release induced by morphine is not additive to the normal release, both stimuli seem to act through a common mechanism. It is tempting to speculate that the liberation of calcium is important in this respect, since effects of morphine, as well as stimulation-induced catecholamine release, are dependent on the presence of this ion (19). Morphine has also been shown to induce a rapid depletion of calcium from brain tissue (20). This might also account for the independent stimulation of DA biosynthesis exerted by morphine (see Introduction), since increased intrasynaptosomal calcium has been shown to activate tyrosine hydroxylase through increased membrane association (21). It is suggested, therefore, that morphine liberates calcium in dopaminergic nerve endings resulting, separately, in increased DA biosynthesis and in release of DA, which is dependent on the impulse flow of the dopaminergic neurons.

Acceptance of this hypothesis,in favour of that advanced in the Introduction, relies mainly on the observation that morphine had no effect when the impulse flow was increased by haloperidol (vide supra). It might be argued that morphine increases DA release by blockade of DA receptors (22). Since haloperidol blocks DA receptors, additional morphine treatment would then have no effect. It was observed, however, that haloperidol accelerated DA decline, in contrast to morphine, when the drug was administered after αMpT. This effect of haloperidol is probably the indirect result of receptor blockade, since haloperidol has been shown to increase impulse flow in the nigrostriatal pathway (23). Moreover, morphine caused release of DA from lesioned neurons, unlike haloperidol. These observations support the suggestion that morphine releases DA by a direct action on dopaminergic neurons rather than by receptor blockade. Thus, morphine does not act through the same mechanism as haloperidol.and this seems to validate therefore, the suggestion that DA release, induced by morphine,is dependent on the impulse flow of the dopaminergic neurons.

The observation that haloperidol accelerated DA decline when administered after αMpT also shows that receptor blockade causes DA release which is independent of the increase in DA biosynthesis (24, 25), since DA biosynthesis was entirely inhibited in the present experiments. Similarly, apomorphine reduced DA release which is independent of its effect on DA synthesis, in contrast to the suggestion of Goldstein et al. (26).

Another conclusion, concerning feedback control of DA release, can be drawn from the present lesion experiment. The observation that there was no release of DA on the lesioned side can be taken as an indication of the effectiveness of the lesion. It also indicates, however, that absence of impulse flow and consequent lack of DA receptor stimulation does not trigger any local feedback stimulation of DA release.Kehr et al.(24) observed feedback regulation of DA biosynthesis in the absence of impulse flow and therefore, postulated a receptor-mediated feedback mechanism in the striatum. The present results suggest that this receptor-mediated feedback only controls DA biosynthesis and not DA release. Moreover, it was

recently observed in vitro that acetylcholine induces release of DA from striatal slices, which are also obviously devoid of impulse flow (27). It was suggested that presynaptic cholinergic receptors may control or modulate DA release. Our results, obtained in vivo, however, do not support this hypothesis. Therefore, DA release, induced by blockade of striatal DA receptors, seems to depend on a striatonigral feedback pathway, like increased tyrosine hydroxylase activity, which is also induced by blockade of striatal DA receptors (28). Our observation is also in accord with the reported increase in the activity of nigral dopaminergic cells after haloperidol injection (23).

The drug effects in the n. accumbens were similar to those in the striatum. Thus haloperidol accelerated DA decline in the unlesioned side, while morphine induced DA release in the lesioned side. This was somewhat surprising since this mesolimbic brain structure might have been expected to react in a manner which was similar to that of the frontal cortex where morphine induces release of DA in unlesioned rats (6, 8). Nevertheless differences seem to exist between both structures, since the effect of haloperidol was stronger in the striatum, while the effect of morphine was more pronounced in the n. accumbens. The reliability of these quantitative comparisons, however, is somewhat weakened by the fact that DA concentrations were assayed by different analytical techniques in both structures.

Taking together the results of this and the preceding report (8), a complex picture of the interaction of morphine with dopaminergic neurons emerges.

1. Morphine seems to stimulate DA biosynthesis directly in most or all dopaminergic structures (8).

2. Morphine stimulates DA utilization (possibly release) in the frontal cortex and tuberculum olfactorium (unpublished results), but not in the striatum and n. accumbens of rats with normal impulse flow.

3. Morphine stimulates DA utilization (probably release) in the striatum and n. accumbens of rats with a decreased impulse flow.

The overall effect of morphine on dopaminergic systems, therefore, can not easily be predicted. It was suggested that the DA releasing effect of morphine in the frontal cortex (point 2) indicates that extrastriatal dopaminergic structures are of more importance than the striatal for morphine-induced effects like catalepsy (8).

This applies when impulse flow in the dopaminergic neurons remains unchanged. When some manipulation of the rats, however, decreases impulse flow in dopaminergic neurons, then morphine will release DA from these neurons (point 3). Such a manipulation is not necessarily a lesion. It might be expected that a noxious stimulus would also decrease impulse flow in striatal dopaminergic neurons. If this were so, analgesia, induced by morphine, may involve DA release in the striatum. This would be in keeping with results implicating stimulation of striatal dopaminergic neurotransmission in morphine-induced analgesia (29), or at least in a particular analgesic response (30). The point to be stressed is that the role of DA in morphine-induced behaviour can not be evaluated when the impact of the relevant behaviour per se on the state of activity of dopaminergic neurons is not known. Morphine, therefore, does not have an independent effect on dopaminergic neurons, but is a modulator of dopaminergic neurotransmission and dopamine-dependent behaviour. Its actual effect on striatal DA neurons depends on the state of activity of these neurons.

ACKNOWLEDGEMENT

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APPENDIX TO PAPER IV

I

In an experiment identical to the one reported in fig. 1 of paper IV the effect of morphine treatment combined with a lesion of the dopaminergic cell bodies was investigated in the frontal cortex. The aim was to assess whether the observed increased loss of DA in the frontal cortex was dependent on the impulse flow.

The experimental procedure was described in paper IV. Frontal cortices were dissected as described in paper III and DA was assayed using the radiochemical procedure outlined in paper IV.

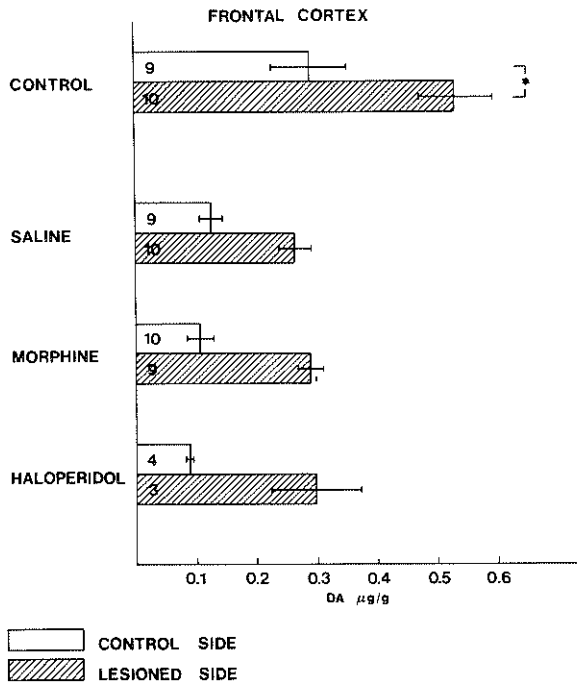

Fig.1. Effect of morphine and haloperidol on the αMpT-induced decline of DA in rats with unilateral lesions of the mesencephalic dopaminergic cell bodies. Values represent mean ± S.E., number of observations are indicated at the base of the columns. DA concentrations in the lesioned side (hatched bars) and control side (open bars) are given at the moment of drug administration (CONTROL, 42' after αMpT) and 2 h later, after saline, morphine (20 mg/kg) or haloperidol (1 mg/kg) treatment. See also Materials and Methods IV

Mean wet tissue weight was 109.1 ± 13.4 mg (± S.D., n = 68).

* $p < 0.05$, Student's t-test.

Fig. 1 shows the result of this experiment. The concentration of DA on the lesioned side strongly increased in control rats when compared with the unlesioned side. The value of 289 ± ng/g DA on the unlesioned side in these rats is in the range normally observed (II, III). The lesion, therefore, induced an almost twofold increase in DA levels in 17 min, an effect not observed in the striatum and n. accumbens (IV). The origin of this effect is obscure, since the prior blockade of DA biosynthesis must be assumed to prevent any increase of DA levels. It has been shown that DA synthesis is completely blocked during the period of observation (II), and this is also true in the frontal cortex (III).

A possible explanation of the observed effect could be that interruption of the impulse flow abolishes the inhibitory effect of the false precursor αMpT on the biosynthesis of DA. This, however, seems very improbable, since it would need a tremendous increase in DA biosynthesis in order to obtain in 17 min DA levels which are twice the normal. Moreover, this would not occur in the dopaminergic neurons projecting to the striatum and nucleus accumbens after the same treatment (III). An artifact has to be considered, therefore, to account for this observation.

Whatever the cause may be for the observed increase in DA levels shortly after a lesion, the effects of drug treatments as shown in fig. 1 can not be interpreted properly due to the observed effect of the lesion procedure itself.

II

It has been shown that morphine does not affect DA release in the striatum of the rat, when the drug was administered 30 min after αMpT. This distinguished the striatum from the frontal cortex, where an increase of DA decline was observed (II, III). A follow-up study showed that morphine also had an effect on DA release in the striatum, but this effect was dependent on the state of activity of the neurons involved (IV). When the decline of DA was blocked by a lesion of the mesencephalic dopaminergic cell bodies or inhibited by pretreatment with apomorphine, morphine induced loss of DA in the striatum, while at normal or increased impulse flow morphine had no effect (IV). In the n. accumbens similar effects of morphine were observed. Whether other dopaminergic brain structures react alike to manipulation of the impulse flow, was not assessed. The present experiments provide a preliminary indication that the effect of morphine in the tuberculum olfactorium (T.O.) may not be dependent on the state of activity of dopaminergic neurons in the same way as in the striatum.

The effect of morphine, in combination with haloperidol or apomorphine, on DA decline in the T.O. was investigated in an experiment identical to those described in paper IV. Pooled olfactory tubercles of 4 rats were analysed for DA content as described elsewhere (III). DA release was increased by haloperidol in the T.O. and striatum resulting in concentrations of DA of 60-70% of controls. Apomorphine, on the other hand, inhibited DA decline, resulting in DA levels of 145-150% of controls (table 1). The change in DA release by haloperidol and apomorphine in striatum and T.O. was similar, therefore. The effect of morphine treatment in relation to pretreatment with haloperidol and apomorphine can be read from the difference between saline and morphine treatment

TABLE 1.

