MECHANISMS OF TOLERANCE TO AND DEPENDENCE ON NARCOTIC ANALGESIC DRUGS

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METHODS OF PRODUCING TOLERANCE AND DEPENDENCE IN LABORATORY ANIMALS

Non-Primates

ROUTES OF ADMINISTRATION Methods for evaluating the degree of tolerance to and/or dependence on narcotic analgesic drugs have been described in various reviews in this series and others (1–5). During the last five years, assays for tolerance and dependence have become quantitative and precise, especially in small laboratory animals. The daily administration of morphine or other opioids to rodents, cats, and dogs in one or more injections per day has been used for many years to produce the tolerant state which develops at a rate dependent on the specific drug, the dosage schedule, the interval between doses, and the sensitivity of the pharmacological assay (6).

Another method of administering morphine to rodents has been described by Huidobro & Maggiolo, who implanted pellets of morphine base subcutaneously (7). The absorption of drug into the soft tissues from 100 mg pellets was uniform over a 30-day period during which tolerance developed (8). The formulation of the pellets was modified by Way and his colleagues by including various filler materials (9). The absorption from these pellets was constant for 2 to 3 days and fell off by the fifth day. The amount of drug released from the pellet in 3 days was about half the original weight of 75 mg of morphine (9, 10). Tolerance was maximal in mice 3 days after the implantation of the pellet, as was dependence (9). Tolerance also has been

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produced in rats (11), guinea pigs (12), and rabbits (13) by the subcutaneous implantation of morphine pellets.

Sustained-release preparations that can be injected subcutaneously have been described by Collier et al (14) and found to induce tolerance and dependence in 24 to 72 hr (15–17). Sustained-release depots of antagonists have also been prepared that produced blockade of agonist activity for a month (18). A drug reservoir implanted under the skin was useful because drug substitution could be easily accomplished if abrupt drug withdrawal or drug replacement was desired (19).

Short-term intravenous infusions of morphine or other opioids produced an acute tolerance and dependence within several hours, when the drugs were infused in doses appropriate for each species: 3 mg/kg per hr for the dog (20), 5 to 10 mg/kg per hr for rats (21), and 35 mg/kg per hr in mice (22, 23).

Early methods of initiating self-administration of opioids by nonprimates included the following: mixing dihydromorphinone with milk (24); adding a potent analgesic drug, etonitazene, to drinking water (25); adding quinine to drinking water so that morphine solution would be a palatable alternate source of fluid (26); and by breeding rats for high consumption of morphine in drinking water (27). A dilute solution of methadone as the only fluid available also induced a slow development of tolerance in rats (28).

The technique of self-administration by intravenous injection triggered by lever pressing was first shown to be effective in producing tolerance and dependence in rats by Weeks & Collins (29) who observed opiate-directed behavior. They found that when lever presses were rewarded at a fixed ratio of one injection per 10 lever presses (FR10), the rate of pressing was proportional to drug concentration in the injection solution over a certain range, and that when the FR was increased to 180, lever pressing behavior was extinguished. Extinction also occurred shortly after the drug was removed from the injection solution (29).

EFFECT OF DOSE AND SCHEDULE Varying suggestions have been made concerning the minimal drug treatment required in order to detect signs of tolerance and/or dependence, based on the following kinds of evidence: a single dose of morphine produced tolerance to the analgesic action of morphine for one year (30); single doses of levorphanol (16) or morphine (31) produced dependence in several hours as shown by naloxone-induced jumping; in the spinal dog, after an 8 hr infusion of morphine, the administration of nalorphine precipitated abstinence (20); one dose of 5 mg/kg morphine each week produced tolerance in rats by the third week, a tolerance maintained by the same dosage schedule (32).

Other aspects of the contributions of dose and interval between doses to the degree of tolerance have already been reviewed (6, 10, 33).