Effect of Morphine in Combination with Haloperidol or Apomorphine on DA Decline in the Tuberculum Olfactorium and Striatum.[a]

Pretreatment	Haloperidol	Saline	Apomorphine
Tuberculum Olfactorium			
Saline	65.0;76.3	100.0±4.8 (3)[b]	133.4;158.0[x]
Morphine	53.1;57.7[x]	88.1±3.1 (3)	128.8;150.7[x]
Saline-Morphine	15.2± 6.2	11.9±5.6	5.9± 16.4
p (Saline vs Morphine)		<0.05	
Striatum[d]			
Saline	61.1±3.0 (8)[x]	100.0±2.4 (12)[c]	150.7±6.7 (7)[x]
Morphine	60.9±2.8 (7)[x]	92.1±3.2 (14)	125.7±3.2 (8)[x]
Saline-Morphine	0,2±4.2	7.9±4.2	25.0±7.2
p (Saline vs Morphine			<0.005

x p <0.05 vs Saline in middle column
a. Values represent DA concentration ± SEM as percentage of controls treated 2½ h before with αMpT only. Other drugs were injected 2 h before decapitation.
b. 100% = 1.51 µg/g DA; DA concentration in untreated rats: 3.43 µg/g.
c. 100% = 2.58 µg/g DA; DA concentration in untreated rats: 5,71 µg/g.
d. from IV.

(Saline-Morphine, table 1). As reported in paper IV, the effect of morphine in striatum increases with decreasing activity of the neurons involved. Thus, in combination with haloperidol no effect was obtained, while in combination with apomorphine a significant increase of DA release by morphine was observed (table 1). In the T.O., however, only a slight increase of DA decline was observed when morphine was administered in combination with apomorphine, while a significant effect was obtained without pretreatment.This effect was even stronger in combination with haloperidol, although it was not statistically significant. ($p \simeq 0.12$). Thus, in the T.O. the effect of morphine does not seem to increase with decreasing impulse flow and the opposite can even not be excluded.

Although it is difficult to draw firm conclusions on the basis of the present data, the observations indicate that it might be worth-while to study the DA releasing effect of morphine in relation to the state of

activity of the dopaminergic neurons in more brain structures than has so far been attempted. It has been suggested that such state-dependent effects of morphine may provide an important clue for the interpretation of contradictory results in different behavioural responses to the drug (IV). If in addition this state-dependency would differ in various brain structures, an accurate analysis of these interactions is indispensible for a correct correlation of cellular and molecular effects of morphine to its behavioural effects.

V

MORPHINE CATALEPSY IN RELATION TO STRIATAL AND EXTRASTRIATAL DOPAMINE

ABSTRACT

When morphine (20 mg/kg i.p.) was administered to rats 30 min after treatment with an inhibitor of dopamine biosynthesis (α-methyl-para-tyrosine), it induced strong catalepsy. However, no effect on the decline of dopamine was observed in the striatum under these conditions. If morphine catalepsy is caused at the level of the striatum only dopamine receptor blockade is compatible with these results. On the other hand did morphine increase the decline of dopamine in the frontal cortex. The results suggest, that mesolimbic dopaminergic systems might be involved in the generation of morphine catalepsy.

INTRODUCTION

One clear-cut result of acute narcotic treatment of the rat is an increase of dopamine turnover in the striatum (1,2). Moreover, evidence has been presented to correlate this effect to a definite behavioural effect of narcotics viz. their cataleptogenic action (3,4). Main arguments for this correlation are an antagonism of morphine catalepsy by dopamine agonists like apomorphine and L-DOPA (3) and the parallelism between neuroleptic and narcotic catalepsy (5,4). Neuroleptics also cause a catalepsy and an increase in dopamine turnover. Since this catalepsy is suggested to result from a blockade of dopaminergic receptores in the striatum (6), narcotics are considered to antagonise dopaminergic transmission in the striatum, too (7,5,3).

However, closer analysis of these facts also reveal a number of discrepancies between the action of narcotics and neuroleptics with respect to catalepsy and dopamine turnover. Narcotic catalepsy is associated with a muscular rigidity while catalepsy induced by neuroleptics is often accompanied by hypotonia. Furthermore, narcotic catalepsy is more effectively antagonised by dopamine agonists than neuroleptic catalepsy (3). As far as dopamine turnover is concerned, several differences between the two groups of drugs at the molecular level are known, including effects on tyrosine hydroxylase, dopamine sensitive adenylate cyclase and quantitative differences with respect to synthesis rate in vivo (8,5). This led to the consideration, that narcotic catalepsy is a dopaminergic phenomenon of striatal origin, but with an underlying mechanism different from neuroleptic catalepsy (3,5,4).

Recent observations in our laboratory, however, led us to reevaluate the role of striatal dopamine in narcotic catalepsy. We studied the effect of morphine on the decline of dopamine after α-methyl-para-tyrosine (αMpT) treatment in striatum and frontal cortex. Morphine was administered to rats 30 min after αMpT when the synthesis of dopamine is completely inhibited,

so that direct or indirect effects on dopamine synthesis are excluded. Under these experimental conditions we observed an enhanced decline of dopamine in the frontal cortex, but, on the contrary, no effect of morphine on dopamine in the striatum could be detected (9). This prompted us to study morphine catalepsy under exactly the same conditions, because, if an increased dopamine turnover in the striatum is a prerequisite for narcotic catalepsy, no catalepsy should be present when morphine is administered 30 min after αMpT.

MATERIALS AND METHODS

Male, albino Wistar rats (TNO, Zeist, The Netherlands) weighing 100-130 g were used. The animals were housed 5 in a cage with ad libitum food and water. Lights were on from 7 a.m. to 9 p.m. Experiments were performed between 9 a.m. and 1 p.m.

α-Methyl-para-tyrosine-methylester.HCl (αMpT, AB Labkemi) was injected in a dose of 200 mg/kg as base. Morphine.HCl, naloxone (Endo Laboratories) or a combination of both was administered 30 min later. Naloxone treated rats received a second injection 60 min later immediately after the first catalepsy test. All drugs were dissolved in 0.9% NaCl and injected intraperitonealy in a volume of 5 ml/kg.

Catalepsy was tested 1 and 2 hr after morphine administration according to Kuschinsky and Hornykiewicz (3). Rats were placed with their forepaws on a 7.5 cm high bar. They were considered cataleptic when they remained in this position for 30 sec.

In a separate experiment rats were decapitated 2 h after morphine treatment (2½ h after αMpT). Brains were removed and striata dissected according to Gispen et al. (10). Striatal tissue was homogenized in 10 ml ice-cold 0.4 N perchloric acid containing 0.5% EDTA and 0.05% $Na_2S_2O_5$ and centrifuged at 25,000 g for 15 min at 2° C. Dopamine was absorbed to Aluminiumoxide according to Weil-Malherbe (11) and eluted with 3 ml of 1 N HCl (containing 1 mg/l ascorbic acid and 10 mg/l EDTA) in a batch procedure. Dopamine was assayed according to Atack (12) in 1 ml aliquots of the eluate.

RESULTS

Morphine in a dose of 20 mg/kg caused a significant catalepsy 60 min after administration (table I). This effect had disappeared after 120 min. When the rats were pretreated with αMpT 30 min before morphine an even greater number of rats showed catalepsy at 60 min and this number decreased but was still significant 120 min after morphine injection. αMpT, therefore, intensifies the catalepsy induced by morphine, while it does not cause any catalepsy per se. Moreover, the cataleptic effect of morphine was blocked by simultaneous treatment with naloxone, indicating the specificity of the effect (Table I).

Administration of graded doses of morphine did not significantly affect the dopamine decline in the striatum of rats pretreated with αMpT 30 min before morphine. Dopamine concentrations declined from 4.99 ± 0.22 µg/g (mean ± S.E., n = 6) at the moment of morphine injection

Table I

Catalepsy Induced by Morphine after Inhibition of Dopamine Synthesis.

Treatment		Catalepsy	
Time -30 min	0 min	60 min	120 min
-	Saline	0/12	0/12
-	Morphine[a]	7/12*	2/12
αMpT[c]	Saline	0/12	0/12
αMpT[c]	Morphine[a]	10/12*	6/12*
αMpT[c]	Naloxone[b]	0/12	0/12
αMpT	Morphine[a] + Naloxone[b]	1/12[xx]	1/12[x]

Fisher exact probability test:

* Different from saline control; $p < 0.01$

x Different from morphine treatment; $p < 0.05$

xx Different from morphine treatment; $p < 0.01$

[a] 20 mg/kg, i.p.

[b] 1 mg/kg i.p. Injection was repeated immediately after first catalepsy test.

[c] α-methyl-para-tyrosine-methylester.HCl, 200 mg/kg as base, i.p.

to 2.1 - 2.5 µg/g (Fig. 1) 2 hr later. Because morphine causes catalepsy at a dose of 20 mg/kg but not at 10 mg/kg or lower, we included lower doses than 20 mg/kg for comparison of cataleptogenic and non-cataleptogenic doses. Besides 40 mg/kg was included to be certain that a marginal effect at 20 mg/kg was not missed. However, with none of these doses an effect on dopamine was observed.

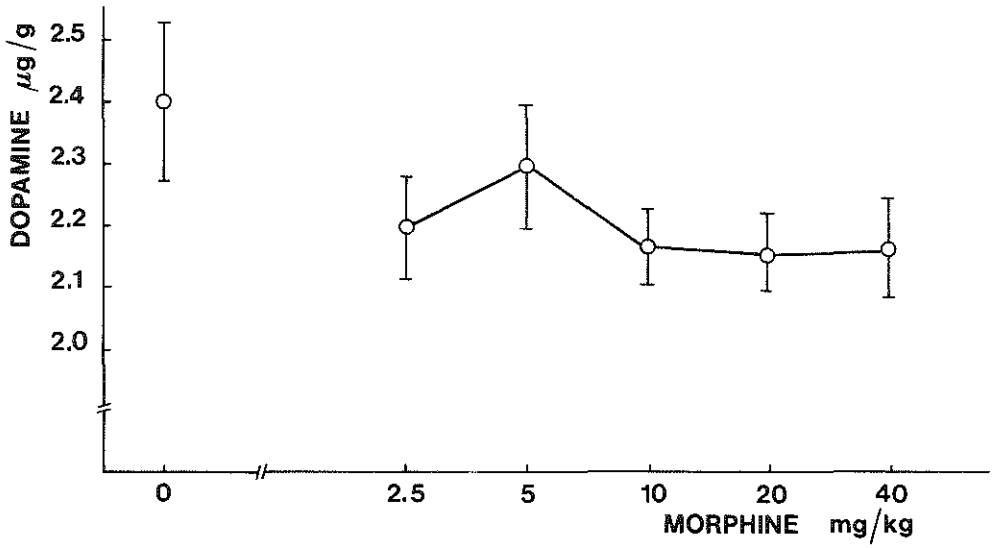

Fig. 1. Dopamine levels in rat striatum 2 h after different doses of morphine, administered 30 min after αMpT treatment. Values represent mean ± S.E. of 7 or 8 determinations. None of the morphine treatments gave a value significantly different from control (0 mg/kg); test of Dunnett (13), for multiple comparison of several treatments with a control.

DISCUSSION

The present results show that morphine can induce catalepsy without concurrent effect on dopamine turnover in the striatum. This indicates that morphine catalepsy is not caused by a presynaptic effect on dopaminergic neurons in the striatum, like diversion of newly synthesized dopamine from storage sites to sites of catabolism as suggested earlier (3). This emerges from two observations. Firstly, αMpT does not induce catalepsy at points of time that dopamine synthesis is completely inhibited. Therefore, a deficiency of newly synthesized dopamine does

not induce catalepsy per se. Secondly, morphine does induce catalepsy even when the synthesis of dopamine is completely inhibited and no dopamine is displaced at all in the striatum. The only mechanism at the level of the striatum by which morphine may induce catalepsy seems to be a blockade of dopamine receptors as suggested by Puri et al. (7). Such a blockade would increase dopamine turnover via a feedback on the biosynthesis of dopamine. After prior synthesis inhibition by αMpT, then, no effect should be found on striatal dopamine turnover as shown in the present experiment.

However, several observations indicate that dopamine receptor blockade does not seem to be the cause of narcotic catalepsy (8) or at least not the only cause (5). Moreover, Papeschi et al. (14, 15) concluded from similar observations as presented here that an interaction of morphine with dopaminergic neurotransmission is not likely to be the cause of catalepsy at all. This, however is not in agreement with the observation that morphine catalepsy is easily antagonized by apomorphine (3), which indicates, that it is a dopaminergic phenomenon. It is therefore, of significance that under these experimental conditions morphine does enhance the rate of dopamine depletion in the frontal cortex, while no effect was detected in the striatum (9). A role of mesolimbic dopaminergic systems in morphine catalepsy has been suggested recently (16) and is in accord with our observations.

CONCLUSION

Although the present experiments do not exclude a blockade of dopamine receptors in the striatum as the cause of morphine catalepsy, an interaction of morphine with dopaminergic neurotransmission in mesolimbic areas of the rat brain should also be considered as the origin of the catalepsy.

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VI

IS MORPHINE-INDUCED CATALEPSY RELATED TO ACTIVATION OF DOPAMINERGIC NEURONS?

ABSTRACT

Administration of morphine (20 mg/kg, i.p.), 30 min after α-methyl-para-tyrosine-methylester (αMpT) resulted in marked catalepsy, which was maximal 40-90 min after injection of morphine. Pretreatment with αMpT did not alter the induction of morphine catalepsy, but prolonged its duration. The effect of several drugs on the catalepsy, induced by morphine, after αMpT pretreatment, was investigated. Naloxone, a specific morphine antagonist, entirely abolished the morphine-induced catalepsy, while apomorphine antagonized this catalepsy for 40 min, when administered simultaneously with morphine. Amphetamine, on the other hand, potentiated morphine-induced catalepsy and also produced catalepsy directly. Moreover, amphetamine-induced sniffing, a major sign of stereotypic behaviour, was antagonized by morphine. Atropine, an anticholinergic agent, and bicucculine, a GABA-receptor blocking agent, were ineffective against morphine-induced catalepsy.

Administration of haloperidol (1 mg/kg, i.p.), 30 min after αMpT, resulted in a gradually increasing catalepsy, after repeated testing of the rats, but no catalepsy was observed after a single test, regardless of the time period between test and haloperidol administration. Thus, repeated testing is an important factor in the induction of catalepsy with haloperidol.

The present results indicate the involvement of two dopamine (DA) systems in the interaction of morphine with dopaminergic agents. It is suggested that morphine may induce catalepsy by activation of a DA system other than those projecting to the striatum and n. accumbens. The implications of this hypothesis are discussed in relation to the interaction of morphine with apomorphine- and amphetamine-induced catalepsy and stereotypic behaviour.

INTRODUCTION

Catalepsy induced by neuroleptics or by cholinergic agents, has been ascribed to an action on the striatum (1, 2). Moreover, it has been suggested that catalepsy, induced by different types of drugs, always involves the dopaminergic (DA) system (3) and that catalepsy is always associated with changes in DA metabolism (4). It has further been suggested that neuroleptics block DA receptors, and the resulting increase in turnover of DA in the striatum can be explained on the basis of a possible feedback mechanism (5, 6). Since opiates increase DA turnover in the striatum and also induce catalepsy, it was suggested that opiates may have a mechanism of action which is similar to neuroleptics (4, 7, 8). The striatal origin of morphine-induced catalepsy, however, has been questioned recently, since lesions of the amygdala were shown to reduce morphine-

induced catalepsy (9). Moreover, it has been reported that morphine does not affect DA release in the striatum (10). In fact, these results raise some doubt as to whether morphine-induced catalepsy is directly related to DA systems . On the other hand, morphine increases DA release in the frontal cortex (10), which would favour the suggestion that morphine-induced catalepsy may involve extrastriatal rather than striatal DA systems (9, 11, 12).

The possible relationship between dopaminergic neurotransmission and morphine-induced catalepsy has been assessed indirectly (11). Thus, when the synthesis of DA was blocked by αMpT, subsequent morphine administration resulted in acceleration of the decline in DA levels in the frontal cortex, but not in the striatum (10, 11), while after the same drug treatments marked catalepsy was observed (11). The aim of the present study was to directly assess whether the morphine-induced catalepsy was related to DA systems or not. For this purpose, the effect of dopaminergic or other agents was investigated on the catalepsy induced by morphine after αMpT treatment.

MATERIALS AND METHODS

Male albino Wistar rats were purchased from T.N.O, Zeist, the Netherlands. The animals were housed 4-6 to a cage, with free access to food and water. The period of light was from 7.00 h to 21.00 h, ambient temperature was ca 20° C. The animals weighed 100-150 g on the day before the experiment. On the day of the experiment the animals were moved to a sound attenuated room with background noise produced by a ventilator. The rats were placed in individual cages at least 30 min before the first drug, which was administered between 10.30 h and 11.30 h.

The following drugs were used: Morphine.HCl, α-methyl-para-tyrosine-methylester.HCl (αMpT, AB Labkemi), Naloxone.HCl (Endo Labs.), Apomorphine.HCl (Sandoz A.G.), Atropine-sulphate, Bicucculine (K & K Labs.), Haloperidol (Janssen Pharmaceutica), and Amphetamine-sulphate. Morphine, naloxone, amphetamine and αMpT were dissolved in 0.9% NaCl; apomorphine in 0.9% NaCl with 0.2 mg/ml ascorbic acid; bicucculine, atropine and haloperidol in 0.9% NaCl with 0.2 mg/ml ascorbic acid acidified to pH = 1.7 with HCl. Where ascorbic acid or acid drug solutions were used, all other drug solutions utilized throughout these particular experiments were also made acidic. All drugs were injected intraperitoneally in a volume of 5 ml/kg. Drug doses refer to the salts. Treatment schedules were randomized. All drugs were administered 30 min after αMpT pretreatment and injection of naloxone was repeated 60 min later.

Catalepsy Test

Catalepsy was measured by the procedure outlined by Kuschinsky and Hornykiewicz (8) immediately followed by a slight modification of the method of Shore and Doris (13). In the first procedure, both forepaws of the rat were placed on a 7.5 cm high bar or peg. The rats were considered cataleptic when they remained in this position for 30 sec. In the second test, each front paw of the rat was placed successively on a 2.5 cm and 7.5 cm high peg. A maximum of three points was assigned to a rat which always remained in that position for 10 sec (see also Shore and Doris, 13).

Since the results of both tests were similar, only the results of the second test are presented. The time-course of drug-induced catalepsy was assessed by repeated testing of the same subjects at indicated time intervals.

Stereotypy

In a double blind experiment, utilizing amphetamine and morphine (table 1), an experienced technician was instructed to note any behavioural signs observed, as well as performing the catalepsy test according to Kuschinsky and Hornykiewicz (8). Straub tail, piloerection and several signs of stereotypic behaviour were observed repeatedly, but only sniffing could be consistently quantified post hoc. Table 1 shows the number of animals in which sniffing was observed.

Statistics

Differences between drug treatments were evaluated by means of the Wilcoxon rank sum test, or Fisher exact probability test. Values of p less than 0.05 were considered significant.

Table 1

Interaction of Morphine and Amphetamine on Catalepsy and Stereotypic Behaviour after αMpT Treatment.[a]

	1 h[b]				2 h[b]			
	catalepsy		sniffing		catalepsy		sniffing	
Amphetamine[c]	-	+	-	+	-	+	-	+
Morphine (mg/kg)								
0	0	5^{x}	0	9^{xx}	0	2	0	6^{xx}
5	0	7^{xx}	0	4^{f}	0	4	0	2
10	0	7^{xx}	0	5^{x}	0	3	1	1^{f}
20	4^{f}	9^{x}	0	1^{ff}	1	7^{x}	1	1^{f}

Fischer exact Probability Test:
x $p< 0.05$, xx $p< 0.01$ vs corresponding control without amphetamine
f $p< 0.05$, ff $p< 0.01$ vs corresponding control without morphine

a. The numbers indicate those animals which exhibited the symptoms shown out of a total of 9 rats.
b. Time after amphetamine and/or morphine administration.
c. 15 mg/kg combined with morphine, injected 30 min after αMpT.

RESULTS

Marked catalepsy was induced by morphine (20 mg/kg) when administered 30 min after αMpT. The catalepsy was maximal 40 to 90 min after morphine administration (fig. 1 & 2) and had disappeared after 180 min (not shown). Pretreatment with αMpT did not change the time of onset of the catalepsy induced by morphine, but attenuated its disappearance (fig. 1). Catalepsy was not observed for up to 270 min after administration of αMpT. The specific morphine antagonist, naloxone, entirely abolished the morphine-induced catalepsy.

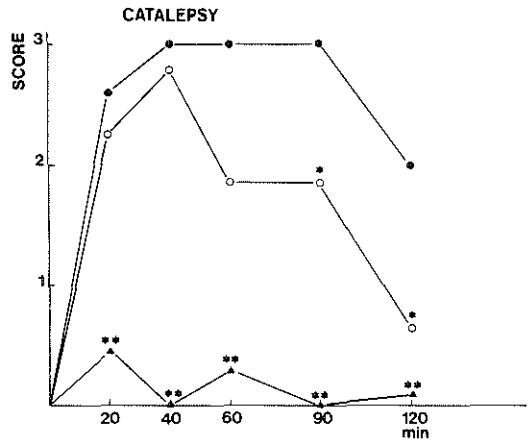

Fig. 1. Catalepsy induced by morphine after αMpT pretreatment and antagonism with naloxone. Morphine (20 mg/kg) and naloxone (1 mg/kg) were administered at t_0, 30 min after αMpT or saline injection. At 60 min a second dose of naloxone (1 mg/kg) was administered. No catalepsy was observed after administration of αMpT alone or in combination with naloxone. Number of animals per treatment group: 10.

●—● αMpT + Morphine
○—○ Saline + Morphine
▲—▲ αMpT + Morphine + Naloxone
* $p < 0.05$, ** $p < 0.01$ vs αMpT + Morphine at the corresponding time of observation.