SIGNS OF TOLERANCE Most of the pharmacological responses to chronic treatment with narcotic analgesics are classed as depressant effects although there are some excitant, and even convulsant effects (34). The classic method of assessing the analgesic response in rats and other small animals by the hot-plate procedure was

described many years ago by Eddy & Leimbach (35). Recent studies have suggested that learning may be involved if hot-plate trials are repeated in the same animals, as when assessing the development of tolerance (36, 37). Experience in the testing procedure contributed significantly to the measured rate of tolerance development in studies in which the control (preinjection) latencies of response were reduced by repeated testing whether or not the animals received morphine in the injection, although the drug-reinforced groups had lower control latencies than those not drug-reinforced (37). Also, the analgesic responses in the daily-tested group of chronic morphine-treated rats were lower than in animals receiving morphine but not tested (37). Repeated hot-plate exposure also reduced the analgesic response to a first injection of morphine (32). "Chronic behavioral tolerance," or the decreased response after repeated drug administration and tests, was differentiated from learning because unreinforced rats showed behavioral tolerance but not learning (32). The development of pharmacological tolerance proceeded at a rate slower than that of chronic behavioral tolerance.

An apparent excitant effect is produced in mice by a single injection of an opioid, a running fit, or stereotyped pattern of automatic running activity (24, 38, 39). Morphine, levorphanol, *l*-methadone, ketobemidone and meperidine (39), and dihydromorphinone (24) all had this effect, while *d*-methadone and dextrorphan did not (39). Although it was first believed that tolerance developed only to depressant effects of opioids (6, 34), tolerance to this drug effect did follow chronic morphine treatment and cross-tolerance was demonstrated between levorphanol and morphine, methadone and ketobemidone (39).

A pair of excitant-depressant responses, catalepsy and stereotypic behavior, seem to be inversely related during tolerance development in rodents (40). In rats, the response to the first injection of morphine was cataleptic at appropriate doses but never stereotypic (41). With chronic administration of morphine (42) or methadone (43), the cataleptic response decreased and stereotypic behavior emerged. Cataleptic behavior was antagonized by naloxone (44), while stereotypic jumping was increased by abrupt agonist withdrawal or naloxone-precipitated withdrawal (45).

In spinal dogs, a course of morphine intoxication began with an injection of 6 mg/kg morphine in the morning and a larger oral dose in the afternoon, with increasing doses until 54 mg/kg per day was reached (46), during which observations of pupillary diameter, pulse rate, respiration, flexor reflex, body temperature, etc were recorded over a 23-week period. The oral doses in the afternoon prevented overnight abstinence.

In cats, the excitant abnormal behavioral activity, which is an acute response to morphine and other agonists (47), seems similar to the hyperexcitability associated with withdrawal in other species (48). Tolerance developed to this excitant behavior upon chronic morphine treatment (49). In other mammalian species including the dog, opioid administration stimulated intestinal motility (50). Tolerance to this stimulatory response also developed during chronic morphine treatment (51).

Convulsive activity was produced by high doses of narcotic analgesics, such as 200 mg/kg morphine in the rat (34). Tolerance did not develop to these convulsive effects (6, 24).

Assays for opiate tolerance using behavioral (52), biochemical (53), or electrophysiological (54) parameters have the greatest usefulness when the assay is related to the phenomenon under study.

SIGNS OF DEPENDENCE The question of whether tolerance and dependence are different aspects of the same physiological phenomenon, or are quite different phenomena, will remain undecided until mechanisms for each have been established. Because tolerance and physical dependence usually develop concurrently and are interrelated with dose and frequency of drug treatment, (6), they are often treated as a single entity. In several experimental conditions, however, the two states have been differentiated. In spinal dogs, nalorphine evoked abstinence before signs of tolerance were discernible during morphine treatment (55), and, in monkeys, tolerance developed to the chronic administration of cyclazocine although the withdrawal syndrome was not precipitated by naloxone administration (56). It is possible that the assays for tolerance and for physical dependence were not equally sensitive in these experiments.

Dependence is measured in tolerant animals by the evocation of abstinence signs by abrupt drug withdrawal or the administration of a narcotic antagonist. The quantitation of abstinence signs in rodents has been examined in several laboratories. Wei and his colleagues have shown that there was a dose-response relationship between the dose of naloxone and selected signs of precipitated abstinence in rats implanted with morphine pellets for 3 days (57). In these animals, the ED_{50} for naloxone for most abstinence signs (diarrhea, ptosis, abnormal posturing, ear blanching, swallowing movements, teeth chattering, and escape attempts) was less than 1 mg/kg, with the precipitation of wet shakes requiring 4 mg/kg (57).

A large number of criteria for weighting abstinence signs in the spinal dog have been described by Martin et al (46) for the quantitation of withdrawal. Precipitated abstinence was compared with withdrawal abstinence in the animals in relation to the level of dependence, and it was found that abstinence signs changed in relation to level of dependence when abstinence was precipitated to a greater extent than when drug use was discontinued (46).