Apomorphine (4 mg/kg), administered simultaneously with morphine 30

min after αMpT, suppressed the morphine-induced catalepsy for 40 min (fig. 2), while apomorphine had no direct effect. Amphetamine, on the other hand, potentiated morphine-induced catalepsy when measured 1 and 2 h after combined administration. Amphetamine, administered 30 min after α MpT, without morphine, also induced catalepsy (table 1).

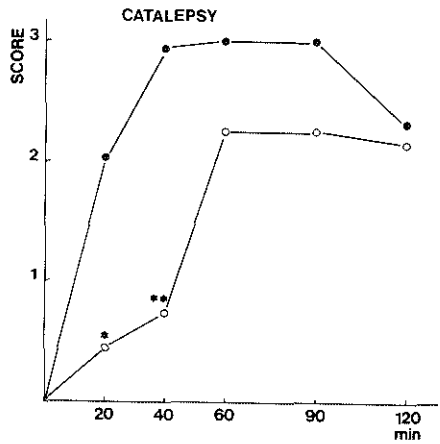

Fig. 2. Catalepsy induced by morphine after αMpT pretreatment and antagonism with apomorphine. Morphine (20 mg/kg) or morphine and apomorphine (4 mg/kg) were administered at t_0, 30 min after αMpT injection. No catalepsy was observed after αMpT + apomorphine treatment. Number of animals per treatment group: 10.
● ——— ● αMpT + Morphine
○ ——— ○ αMpT + Morphine + Apomorphine
* $p < 0.05$, ** $p < 0.01$ vs αMpT + Morphine at the corresponding time of observation.

Besides catalepsy, amphetamine administration 30 min after αMpT also induced sniffing, a sign of stereotyped behaviour, which could be antagonized by morphine (table 1).

Neither atropine (3 mg/kg) nor bicucculine (2 mg/kg) had any effect on morphine-induced catalepsy nor did either of these drugs exert a direct effect of their own (not shown).

When haloperidol (1 mg/kg) was administered 30 min after αMpT treatment, a gradually increasing catalepsy was observed. The catalepsy was maximal at about 120 min after haloperidol injection. No difference was observed in haloperidol-induced catalepsy after pretreatment with αMpT when compared with saline pretreatment (not shown). When the rats were tested once only, however, no catalepsy was observed even 120 or 180 min after haloperidol treatment. Repeated testing, therefore, seems a major factor

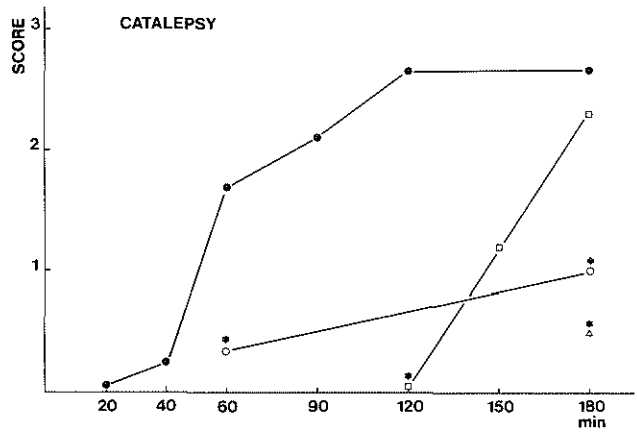

Fig. 3. Catalepsy induced by haloperidol after αMpT pretreatment and the effect of repeated testing. Haloperidol (1 mg/kg) was administered at t_0, 30 min after αMpT injection. Animals were first tested for catalepsy at 20, 60, 120 or 180 min after haloperidol treatment and tested again at the time intervals indicated. Number of animals per group: 10. * $p < 0.05$ vs the group tested for the first time at 20 min after haloperidol injection.

in the induction of catalepsy after haloperidol treatment. This is also evident from the fact that no significant differences were observed between the first, second or third catalepsy tests, regardless of the time period between test and haloperidol administration (fig. 3). Repeated testing of rats treated with αMpT only, however, never resulted in positive catalepsy scores.

DISCUSSION

The present experiments show that catalepsy induced by morphine after α MpT pretreatment, is closely related to the activity of DA systems. Thus, morphine-induced catalepsy was antagonized by apomorphine, which is in agreement with the reported antagonism produced by apomorphine on morphine-induced catalepsy without αMpT pretreatment (8, 12). Recently,

a relation between GABAergic systems and neuroleptic-induced catalepsy has been reported, based on the findings that an increase in brain GABA concentrations results in potentiation of haloperidol-induced catalepsy (14). If such a relationship also exists for morphine-induced catalepsy, the GABA receptor blocking agent bicucculine would be expected to antagonize morphine-induced catalepsy. Such an antagonism, however, was not observed in the present experiments. Also the anticholinergic drug atropine did not influence the morphine-induced catalepsy, indicating that muscarinic receptors are not involved in morphine-induced catalepsy, which is in keeping with other reports (9, 12, 15). Although serotonergic mechanisms may affect morphine-induced catalepsy (16), it was suggested that a direct action of morphine on serotonergic systems is unlikely to account for its cataleptic activity (17). Morphine-induced catalepsy is most easily altered by dopaminergic agents and may be most closely related, therefore, to DA systems, a conclusion which also applies after αMpT treatment.

The DA systems involved in morphine-induced catalepsy, however, are unlikely to be those which project into the striatum or n. accumbens, as shown by Costall and Naylor (9, 18), using selective brain lesions. This suggestion is substantiated by the observation that morphine does not affect DA release in these structures (10, 19), while blockade of DA receptors by morphine in the same structures also seems to be unlikely (8, 20, 21). Since lesions of the central amygdaloid nucleus prevent morphine-induced catalepsy, it was suggested that this structure may mediate the catalepsy induced by morphine rather than the striatum or n. accumbens (9, 22). We observed increased release of DA in the frontal cortex and tuberculum olfactorium (10, 23), while no biochemical data are available concerning the amygdala. Thus, these three structures may be involved in morphine-induced catalepsy in contrast to the striatum and n. accumbens.

Haloperidol-induced catalepsy was also studied after αMpT pretreatment to compare its effects with those of morphine. Under these experimental conditions, haloperidol treatment, unlike morphine treatment (19), resulted in increased release of DA in both the striatum and the n. accumbens. This observation is in agreement with previous studies indicating that haloperidol-induced catalepsy is mediated by striatal dopaminergic mechanisms (1, 9, 12), in contrast to morphine-induced catalepsy. No potentiation of catalepsy was observed after injection of 1 mg/kg haloperidol into rats pretreated with αMpT. Shore and Doris (13), however, reported that αMpT potentiated the catalepsy induced by 0.25 mg/kg haloperidol, a dose which was without effect in our hands, although the same test for catalepsy was used.

Recently Stanley and Glick (24) reported that catalepsy scores, obtained in haloperidol treated rats, were much higher when the subjects were tested repeatedly, instead of once only. Since this observation was made while using a rather different testing procedure, we investigated the influence of repeated testing with the present procedure. The results are in agreement with those reported by Stanley and Glick (24), and show that 1 mg/kg haloperidol did not produce any catalepsy in αMpT pretreated rats, unless the animals were tested repeatedly. In fact, the number of tests seemed to be of more importance than the time which elapsed between haloperidol treatment and the catalepsy test. The catalepsy observed after repeated testing was, nevertheless, related to haloperidol itself, since catalepsy was not observed after repeated testing of rats treated with α MpT alone. These observations again point to a difference between neuroleptic- and morphine-induced catalepsy. In the latter case, catalepsy

was almost maximal during the first test, 20 min after injection of 20 mg/kg morphine, while at a lower dose of morphine (10 mg/kg) no catalepsy was observed, not even after repeated testing.

Some observations seem to suggest that morphine-induced catalepsy is related to diminished dopaminergic activity. Thus, apomorphine antagonized morphine-induced catalepsy and αMpT pretreatment prolonged its duration. On the other hand, amphetamine and morphine acted synergistically in inducing catalepsy. In this context, it is significant, that amphetamine antagonizes the catalepsy induced by neuroleptics (1, 3), a phenomenon, which has been related to inhibition of DA activity. Moreover, the present results show that administration of amphetamine alone to αMpT pretreated rats results in catalepsy. It is suggested, therefore, that the catalepsy induced by both drugs is the result of an activation of DA systems. This hypothesis is in agreement with the observation that morphine increases DA release in the frontal cortex and tuberculum olfactorium (10, 23), which does not seem compatible with an antagonism of dopaminergic neurotransmission. It is possible therefore, that catalepsy is brought about, on the one hand, by decreased activity in the striatal system (cf neuroleptic catalepsy) and on the other hand, by activation of DA systems in some extrastriatal structures (cf narcotic catalepsy).

While amphetamine and morphine potentiated each other in the test for catalepsy, morphine antagonized the amphetamine-induced sniffing. Sniffing, a major sign of amphetamine-induced stereotypic behaviour (1,25), has been related to activation of dopaminergic neurotransmission particularly in the striatum (1, 25-28). Thus, two dopaminergic systems seem to be involved in the interaction of amphetamine and morphine. The synergism of both drugs in the induction of catalepsy may involve the extrastriatal dopaminergic structures, mentioned above, while the amphetamine-induced stereotypic behaviour involves striatal DA systems and can be antagonized by morphine. The existence of two DA systems mediating opposite effects has also been suggested from experiments using a rather different approach (29, 30). Locomotor stimulation in the rat, for example, may be induced by the activation of a postulated excitatory DA system, but also by inhibition of a postulated inhibitory DA system (30) and a balance between both systems would be essential for normal psychomotor functioning (29). In this model, haloperidol and apomorphine were characterized as a specific antagonist and an agonist, respectively, of the excitatory DA system, while amphetamine acted as an agonist in both systems (29). These features also fit with the present data, if it is valid to assume that morphine activates the DA system which is unaffected by haloperidol and apomorphine. Accordingly, haloperidol may induce catalepsy by inhibition of the excitatory DA system and morphine by activation of the inhibitory DA system. Since a balance would exist between both systems, apomorphine may antagonize morphine-induced catalepsy by activation of the former system, thus restoring the balance. This would not apply for amphetamine, since this drug would also activate the second system, thus accounting for amphetamine induced catalepsy and synergism with morphine. Activation of the excitatory DA system by amphetamine would account for its stereotypic properties, which effect may be counteracted by morphine, through activation of the inhibitory DA system. However, since amphetamine may simultaneously induce catalepsy and stereotypic behaviour, the two DA systems may not directly antagonize each other, but may rather mediate opposite responses. This "physiological antagonism" may lead to blockade of one behavioural response in favour of the other (cf stereotypic behaviour and catalepsy).

In conclusion, the present results suggest that morphine may induce

catalepsy through a DA system located in structures outside the striatum and n. accumbens. Moreover, it is suggested that the effects of morphine are the result of activation of dopaminergic neurotransmission rather than inhibition. This is in contrast to haloperidol, which presumably induces catalepsy through inhibition of striatal systems (1, 9, 12). The existence of two pharmacologically and anatomically distinct DA systems, as suggested here, may parallel the concept of two distinct types of DA receptors as suggested by Cools et al. (29, 30), though it has yet to be shown whether these classifications are mutually compatible or not.