The individual signs of abstinence have been examined in rats by Bläsig and co-workers, as related to the number of morphine pellets implanted, the duration of drug exposure, and the frequency of pellet implantation (10). The frequency of such signs as exploring, jumping, and teeth chattering increased as the number of pellets and the duration of drug exposure were increased. In contrast, writhing and wet dog shakes were predominant at a medium degree of tolerance and decreased as other signs such as jumping and "flying" became predominant with increasing dependence. A similar quantitative shift of signs was produced by increasing the dose of antagonist. These workers concluded that jumping was the most suitable sign for measuring abstinence quantitatively because jumps are easily counted, and

the jumping rate increased when dependence increased or dose of antagonist increased (10). Signs of aggressive behavior evoked by nalorphine administration to morphine-tolerant rats have been quantitated by Puri & Lal (58).

When naloxone-precipitated jumping in mice was combined with a test for agonist activity (59), opioids with mixed agonist/antagonist activities could be assayed for agonist activity. Pentazocine reacted like the purer agonists in these tests, but could be distinguished from the agonists by the lack of withdrawal signs when nalorphine was administered. Mice treated chronically with nalorphine, however, jumped in response to naloxone administration (59). Nozaki & Hosoya have classified opioids into three groups on the basis of body weight changes in response to chronic injections, abrupt withdrawal, naloxone challenge, and agonist substitution (60). Using this scheme, Nozaki & Hosoya separated the mixed agonists pentazocine and nalophine into one group because they caused a weight gain after withdrawal and a weight loss when substituted for morphine.

A sensitive single-dose suppression test for agonist activity in dependent mice has been described by Takemori et al (61). The mice were treated chronically with morphine and then withdrawn for 4 to 6 hr. The test drug was administered and the percentage of mice jumping and the number of jumps per mouse were measured. Active agonists suppressed abstinent jumping; counting the number of jumps per mouse was the more sensitive measure of agonist activity (61). The method of counting jumps per mouse was used earlier to quantitate the withdrawal syndrome in mice (62).

Several caveats have been offered concerning the specificity and reproducibility of using abstinence signs to quantitate dependence. Some signs such as wet shakes and aggression are not specific for narcotic abstinence, because they also occurred after withdrawal from neuroleptics, whereas weight loss, writhing, and hypothermia were not observed after neuroleptics (63). In selecting a single sign as an assay for dependence, one must consider that any experimental manipulation of the dependence state may selectively affect that sign and not the whole syndrome (57).

The rate of development and the degree of tolerance and physical dependence seem to run parallel (9). The relationship between the pharmacological responses of analgesia and locomotor activity and the number of agonist injections or the duration of pellet implantation (tolerance) and the relationship between abstinence signs and dose schedules of agonist administration before withdrawal (dependence) have shown the same proportionality in many studies (9, 10, 32, 38, 64). When the interval between injections was fixed, both tolerance and dependence depended on the amount of drug administered in each injection (10, 32, 38, 62). For a large number of agonists, similar manifestations of abstinence signs was precipitated by the same dose of naloxone (65). However, various agonist responses required different amounts of naloxone for suppression of response, showing that the antagonist activity of naloxone reflected the intensity of the stimulus in the assay (66).

In animals exhibiting abstinence signs after abrupt withdrawal from narcotic drugs, the administration of the same drug or another agonist reduced the severity

of the abstinence. However, in precipitated abstinence, even large doses of narcotic agonists did not always suppress abstinence. Kaneto et al (23) and Kamei et al (31) have reported that naloxone-induced withdrawal was difficult to overcome by agonist treatment. However, a large dose of heroin blocked withdrawal signs in rats challenged with naloxone (14). Evidently the agonist/antagonist ratio became important in those experiments.

INFLUENCE OF PREVIOUS CYCLE OF DEPENDENCE Long-term effects of chronic drug administration such as the persistence of abstinence signs or of tolerance (36, 30, 59), or the retention of morphine or methadone in brain (67, 68) have been observed. In spite of these data, most observers feel that the previous drug history of the animal does not influence the subsequent development of tolerance to and dependence on a second cycle of drug treatment. Cheney & Goldstein have found that physical dependence, like tolerance, was completely and rapidly reversible, and that one cycle of dependence and recovery did not modify the course of the next cycle, as measured by the ED_{50} values for naloxone-precipitated jumping in morphine-dependent mice (16). Wei & Loh also measured the effect of previous experience on dependence and concluded that dependence was neither enhanced nor lessened by a second drug treatment cycle, as measured by an abstinence ranking including weight loss and behavioral signs (69). It is possible, of course, that alterations due to drug history will become discernible as assay procedures become more sensitive and specific. Indeed, by the use of the EEG (70) or secondary reinforcers (71), postaddict monkeys have been distinguished from naive animals.

SITES IN BRAIN FOR TOLERANCE AND DEPENDENCE Attempts have been made to find sites in the central nervous system specific for tolerance and dependence. The introduction of morphine by the intracerebral or intraventricular route of administration produced pharmacological responses (72, 73). Repeated injections of morphine intraventricularly induced tolerance, as measured by analgesia (73), or by a depression of a behavioral response (food-reinforced lever pressing) (74). In these animals, after abrupt drug withdrawal, the systemic injection of antagonist-evoked abstinence with the same signs as in animals made tolerant by morphine injection systemically (73, 74). The similarity of abstinence signs was also observed in the reverse situation, agonist administered by systemic route and antagonist by the intracerebral route (11, 73, 74).

Lesioning experiments, in which a small portion of a brain area is removed, have been used to demonstrate the nerve pathways involved in the pharmacological responses to acute narcotic analgesic administration. However, sites needed for the development of tolerance and dependence to chronic opioid use have not been explored to the same degree. In one series of experiments, lesions of the ventromedial hypothalamus influenced the rate of tolerance development (76). In another series of experiments, rats with lesions in the posterior aspect of the medial forebrain bundle showed fewer signs of abstinence upon withdrawal than did sham-operated control animals (77). The lesioned animals also self-administered morphine more readily than control animals, leading the investigators to suggest that

the addiction process might be separated from the dependence process (77). Two-stage bilateral lesioning of the ventral lateral hypothalamus near the fornix prevented rats from drinking morphine solution, although the animals drank water and quinine solutions when offered (78). Self-stimulation in the lateral hypothalamus by rats was both enhanced and suppressed by morphine administration, dependent on time and dose (79). Tolerance developed only to the depressant effect of morphine in this experiment (79).

The microapplication of naloxone into parts of the pontine area of the brainstem (75) or into the medial thalamus and medial areas of the diencephalon (76) elicited signs of abstinence in tolerant animals. In experiments involving microablation or microinjection, in which it is always difficult to separate effects on effector pathways needed for the manifestation of one response from effects on pathways intrinsically involved in dependence, the evocation of withdrawal signs by naloxone largely overcomes the difficulty.

Primates

ASSESSMENT OF ADDICTION LIABILITY Before 1950, new compounds were evaluated for addiction liability in man at the Addiction Research Center in Lexington (80). In 1950 Seevers received support from the Committee on Drug Addiction of the National Research Council to establish opiate addiction in monkeys. In Seevers' primate laboratory at the University of Michigan, the fundamental principles relating to dose-response curves, duration of action, and rate of dependence development were established in the monkey for the various chemical classes of narcotic analgesic drugs. The validity of single-dose suppression tests and direct addiction techniques as well as antagonist activity assays were established in the major preclinical trials of new narcotic drugs (80). Since 1957 nearly 900 new drugs have been evaluated in the monkey for agonist and/or antagonist activity. The results of these studies have been summarized by Villarreal (56).

The introduction of self-administration by the intravenous route in monkeys has added behavioral aspects both to the drug evaluation studies (81) and to basic studies on dependence to narcotic analgesic drugs (82, 83).

SELF-ADMINISTRATION OF OPIATES The technique of implanting monkeys with chronic intravenous catheters that permit the immediate delivery of a drug solution (81) has led to the assessment of various pharmacological and behavioral variables which affect self-administration. A basic premise, that narcotic agonists act as reinforcers, has been confirmed. Morphine (84, 85), codeine (86), methadone (87), pentazocine (86), and propoxyphene (86) acted as positive reinforcers in the self-administering monkey. Agonist-antagonists such as nalorphine and cyclazocine acted as negative reinforcers in these animals (88, 89).