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RESULTS AND DISCUSSION

αMpT-METHOD

Various experimental approaches to study drug effects on dopaminergic neurons have been reviewed in Part I. In the present experiments a modification of the αMpT method was used. Drug induced changes, observed with other methods, are strongly dependent on changes in DA biosynthesis, while those observed with the synthesis inhibition method, as used here, are related to DA disappearance. Whether this disappearance can be understood as release of DA onto its physiological receptors, is not certain (Part I). It is imperative, however, to analyse the effect of drugs with this method during the period of complete synthesis inhibition. For that reason the time course of DA synthesis inhibition was investigated.

It was observed that αMpT completely inhibited DA biosynthesis, as measured in whole rat brain, from 30 min onwards, 5 h being the longest period of observation (II, table 1). Drugs were therefore administered in the following experiments 30 min after injection of αMpT.

The observation of differential effects of morphine in frontal cortex and striatum made it necessary to establish whether the previous observation of total inhibition of TH in whole rat brain also applied to these structures separately. Indeed, maximal inhibition of DA biosynthesis was achieved 30 min after αMpT administration in both structures (III, table 2).

Table 1.

DA Concentrations in Frontal Cortex and Striatum 2½h after αMpT Treatment

	% DA[a]	$k(h^{-1})$[b]	$k(h^{-1})$[c]
frontal cortex	44 ± 9 (3)	0.328	
striatum	48 ± 5 (6)	0.294	0.30

a. DA as percentage of the concentration at the moment of αMpT injection. Values represent mean ± S.D., the number of series of experiments is given in parantheses. Series of experiments from paper II, table 3; paper III, table 3 & 4; paper IV, fig. 1a, 2 & 3.
b. Corresponding mean fractional turnover rate.
c. From Doteuchi et al. (1).

In rats treated 2½ h with αMpT a rather reproducible decline of DA was observed throughout several series of experiments, performed over an extended period of time (table 1). DA levels dropped 2½ h after αMpT to 44% in the frontal cortex and 48% in the striatum.

Fig. 1 shows the relative concentrations of DA, HVA and DOPAC in the striatum 30 and 150 min after αMpT administration. At 30 min DA levels were significantly lowered, but HVA and DOPAC levels were not. Moreover, the percentage DOPAC was significantly above that of DA. No differences

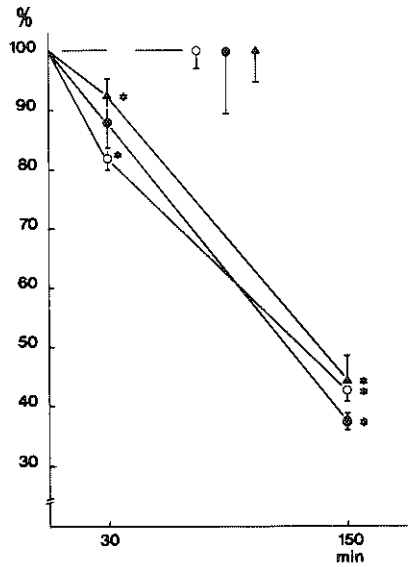

Fig. 1. αMpT-induced decline of DA, HVA and DOPAC in the striatum Concentrations were calculated relative to the level at the moment of αMpT administration (t_o).

○——○ DA, 100% = 5.11 µg/g
●——● HVA, 100% = 0.38 µg/g
▲——▲ DOPAC, 100% = 0.73 µg/g

✱ $p < 0.05$ vs respective 100% value
✲ $p < 0.05$ vs respective % of DA
Number of observations was 3-7.

were observed by 150 min. This could point to the fact that αMpT releases DA during the first 20-40 min after administration (1), although elevation of the catabolite levels above 100% may have been expected then at 30 min. These results confirm the previous observation that DA biosynthesis is not instantaneously inhibited.

In most experiments morphine was administered 30 min after αMpT and the rats were decapitated 2 h later. Several considerations led to this experimental set up:

1. The aim was to assess drug effects as shortly as possible after αMpT-treatment, since the rapid decline of DA, induced by the synthesis inhibitor (vide supra), might result in changes in neuronal activity (Part I). Since complete inhibition of synthesis is essential, morphine was administered 30 min after αMpT. Moreover, effects of αMpT or its metabolites on DA release have ceased by then (1).

2. The analgesic and cataleptic effects of 20 mg/kg morphine i.p., last for about 2 h (3, VI), corresponding roughly to the time course of morphine brain levels (4, 5). Rats were decapitated therefore 2 h after morphine administration.

3. Although originally the decline of DA is assessed at several time points after αMpT administration (6), we decided to restrict the analysis to one time point, i.e. 2 h after drug treatment. When intermediate time points are added, it is possible to evaluate the linearity of the regression of log(DA) vs time. Since the errors of the mean DA concentrations are such that only gross deviations from linearity will be detected, it was preferred to increase the accuracy of the determination of DA decline by concentrating all observations at the ends of the time period to be covered. The consequence of this approach is that biphasic effects will escape attention at all events.

The tables as presented in paper III and IV not only contain values for DA concentrations, but also rate constants of DA loss (k, fractional turnover rate: 6). In a strict sense the presentation of the fractional turnover rate may not be justified, since it is based on the assumption of a steady state. Since morphine is injected during the period of observation and morphine levels, and presumably morphine effects, thereafter are not constant with time, this condition will not be met. However, because the decline of DA after αMpT treatment is exponential, the mere presentation of final DA concentrations was judged an insufficient description of the effects obtained.

DA, HVA AND DOPAC ASSAY IN BRAIN TISSUE

To evaluate the reliability of the assay of DA, HVA and DOPAC after decapitation of the rat and dissection of the brain tissue, the time course of their concentrations at postmortem was investigated. Effects were observed 7½ min after decapitation for DOPAC, but not for DA and HVA (I). In all subsequent experiments brain tissue was frozen in liquid nitrogen within 4 min after decapitation, when no postmortem effects were apparent.

EFFECT OF MORPHINE IN DIFFERENT DOPAMINERGIC BRAIN REGIONS

Table 2 summarizes the effects of morphine in the frontal cortex and striatum, obtained in several series of experiments. The αMpT-induced DA decline was accelerated significantly by morphine in the frontal cortex, but not in the striatum.

The increase of DA loss in the frontal cortex was prevented by naloxone, a specific morphine antagonist (III, table 4). Specific opiate receptors, therefore, seem to be involved rather than aspecific membrane interactions. The frontal cortex was subdissected to localize the dopaminergic neurons involved. It was anticipated that the cortex rostral to the corpus callosum and/or the anterior cingulate cortex would account for most of

Table 2

Effect of Morphine (20 mg/kg) on the Concentration of DA in the Frontal Cortex and Striatum after αMpT Treatment.[a]

Frontal Cortex	p[b]	Striatum	p[b]	Reference
78 %	<0.01	99%	n.s.	II, table 1
78 %	<0.01	100%	n.s.	III, table 3
72 %	<0.02	86%	<0.02	III, table 4
		105%	n.s.	IV, fig. 1
		90%	n.s.	IV, fig. 2
		94%	n.s.	IV, fig. 3
		90%	n.s.	V, fig. 1

a. Values represent the concentration of DA (2½ h after αMpT and 2 h after morphine treatment) as percentage of the DA concentration in saline treated rats. See for details the papers referred to.
b. p Values for difference of morphine treatment from saline treatment.

the DA present, since these areas contain the most important cortical DA projections in the telencephalon (7). Most DA (80%), however, was localized in another area (III, fig. 1, table 6), a part of the basal forebrain. The morphological implications of this observation are not entirely clear (III).

A positive drug effect can be proven beyond reasonable doubt. The absence of an effect, however, can only be made more probable by confirmation via different approaches. We analysed therefore, the concentrations of the DA catabolites HVA and DOPAC in the striatum in an experiment identical to those reported in table 2, and found no effect of morphine (III, table 5). Furthermore a dose range of morphine (2½, 5, 10, 20 and 40 mg/kg) was administered and the decline of DA analysed. Again no effect was observed (V, fig. 1). This last experiment also shows that the dose of 20 mg/kg is not just below the limit of sensitivity of the nigrostriatal dopaminergic neurons.

It has been discussed that possible biphasic effects will remain unnoticed with the present method. Since biphasic responses to morphine are known (Part I), it may be suggested that an inhibition and subsequent stimulation by morphine of DA release, just happens to result in the absence of a difference in DA concentrations at the moment of decapitation of the rats in the present experiments. The results with different doses of morphine, however, make this seem unlikely. The duration of depressant and stimulant effects of morphine differs considerably with different doses. It would have been expected therefore, that a biphasic effect on DA decline would have resulted in an obvious difference in DA concentrations with one dose or another.

In other laboratories an increased decline of DA levels in the

striatum was observed when morphine was administered before αMpT (8, 9, 10). It has been discussed in Part I that this procedure (conventional synthesis inhibition method) precludes an unequivocal interpretation of the drug effects obtained. To ascertain that our different results in the striatum with the modified synthesis inhibition method were not due to trivial factors (e.g. strain differences, analytical techniques etc) I reproduced these experiments. Indeed an enhanced decline of DA was observed in the striatum when morphine was administered 30 min before αMpT (III, table 3). It was concluded that morphine accelerates DA biosynthesis, but this does not seem to be of functional relevance (III).

Other dopaminergic brain structures have also been studied. The n. accumbens seems to react to morphine as does the striatum (III, fig. 1b), while the tuberculum olfactorium resembles the frontal cortex with the observation of an increased loss of DA (IV). A dissociation of the effects of morphine in terms of nigrostriatal and mesolimbic/mesocortical dopaminergic systems does not appear, therefore, from these data.

The effect of morphine on the decline of DA after interruption of the impulse flow (IV) was investigated for several reasons:

1. To evaluate whether the observed enhanced decline of DA in the frontal cortex was a direct effect on dopaminergic neurons or the result of modification of an input to this system. It was expected that elimination of the impulse flow would antagonize the effect of morphine in the frontal cortex in the latter case but not in the former.

2. To test the hypothesis that morphine induces DA release in the striatum, as in the frontal cortex, but simultaneously inhibits the same neurons. These effects would cancel each other out in normal rats. After a lesion the releasing effect should be uncovered (see IV for a more extensive account of this hypothesis).

3. To compare the effect of the neuroleptic haloperidol with the effect of morphine in the striatum under these conditions.

As can be seen from the appendix to paper IV, point 1 can not be clarified due to an effect of the lesion procedure itself, probably an artifact. Concerning point 2 it was observed, that morphine induced DA release in the striatum after interruption of the impulse flow, in contrast to the striatum of the intact brain side (IV, fig. 1a). This is contrary to haloperidol, which drug induces an acceleration of DA decline in the striatum of the intact brain side. This effect of haloperidol was abolished by the lesion (IV, fig. 1a). Similar effects of both drugs were observed in the mesolimbic structure, the n. accumbens (IV, fig. 1b), when compared with the striatum.