The possibility of altering the reinforcing properties of narcotic agonists has been explored in several aspects: (*a*) substitution, in which a drug with less ability to reinforce drug-seeking behavior is substituted for a more potent drug (87); (*b*)

manipulation of behavioral variables, such as schedules of reinforcement or aversive stimuli (83) or the administration of other drugs such as 2-methyltyrosine or haloperidol which affect behavior (90); or (*c*) blockade of reinforcer activity by extinction with narcotic antagonists (91) or immunochemical blockade by the production of antibodies to the drug (92). The potential therapeutic value in man of such studies is obvious. The studies have been reviewed elsewhere in excellent detail (83, 87, 89, 91, 93).

POTENTIAL MECHANISMS OF TOLERANCE AND DEPENDENCE

Memory Coding by RNA and Protein

Several theories on the mechanisms of tolerance and dependence include a requirement for coding the initial drug: tissue interaction, presumably by macromolecular biosynthesis. Collier (94, 95) has suggested that silent (inactive) receptors are induced by opiate exposure, with the formation of drug: inactive receptor complexes, which reduce the pharmacological effects of the drug (=tolerance). In other theories, which postulate an expansion or a derepression of some biosynthetic enzymes involved in receptor activity, thereby increasing the receptor capacity of the tissue, there would seem to be a requirement for protein synthesis (96, 97) as also in the elaboration of antibodies to opiates. Some evidence for the existence of a memory peptide for morphine tolerance has been developed by Ungar & Galvan (98). In general, recent studies have not contributed definitive answers to the question of the participation of macromolecular biosynthesis in tolerance and dependence. The best evidence that macromolecular biosynthesis might be required, especially in the CNS, for the development of tolerance comes from earlier studies in which inhibitors of RNA or protein synthesis were shown to block or delay the development of tolerance to chronic opiate treatment, (reviewed in reference 99). Huidobro (8) has confirmed that an inhibitor of protein synthesis, cycloheximide, blocks the development of tolerance in mice implanted with morphine pellets, and, in addition, has shown that the inhibitor also decreased the tolerance induced by a single injection of morphine. Feinberg & Cochin (100) also have explored the effect of cycloheximide on single-dose tolerance to morphine by administering the inhibitor to rats 1 hr prior to each weekly injection of morphine, finding that development of analgesic tolerance to this treatment schedule appeared at the fifth week of treatment and lasted for 17 weeks.

Any expansion of the opiate receptors should take place at the site of opiate-receptor interaction, presumably at the neuronal synaptic junction. In an attempt to detect new protein synthesis after morphine treatment, protein biosynthesis has been examined in various preparations of nerve-ending particles and synaptic plasma membranes. Franklin & Cox (101) measured the rate of protein synthesis from radiolabeled precursor amino acids administered intraventricularly into synaptic plasma membrane fractions isolated from the brains of rabbits perfused with morphine for 3 hr, or injected with morphine over a period of 2 days, and found

no differences in the incorporation of radioactivity between morphine-treated and control rabbits. Similarly, Hahn & Goldstein (102) injected radioleucine intracerebrally into mice implanted with morphine pellets and found no change from control in the rates of biosynthesis of protein solubilized from brainstem by detergents. Our earlier report (103) that single, depressant doses of morphine depressed protein synthesis in brain has been confirmed (104). It is likely that this transient inhibition of protein biosynthesis is not a specific opiate effect but is, instead, related to a general depression of metabolism in brain (105), because even 5 min of anoxia produced a prompt inhibition of protein synthesis and RNA polymerase activity in rabbit brain (105).

The enzymatic steps involved in the metabolism of nucleic acids have been examined in the brains of morphine-treated animals in the hope that an increased enzyme activity or a new RNA species might be detected. One hour after an injection of morphine, the rate of incorporation of labeled thymidine into rat brain DNA was decreased below normal, a depression attributed to the metabolic depression in the CNS of the animals (106). However, in the same experiments, the amount of radiolabel from precursor orotic acid in brain RNA was considerably above control at later times with the maximal difference 48 hr after the injection of morphine. This effect could be attributed either to an increased synthesis or a decreased catabolism of the RNA fraction. Support for the former mechanisms comes from the work of Lee et al (107), who found an increase in chromatin template activity in the brains of mice implanted with morphine pellets. The chromatin isolated from the brains of morphine-treated animals had greater template activity in synthesizing RNA than chromatin isolated from the brains of placebo pellet-implanted mice. When both kinds of chromatin were washed excessively, the control template activity increased to that of the morphine-treated sample, suggesting that normal chromatin was partially repressed, and was derepressed in morphine-tolerant animals. On the other hand, in a series of experiments, Datta & Antopol have examined various aspects of RNA metabolism in the brains of morphine-treated mice and have found decreased RNA synthesis (108), decreased RNA polymerase activity (109), and decreased aminoacyl-tRNA synthetase activity (110) in the brains of the treated animals, again suggesting a general reaction to the depressant effects of morphine. Johannesson et al (111) found that the amount of RNA and DNA in brain or in brain nuclei from pregnant rats was not affected by acute morphine treatment.

Acting on the hypothesis that, if an enhanced RNA synthesis was required during the development of tolerance, then a supply of RNA precursors might influence the rate of tolerance development, Grecksch et al (112) injected orotic acid into rats prior to and during morphine treatment and found that tolerance developed more rapidly to the analgesic and catatonic responses with orotic acid supplementation, although there was no effect on the appearance of the stereotypic behavior.

Further studies on the effect of opiates in inhibiting RNA and protein synthesis in single cells in culture (reviewed in reference 113) have again focused on the cell membrane as a site of drug action. When levallorphan was added to *E. coli* cells

at 1 m*M* concentration, the uptake of arginine was inhibited (114). At higher drug concentrations, the cells became permeable as the transport system was blocked. Stephens & Zimmerman (115) attempted to relate the inhibition of one species of macromolecule to levallorphan inhibition in synchronized *Tetrahymena* cells, and found that RNA, DNA, and protein synthesis were all depressed in a dose-dependent way as cell division was delayed. In dorsal root ganglia in organotypic culture, the addition of $10^{-4}M$ methadone had toxic effects but the cultures survived lower concentrations of the drug (116).

The focus on cell membranes as a possible site of opiate action has prompted studies on membrane components other than proteins, such as phospholipids and cerebrosides. In *S. aureus,* the incorporation of one precursor, acetate, into phospholipids was depressed while the incorporation of another precursor, glycerol, was stimulated by the addition of various morphine congeners to the incubation medium (117). The phospholipid with the highest radioactivity in cells grown in the presence of an opiate was cardiolipin, possibly reflecting an increased phosphatidylglycerol turnover. In *E. coli,* the incorporation of labeled precursors was inhibited, with synthesis diverted from phosphatidylethanolamine to cardiolipin, when the cells were grown in the presence of 1.35 m*M* levorphanol (118). In nervous tissue, the squid axon, the concentration of levorphanol that inhibited electrical conduction, 1 m*M*, also decreased P^{32} incorporation into phosphatidylcholine and -ethanolamine, and increased the amount remaining in phosphatidic acid (119). The acute administration of morphine to guinea pigs produced profound and complex changes in radiolabeling patterns of phospholipids isolated from cerebral cortex (120). Membrane cerebrosides have been reported to bind opiates stereospecifically (121).

Because sialoglycolipids and sialoglycoproteins are important components of synaptic membranes, the content of sialic acid in whole rat brain was examined in preliminary experiments after acute or chronic morphine treatment (122). There was a slight yet significant fall in sialic acid levels after the acute dose of morphine but not after chronic treatment.

An extension of the hypothesis that the synthesis of proteins in brain is required for the development of tolerance to chronic morphine treatment is that the newly synthesized substance can be isolated from brain and injected into naive animals with the transfer of tolerance (98). The extensive reports of Ungar and his colleagues on the transfer of morphine tolerance by the injection of brain extracts from tolerant animals into naive animals, usually of another species (123, 124), have been confirmed in one laboratory (125). In addition, Huidobro & Miranda reported that substances, possibly polypeptides, which decreased abstinence signs and prolonged morphine analgesia, were obtained from whole mouse extracts (126). Most attempts to repeat the transfer of morphine tolerance have, however, been unsuccessful (127, 128). Antimorphine effects of polypeptides have recently been described. An analog of vasopressin facilitated a resistance to the analgesic action of morphine (129) and the β-melanocyte stimulating hormone and B^{1-24} corticotropin prevented the depressant effects of morphine on synaptic reflex activity in spinal animals (130).