An electrolytic lesion is quite a radical manipulation. It is not exactly known in what condition the nerve terminals are after a lesion of the neuronal cell bodies. Therefore, the effect of morphine was also studied after decreasing the DA release to about 50% by administration of apomorphine. Morphine accelerated the loss of DA under these conditions, but not in the striatum of control rats (IV, fig. 2). When the decline of DA was accelerated by haloperidol, however, morphine exerted no effect (IV, fig. 3). It appears therefore, that morphine releases DA from the inactive neuron. This effect is progressively reduced in neurons with increasing activity, which results in a slight but insignificant effect at normal activity and the total absence at increased activity (IV, fig. 4). This observation may account for the incidental observation of an increased DA decline in the striatum in one out of seven experiments (table 2), since in this experiment a lower basal activity of the striatal neurons was observed (III).

The suggestion that the observed activity-dependent effect of morphine in the striatum also applies to other dopaminergic systems does not seem justified, since the same relation did not appear from preliminary observations in the tuberculum olfactorium (appendix to paper IV). These results are far from conclusive because of a minimal number of observations. It is necessary therefore, to extend these observations and to evaluate the effect of morphine after interruption of the impulse flow in these dopaminergic neurons to substantiate possible differences. The observed impulse-flow dependency in the striatum, however, can certainly not be extrapolated to other structures without positive indications.

MORPHINE-INDUCED CATALEPSY

Catalepsy is considered to be always accompanied by an increased DA turnover in the striatum (8, 11, 12). The observation that morphine has no effect on αMpT-induced DA decline in the striatum (vide supra) suggested that this may not always be true. Morphine-induced catalepsy was measured therefore, in rats pretreated with αMpT. This allowed a direct comparison to be made with the experiments described in the previous section. Pretreatment with αMpT even prolonged morphine-induced catalepsy (V, table 1; VI, fig. 1). Naloxone antagonized this catalepsy which shows that the catalepsy is specific for morphine. It was concluded therefore, that changes in striatal dopaminergic activity do probably not underlie morphine-induced catalepsy (V).

Experiments with apomorphine, amphetamine, atropine and bicucculine showed that dopaminergic neurons, nevertheless, play a critical role in the observed catalepsy (VI). Apomorphine antagonized, while amphetamine enhanced morphine induced catalepsy. A simple interaction between dopaminergic agents and morphine-induced catalepsy does not, therefore, seem to exist. Since morphine may rather stimulate than inhibit dopaminergic neurotransmission, it was suggested that morphine-induced catalepsy may be connected with stimulation of extrastriatal dopaminergic neurons (VI). Catalepsy can also result from inhibition of dopaminergic neurons in the striatum, viz. by neuroleptics. Catalepsy may therefore, be mediated by two dopaminergic systems, i.e. by stimulation of an extrastriatal or inhibition of the striatal system. The existence of two contrary acting DA systems could account for the complicated effects of DA agonists on morphine-induced catalepsy (VI).

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PART III

AN ANALYSIS OF SUBCELLULAR MECHANISMS

Almost all possible mechanisms at the subcellular level have at some time been proposed as a way of morphine action when interpreting specific results. Before propounding another view derived from the present results an analysis of facts seems relevant.

Morphine stimulates DA biosynthesis (1-6) and increases the levels of the dopamine catabolites HVA (7-10) and DOPAC (11) in vivo. When the synthesis of DA is increased in one way or another, this inevitably results in increased catabolite levels at a given moment. This is observed after treatment with neuroleptics (12-16), electrical stimulation of dopaminergic neurons (17-19), or inhibition of impulse flow by GHB (20-22). Whether the increased catabolite levels are dependent on increased DA biosynthesis is not assessed. Reserpine, for instance, may increase catabolite levels (23) and decrease DA biosynthesis (24) although the different time intervals investigated in these studies, leave the question still open. Nevertheless, there are a number of indications that increased catabolite levels are secondary to increased DA biosynthesis, when morphine is administered.

30 Min after administration of morphine, DA concentrations are elevated (2, 25, 26). Moreover, we did not observe increased catabolite levels when the biosynthesis of DA was blocked by αMpT prior to morphine treatment (III). Both observations would point to an increased DA biosynthesis preceding increased catabolism rather than the reverse. The latter observation could be explained by assuming that newly synthesized DA is preferentially catabolized (7, 26). It has been suggested that αMpT not only blocks DA biosynthesis, but also amine transfer from a general store to releasible sites (27). Westerink and Korf (28), however, observed that the increase of catabolite levels induced by morphine was prevented by transsection of the nigrostriatal bundle. Although these authors inferred from this observation that the effect of morphine is impulse flow dependent, another explanation is possible.

Thus, elevated DA concentrations after the transsection indicate that DA biosynthesis may be stimulated to the extent that product feedback inhibition becomes the rate limiting factor. Under these circumstances morphine may not be able to stimulate DA biosynthesis any more and this can account for the prevention of elevated catabolite levels. In this situation there is presumably enough newly synthesized DA which could be catabolized, but morphine has no effect. Increased DA catabolism may be secondary to increased DA biosynthesis induced by morphine. These observations seem to indicate that the hypothesis of Sharman (29) for etorphine (a morphine analogue) as elaborated by Kuschinsky and Hornykiewicz (7) does not hold true. These authors suggested that morphine may displace newly synthesized DA from storage sites to the sites of catabolism. Nevertheless, the basis of this hypothesis merits more attention. At the molecular level it was based on the observations already described, viz. increased DA biosynthesis and catabolism. Neuroleptics exhibit similar presynaptic effects and in addition induce catalepsy in rats as morphine does. Since neuroleptics are considered to produce catalepsy by blockade of striatal DA receptors, it was suggested that morphine may do that too (30). Kuschinsky and Hornykiewicz (7) judged this possibility unlikely, since naloxone antagonizes these effects of morphine but not those of neuroleptics and also because morphine-induced catalepsy is easily

antagonized by DA receptor agonists unlike neuroleptic-induced catalepsy. It has been shown more recently that a number of other parameters connected to DA receptor activity were also unaffected by morphine, in contrast to neuroleptics (3, 6, 31). Thus, neuroleptics change the kinetic parameters of TH in vitro, inhibit DA-stimulated adenylate cyclase, increase striatal Ach turnover and inhibit the increase of striatal cAMP levels elicited by apomorphine or amphetamine, but morphine has no effect on any of these parameters. To account for the morphine-induced catalepsy the hypothesis was put forward then that morphine may displace newly synthesized DA from storage sites to the sites of catabolism, thus resulting in a lack of DA at receptor sites (7). This presynaptic action would then be an indirect DA receptor blockade.

Since recent studies show that morphine may not induce catalepsy at all, via striatal dopaminergic mechanisms (32, 33, II, V, VI), it does not seem necessary to account for any lack of DA at striatal receptor sites. It should be stressed that the observed catalepsy was the only instigation for such considerations.

Since morphine does not block DA receptors and since there is no analogy with neuroleptic mechanisms at the subcellular level, the observed effects of morphine more probably would point to an activation of dopaminergic neurotransmission, i.e. increased biosynthesis and catabolism. This presynaptic stimulation, however, does not result in stimulation of DA receptors, since it did not enhance striatal cAMP levels in vivo as did apomorphine and amphetamine (3). It was observed that morphine had no effect on αMpT-induced DA decline in the striatum (II, III, V). Since the biosynthesis of DA is entirely inhibited and since all the evidence presented above only shows that morphine stimulates DA biosynthesis, it was suggested that the main effect of morphine in the striatum may be just this stimulation of DA synthesis without further functional consequences (III), i.e. release.

This seems to apply, however, only to dopaminergic neurons with a normal impulse flow. When the impulse flow is decreased or entirely impaired, morphine increases or induces release of DA in the striatum (IV). In these experiments the biosynthesis of DA was also inhibited. A direct effect on dopaminergic neurons seems to be implicated, since all input from other neuronal systems is most probably eliminated by a lesion. Blockade of only presynaptic receptors (and not postsynaptic) could account for DA release. These receptors, however, are already entirely deprived of DA, since no DA is released when the neurons are lesioned. There is therefore, no activated receptor to block and, in fact, the superfluous addition of the DA receptor blocker haloperidol has no effect in this situation (IV). I concluded that a molecular mechanism is involved which causes direct release of DA. Furthermore this mechanism becomes decreasingly effective with increasing impulse flow, suggesting that a common link in morphine- and impulse-flow-induced release is involved.

AN UNDERLYING MECHANISM FOR STIMULATION OF DA BIOSYNTHESIS AND ACTIVITY-DEPENDENT DA RELEASE IN THE STRIATUM BY MORPHINE

Morphine induces a rapid depletion of calcium from a number of discrete brain regions (34). This seems to be a specific opiate effect, since it is antagonized by naloxone and not observed with inactive stereoisomers

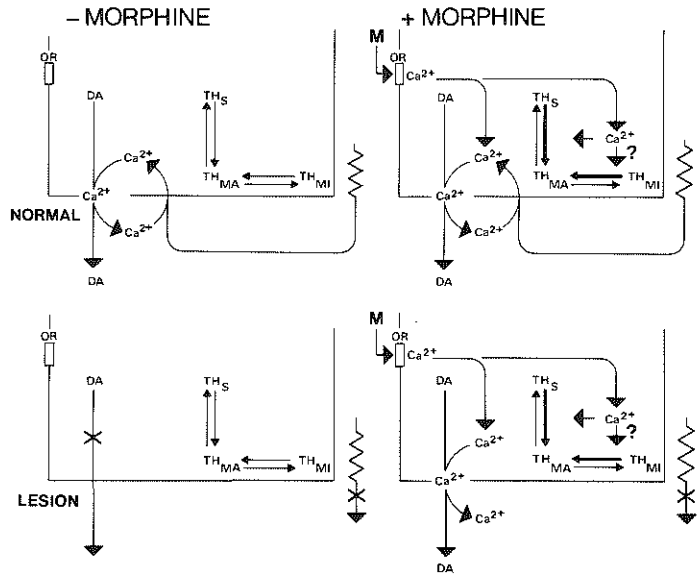

Fig. 1. Liberation of intracellulair calcium by morphine as an underlying mechanism of changes in DA biosynthesis and DA release. In the normal situation the action potential causes a flux of Ca^{2+} into the neuron, which is necessary for DA release. A lesion of the dopaminergic cell bodies abolishes impulse flow and thus Ca^{2+} entry and DA release.

Furthermore, tyrosine hydroxylase (TH) is present in three configurations: a soluble form (TH_S), an activated membrane-bound form (TH_{MA}) and an inhibited membrane-bound form (TH_{MI}). The enzyme activity may be regulated inter alea by shifts between the different conformations, and Ca^{2+} induces such shifts (40). It is not known how the regulations of the enzyme activity is related to the impulse flow.

Morphine stimulates an opiate receptor (OR) which results in increased intracellular Ca^{2+} levels. In the normal neuron this does not result in increased DA release. However, when Ca^{2+} entry is abolished by a lesion, the Ca^{2+}, liberated by morphine, induces DA release. Note that, in this situation Ca^{2+} only leaves the neuron, which may account for Ca^{2+} depletion, induced by morphine. Increased intracellular Ca^{2+} also results in a shift of TH to the activated membrane-bound conformation. This mechanism may account for increased DA biosynthesis induced by morphine. The relation to impulse-flow-induced changes in DA biosynthesis is unknown.

Abbreviations: OR: opiate receptor; DA: dopamine; TH_S: tyrosine hydroxylase, soluble form; TH_{MA}: tyrosine hydroxylase, activated membrane-bound form; TH_{MI}: tyrosine hydroxylase, inhibited membrane-bound form.

of opiates. Moreover, the calcium depletion induced by morphine can be distinguished from a similar effect by reserpine or pentobarbital (34, 35). It was suggested therefore, that binding of morphine to specific opiate receptors (36) will induce a calcium-dissociated state resulting in calcium depletion. Moreover, a specific calcium pool on or within the synaptosomal fraction would be involved (35, 37).

The intracellular release of calcium from presynaptic membranes may account for the observed effects of morphine on dopaminergic neurons, as indicated schematically in fig. 1. In dopaminergic neurons with a normal impulse flow morphine only stimulates DA biosynthesis (111). It has been shown that TH exists in a soluble and membrane bound form (38, 39). The latter form exhibits affinities for the pterin cofactor and for tyrosine which are 15 and 7 times higher, respectively, than observed with soluble TH. Moreover, calcium ions facilitate association of TH with striatal nerve ending membranes and concommitantly increase the total enzyme activity (40). It is tempting to speculate, therefore, that morphine stimulates DA biosynthesis, because liberated calcium ions activate TH by increasing membrane association.

The same intracellular mobilisation of calcium by morphine may account for DA release from inactive dopaminergic neurons. The influx of calcium ions is necessary for the impulse-flow-induced release of transmitters, including DA (41). In the absence of impulse flow morphine may substitute to a certain extent the calcium influx by releasing intracellular calcium. When the impulse flow is increased considerably (e.g. with haloperidol) the calcium, liberated by morphine, may have no effect. This appears from the following considerations. Calcium does not mediate transmitter release simply by opening the presynaptic membrane to let through the neurotrans mitter. Calcium induces instead the fusion between transmitter-containing vesicles and the synaptic membrane (42) by binding to specific membrane components. When fusion is effected in this way, the transmitter is released into the synaptic cleft and the emptied vesicle removed. When the resting state is re-established, a new fusion and transmitter release may start. At maximal impulse flow this chain of events may operate at maximal speed and additional activation by e.g. liberation of intracellular calcium by morphine may have no effect. This mechanism may therefore, account for the impulse-flow-dependent release of DA by morphine. Thus, in the absence of impulse flow morphine will induce DA release and with increased impulse flow morphine will have no additional effect. At normal impulse flow, however, morphine does not release DA either, though the physiological release is not maximal. This may indicate that the calcium liberation by morphine is not as effective as with the physiological mechanism.

Whether the activation of TH by morphine is similarly dependent on impulse flow, is not known. It has been shown that DA biosynthesis is increased during electrical stimulation of the nigrostriatal neurons (43). Thus, DA biosynthesis may be correlated with nervous activity. The mechanism of this activation is not known, but may involve also calcium (40). If so, the activation of DA biosynthesis by morphine may also be inversely related to impulse flow. The regulation of DA biosynthesis, however, is a lot more complicated. DA biosynthesis is increased by electrical stimulation, but also by lesions of the nigrostriatal pathway, i.e. by total impairment of impulse flow (44-46). Furthermore, activation of presynaptic receptors by released DA (47) and accumulation of intraneuronal DA (48) may decrease DA biosynthesis. Physiological changes in DA biosynthesis, therefore, are not simply related to impulse flow and are omitted from fig. 1, although they may interfere with the stimulation by morphine of DA biosynthesis.

Thus, the stimulation of DA biosynthesis and DA release by morphine may be regarded as unrelated consequences of the same molecular event. This may explain why in the striatum DA biosynthesis is stimulated by morphine without corresponding increase in DA release. Nevertheless, the increase in DA biosynthesis may intensify the DA releasing effect of morphine at decreased impulse flow, because more DA may be available for release. In regard to this it can not be concluded from the present experiments that the increase in DA biosynthesis is always without functional consequences, although that may be true at normal impulse flow in the striatum.

The presented mechanism of action of morphine on dopaminergic neurons is consistent with a number of other observations. The action of morphine seems, in fact, to be presynaptic as concluded from its accumulation in presynaptic elements (49) and the presynaptic localisation of its specific receptor (50, 51) and from its interaction with synaptic mechanisms of various neurotransmitters (52, 53). The role of calcium in the effects of morphine is illustrated by the observation that the analgesic response is antagonized by calcium (54, 55). Moreover, ionophores for bivalent cations antagonized the effects of morphine,which may suggest that increase in intracellular calcium levels is involved (51, 55).

The characteristics of the mechanism proposed are not specifically related to dopaminergic neurons. The observed calcium depletion is rather uniform throughout several discrete brain regions, some devoid of dopaminergic neurons (34). The regional distribution of opiate receptors show high concentrations in regions with dopaminergic cell bodies or nerve terminals such as caudate-putamen, substantia nigra pars compacta and amygdala, but also in regions without dopaminergic neurons such as locus coeruleus, periaqueductal gray and medial thalamus (36, 56, 57). The underlying mechanism of calcium liberation, therefore, may apply to all central effector sites of morphine . If so, the observed effects on dopaminergic neurons are a specific manifestation of this general mechanism. Nevertheless, the action of morphine does not seem to be so general that all central neurons are affected. Whether a certain neuronal system is modulated by morphine, may depend on the presence of opiate receptors and on whether the liberation of calcium by morphine results in a functional change of the neuron.

In conclusion, a general molecular mechanism is proposed for the modulation of neurotransmission by morphine. The modulation of dopaminergic neurotransmission as investigated here, is a specific example then of the consequences of this general mechanism. Because of the characteristic regulation of dopaminergic neurotransmission, this example can not be applied to other neurotransmitter systems without specific information.

IMPLICATIONS OF THE MODULATION OF DOPAMINERGIC NEUROTRANSMISSION BY MORPHINE

As discussed above morphine may activate dopaminergic neurotransmission under certain circumstances, while an inactivation is never observed. This agrees with most pharmacological information about morphine. A large body of literature has been reviewed by D.H. Clouet, which shows that pharmacological manipulations presumed to alter DA levels at its receptor "either antagonize or potentiate the pharmacological responses to acute administration of morphine or other opiates according to the direction of change in the levels of biogenic amine" (49). In other words: increased DA

receptor stimulation may potentiate and decreased DA receptor stimulation may antagonize effects of morphine related to DA activity.

The activation of dopaminergic neurotransmission by morphine, however, is not uniform in different brain regions and not uniform in dopaminergic neurons with varying impulse flow. Some implications for the behavioural effects of morphine will be discussed.

When morphine is administered, DA is released only in frontal cortex and tuberculum olfactorium, but not in nucleus accumbens and striatum (II, III, IV). This may indicate that behavioural responses induced by morphine are mediated via tuberculum olfactorium or frontal cortex, rather than via n. accumbens or striatum, if they are dependent on DA neurotransmission. The role of other brain regions, not investigated here. (e.g. amygdala), remains to be established. Such DA-dependent responses may include catalepsy (V, VI), but also locomotor stimulation and grooming (58).

Other responses to morphine, however, also connected to dopaminergic neurotransmission, are not necessarily mediated via the same systems. This concerns responses to morphine of an essentially different category. The former responses (catalepsy, locomotor stimulation, grooming) are directly elicited by morphine. The responses discussed now are alterations by morphine of behaviour induced by another external stimulus. In an analgesic test, for instance, first a response is evoked to an external, i.e. noxious, stimulus and then the effect of morphine on this imposed response is assessed. In this case one can not depart from the normal animal in analysing the effect of morphine, since it may very well be that the imposed response itself involves changes in dopaminergic activity. It is difficult to settle this point, because it does not seem to have been looked at in this way. In several reports in which the role of DA in the analgesic response to morphine was evaluated, no controls were included to evaluate the role of DA in the algesic response per se (59, 60). If we assume that these algesic responses involve decreased dopaminergic neurotransmission in the striatum, morphine will release DA in the striatum and this could account for the involvement of DA in the analgesic activity of morphine. In fact, destruction of striatal DA neurons abolishes the analgesia induced by morphine, while the catalepsy -not mediated by striatal neurons- is increased (59).

Though purely speculative for the examples put forward, the point to be stressed is that it is only possible to evaluate the role of DA in particular responses to morphine, when one is aware of the state of activity of the neurons involved. Moreover, the DA releasing effect of morphine is not only different in various brain regions, but in addition it is not certain whether the dependency on the state of activity is uniform in all regions. Preliminary results suggest that this may not be the case in the tuberculum olfactorium when compared with the striatum (IV).

To state the obvious, these implications for behavioural effects of morphine are only concerned with effects dependent on dopaminergic neurotransmission and only so far as dependent on dopaminergic neurotransmission. It is well-documented, for example, that the serotonergic system plays a most important role in the analgesic response to morphine (61-63). Dopaminergic systems, however, also seem to be involved, possibly only in particular responses to noxious stimulation (60).

CONCLUSIONS

1. Morphine may stimulate, but not inhibit dopaminergic neurotransmission.
2. In normal rats morphine induces release of DA in the frontal cortex and tuberculum olfactorium but not in the striatum and nucleus accumbens.
3. Whether the release of DA in the frontal cortex and tuberculum olfactorium is the result of a direct effect on dopaminergic neurons is not assessed.
4. Morphine induces release of DA in the striatum and nucleus accumbens by a direct effect on the dopaminergic neurons with reduced impulse flow.
5. Morphine increases DA biosynthesis by a direct effect in most or all brain regions which contain dopaminergic neurons, independent of possible DA release.
6. An underlying mechanism for the stimulation of DA biosynthesis and DA release by morphine involving intraneuronal liberation of calcium is proposed.
7. Morphine-induced catalepsy in rats may not be related to inhibition of dopaminergic neurotransmission in the striatum or nucleus accumbens, but rather to release of DA in frontal cortex, tuberculum olfactorium or other brain regions not studied here.
8. Behavioural responses involving decreased dopaminergic neurotransmission in the striatum or nucleus accumbens, may be antagonized by morphine through DA release in the striatum or nucleus accumbens.

Wenn wir diese unsre Anschauung auch zum höchsten Grade der Deutlichkeit bringen könnten, so würden wir dadurch der Beschaffenheit der Gegenstände an sich selbst nicht näher kommen.

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SUMMARY

The aim of this thesis was to investigate the effects of acute morphine administration on dopaminergic neuron systems in the rat brain.

The literature on the acute effects of morphine in the central nervous system is reviewed in Part I. This section also contains a description of the dopaminergic neuron systems and an extensive survey of methods used to study the dynamics of dopaminergic neurotransmission in vivo.

The experimental work is described in Part II. First the postmortem changes in the concentrations of dopamine and its acidic metabolites, dihydroxyphenylacetic acid and homovanillic acid, were analysed. It was observed that the levels of dihydroxyphenylacetic acid changed considerably postmortem, 7½ min and more after decapitation of the rat.

In paper II the effect of morphine on the αMpT-induced decline in dopamine levels is described. In the frontal cortex an increase in dopamine release was observed, while morphine had no effect in the striatum. Moreover, it was shown that the synthesis of dopamine was completely inhibited by αMpT during the period of observation.

The differential effect of morphine in the frontal cortex and striatum was further analysed in paper III. It was shown that the increased dopamine release in the frontal cortex was not due to differential inhibition of dopamine synthesis by αMpT in the frontal cortex and striatum. Since the effect was antagonized by naloxone, it was concluded that a specific opiate action was involved. The frontal cortex was subdissected to analyse the localisation of the dopaminergic neurons involved. Most of the dopamine (80%) was present in basal parts of the forebrain. The absence of changes in dopamine release in the striatum was further substantiated by the observation that morphine had no effect on the levels of homovanillic acid and dihydroxyphenylacetic acid after prior αMpT treatment.

In paper IV is shown that morphine also releases dopamine in the striatum, but only when the activity of the nigrostriatal neurons is decreased by lesion of the dopaminergic cell bodies in the mesencephalon or by prior treatment with apomorphine. Haloperidol, on the other hand, increased dopamine release in the striatum of normal rats, but had no effect after lesion. Similar effects were observed in the n. accumbens. It was concluded that the morphine-induced dopamine release in the striatum is dependent on the state of activity of the neurons involved. It was also suggested that haloperidol-induced dopamine release is mediated by a striatonigral feedback pathway. The implications of the effects of morphine on dopaminergic neurons for its behavioural effects are discussed.

The preliminary results reported in the appendix to paper IV suggest that the impulse-flow-dependent effect of morphine in the striatum may not occur in all dopaminergic brain structures.

In paper V is shown that morphine can produce catalepsy without concurrent changes in dopaminergic neurotransmission in the striatum. It is suggested that morphine-induced catalepsy is related to extrastriatal dopaminergic systems.

The catalepsy induced by morphine is further analysed in paper VI. Pretreatment with αMpT prolonged the morphine-induced catalepsy, while naloxone entirely abolished it. Experiments with atropine, bicucculine, apomorphine and amphetamine suggested that dopamine plays a crucial role

in the catalepsy induced by morphine, although this may not involve dopaminergic systems in the striatum and n. accumbens. It is suggested that two dopaminergic systems are involved in the interaction of morphine with dopaminergic agents. The implications of this hypothesis are discussed.

The results are summarized and technical details are discussed in the Results and Discussion section.

Part III contains the General Discussion. First, the evidence indicating that morphine may block dopaminergic neurotransmission in the striatum is reviewed. It is concluded that many results argue against such a mechanism of opiate action and that the results of experiments used as evidence for this hypothesis can also be interpreted in another way. It is concluded that morphine does not inhibit but rather stimulates dopaminergic neurotransmission under certain conditions and also stimulates dopamine biosynthesis independently. A molecular mechanism is suggested which may account for the stimulation of dopamine biosynthesis and dopamine release induced by morphine. It is suggested that this mechanism involves liberation of intracellular calcium. The implications of these results for the interpretation of the behavioural effects of morphine are discussed in relation to dopamine systems.

Finally, the conclusions of this thesis are briefly summarized.

SAMENVATTING

Morfine en andere opiaten worden vooral gebruikt voor het draaglijk maken van zware pijn en voor het opwekken van een algemeen gevoel van welbehagen (euforie). Deze effekten berusten op een werking in het centrale zenuwstelsel, waarvan de mechanismen echter nog niet bekend zijn. Als morfine herhaaldelijk wordt toegediend, ontstaat een ongevoeligheid voor deze effekten en de dosis moet worden verhoogd (tolerantie). Dit leidt echter tot afhankelijkheid, die voor een deel veroorzaakt wordt door een aanpassing van de cellen aan de steeds hogere doses morfine. Indien de toediening op zo een moment wordt gestaakt, treden onthoudingsverschijnselen op, die onaangenaam en vaak gevaarlijk kunnen zijn.

De direkte effekten van morfine (pijnstilling en euforie) worden veroorzaakt door een complex van inwerking van morfine op de zenuwcellen en van de reaktie van het lichaam hierop. Om te begrijpen hoe die effekten ontstaan, onderzoekt men hoe morfine op cellulair en molekulair nivo in het centrale zenuwstelsel werkt. De veranderingen op dit nivo moeten daarna weer in verband worden gebracht met de effekten zoals pijnstilling en euforie.

Het onderzoek beschreven in dit proefschrift gaat over de effekten van morfine op dopaminerge systemen in de hersenen van de rat. Dopamine is één van de stoffen die in de hersenen zorgt voor de overdracht van impulsen van de ene zenuwcel naar de andere, en wel in bepaalde delen van de hersenen. Dopamine speelt een belangrijke rol bij de integratie van emotionele reakties en van lichaamsbewegingen die hiermee samenhangen. Omdat pijnstilling en euforie, veroorzaakt door morfine, o.a. met zulke reakties samenhangen, is onderzocht of en hoe morfine de prikkeloverdracht door dopamine beinvloedt. Ik heb gepoogd de gevonden effekten te relateren zowel aan molekulaire mechanismen die eraan ten grondslag kunnen liggen als aan gedragseffekten die eruit voort kunnen komen.

In Part I wordt eerst een kort overzicht gegeven van een aantal effekten van morfine in het centrale zenuwstelsel. Ook zijn de dopaminerge zenuwbanen kort beschreven. Voorts bevat dit deel een uitgebreid overzicht van technieken die men bij dierexperimenteel onderzoek kan gebruiken om de prikkeloverdracht door dopamine te bestuderen.

Vervolgens zijn de experimenten beschreven in Part II. In artikel I wordt de betrouwbaarheid van de bepaling van dopamine en zijn zure metabolieten in hersendelen van de rat, zoals die in verdere proeven zijn toegepast, onderzocht. In artikel II wordt beschreven dat morfine de prikkeloverdracht door dopamine verschillend beinvloedt in twee verschillende hersen-strukturen. In de frontale cortex wordt deze prikkeloverdracht door morfine waarschijnlijk geaktiveerd, terwijl dat in het striatum niet zo is. Deze gegevens worden verder uitgewerkt in artikel III. De specificiteit van het effekt in de frontale cortex wordt o.a. aangetoond. Voorts werd de lokalisatie van de zenuwcellen die bij dit effekt betrokken zijn, onderzocht. Het blijkt dat deze cellen niet behoren tot de reeds nauwkeurig omschreven dopaminerge systemen. In artikel IV wordt de werking van morfine vergeleken met haloperidol, een "tranquilizer" met bekende effekten op dopaminerge zenuwcellen. Voorts wordt aangetoond dat morfine onder normale omstandigheden geen effekt heeft op de dopaminerge prikkeloverdracht in het striatum, maar wel als de aktiviteit van de zenuwcellen van te voren veranderd is. De konklusie wordt getrokken, dat morfine de dopaminerge prikkeloverdracht in het striatum misschien niet direkt bein-

vloedt, maar veranderingen in die overdracht dempt. In de appendix van artikel IV wordt besproken dat deze relatie misschien niet voor alle dopaminerge zenuwcellen geldt.

Vervolgens is de relatie onderzocht tussen de bovenstaande effekten op dopaminerge zenuwcellen en een bepaald gedragseffekt van morfine in ratten, namelijk katalepsie, een vorm van bewegingsverstarring. In artikel V en VI wordt aangetoond, dat dit verschijnsel inderdaad samenhangt met veranderingen in de dopaminerge prikkeloverdracht. Voorts wordt gesuggereerd dat katalepsie samenhangt met een effekt van morfine op dopaminerge zenuwcellen in bepaalde hersendelen, namelijk frontale cortex, tuberculum olfactorium en/of amygdala, maar niet striatum en nucleus accumbens. Tenslotte worden de resultaten samengevat en besproken.

In Part III worden de resultaten getoetst aan gegevens en hypothesen uit de literatuur. De hypothese wordt opgesteld en besproken, dat het werkingsmechanisme van morfine zou berusten op het vrijmaken van calciumionen in dopaminerge zenuwcellen. Voorts wordt het verband tussen dit werkingsmechanisme van morfine en bepaalde gedragseffekten besproken. Tenslotte worden de konklusies kort samengevat, namelijk:

1. Morfine kan de dopaminerge prikkeloverdracht stimuleren, maar niet remmen.
2. Onder normale omstandigheden verandert morfine de dopaminerge prikkeloverdracht in frontale cortex en tuberculum olfactorium, maar niet in striatum en nucleus accumbens.
3. Het is niet duidelijk of de verandering in dopaminerge prikkeloverdracht in frontale cortex en tuberculum olfactorium berusten op een direkt effekt op die zenuwcellen of niet.
4. Morfine veroorzaakt stimulatie van de prikkeloverdracht door dopamine in striatum en nucleus accumbens, als de aktiviteit van de dopaminerge zenuwcellen verminderd is.
5. Morfine stimuleert de biosynthese van dopamine, waarschijnlijk in alle dopaminerge zenuwcellen, onafhankelijk van bovengenoemde effekten.
6. De hypothese is opgesteld, dat de verkregen effekten van morfine berusten op vrijmaking van intracellulair calcium.
7. Katalepsie veroorzaakt door morfine, zou berusten op aktivering van dopaminerge prikkeloverdracht in frontale cortex, tuberculum olfactorium of andere hier niet onderzochte strukturen.
8. Gedragseffekten die samengaan met vermindering van dopaminerge prikkeloverdracht in het striatum kunnen door morfine wel in die struktuur beinvloed worden in tegenstelling tot katalepsie.

CURRICULUM VITAE

In 1966 deed ik eindexamen gymnasium β aan het Revius Lyceum te Doorn. Daarna heb ik scheikunde gestudeerd aan de R.U. te Groningen, waar ik in 1970 kandidaatsexamen deed. De studie heb ik vervolgens in Utrecht voortgezet. Het doctoraalexamen met hoofdvak biochemie (Prof. L.L.M. van Deenen) en bijvak farmakologie (Prof. D. de Wied) heb ik in oktober 1972 afgelegd. Vanaf 1 maart 1973 tot 1 januari 1977 heb ik in dienst van FUNGO op de afdeling farmakologie van de Erasmus Universiteit het onderzoek verricht dat tot dit proefschrift leidde. Sinds 1 januari 1977 ben ik in dienst van het Academisch Ziekenhuis Dijkzigt als farmakoloog bij de afdeling Psychiatrie.