Immune Mechanisms

While adaptation in the central nervous system is generally considered the mechanism of tolerance development, a number of observations have suggested that immunoreactions may be involved in opiate tolerance (131). The coupling of 3-carboxymethylmorphine to bovine serum albumin formed an antigen which, when injected serially with Freund's adjuvant, elicited the formation of antibodies specific for opiates in rabbits (132–134) and mice (135). Antigens combining bovine serum albumin with morphine-3-hemisuccinate (136) or morphine-6-hemisuccinate (92) also produced antibodies in rabbits (136) and monkeys (92). The specificity of the antibodies for the various opiates was related to the similarity of structure of the narcotic hapten to the opiate, i.e. antibodies to 3-carboxymethylmorphine reacted with codeine > morphine > nalorphine, with no interaction with methadone (132). Antibody affinity and specificity were related to hapten structure as well as to the number of hapten molecules bound per molecule of carrier (136).

There is some evidence that during chronic opiate treatment, serum proteins develop stronger affinity for the opiates: in the serum of some heroin addicts, proteins were found which bound opiates (137), and in rabbits, the implantation of morphine pellets resulted in an increase in the capacity for opiate binding in serum proteins as much as 100-fold (13, 138).

When mice were immunized with a morphine immunogen, the levels of opiate binding capacity were 100-fold that of adjuvant-treated controls 5 hr after the injection (139). In these animals, the analgesic response to morphine was diminished, suggesting that antibody-bound opiate persists for a longer time in serum, but is less active pharmacologically (139). Monkeys immunized with a morphine immunogen after self-administration trials with cocaine and heroin had the same reinforcement for cocaine as preimmunization, but extinguished heroin self-administration until the heroin concentration in the infusion solution was increased 16-fold (92). These results indicate that anti-opiate antisera may specifically antagonize opiate-seeking behavior. However, the role, if any, which the immune response plays in the development of tolerance and dependence remains to be defined, as does the potential therapeutic usefulness of immunization against drugs that induce dependence.

Neurohormonal Adaptations

Himmelsbach in 1943 proposed the homeostatic counter adaptive theory of morphine tolerance and physical dependence. In this theory, the effect of opiates on hypothalamic centers disturbs homeostasis and leads to physiological adjustments in order to attain a new level of homeostasis (140). More recent hypotheses have suggested some mechanisms whereby this biochemical adaptation to opiates is accomplished. In his enzyme induction hypothesis Shuster has suggested that opiates act both at the neurotransmitter synaptic level and at the enzyme induction level (97). Goldstein & Goldstein have suggested that opiates act to inhibit the synthesis of some of the enzymes that catalyze the biosynthesis of neurotransmitters,

resulting in lower concentrations of neurotransmitters, which in turn would induce the synthesis of the inhibited enzymes (96). Another hypothesis, proposed by Collier, suggests that it is not the neurotransmitter system which adapts, but the receptor itself which is transformed from silent to active by the continued presence of drugs (94, 141).

Although the neurotransmitter systems are interdependent, catecholamines, acetylcholine, and serotonin are examined separately in this review, as are the hypothalamic pituitary hormones.

CATECHOLAMINES The relation between brain catecholamines and the pharmacological responses induced by morphine and other narcotic analgesic drugs has been investigated extensively in order to determine whether brain amines play a role in the responses to acute drug treatment and to examine the possibility that catecholaminergic mechanisms in the CNS are involved in the development of tolerance to chronic drug treatment.

One method to establish the relationship between catecholamines and opiate action is to alter the levels of the amines in the CNS by other drugs and examine the effect on opiate responses. Monoamineoxidase (MAO) inhibitors increase the levels of brain catecholamines by inhibiting their catabolism. Pretreatment of experimental animals with pargyline, tranylcypromine, or iproniazid increased the acute pharmacological effects of opiates (142–147). In opiate-dependent animals, the administration of MAO inhibitors enhanced the pharmacological response and reduced the degree of tolerance (148, 149), and in abstinent animals the inhibitors exacerbated the withdrawal signs (149, 150). Reserpine and other amine depletors have time-dependent effects on catecholamine levels in brain and thus temporal dimensions in their antagonism to opiates (47, 151, 152). The usual effect of prior inhibition of the biosynthesis of brain catecholamines is inhibition of the acute effects of opiates (142, 153). The development of tolerance in mice was blocked by the simultaneous administration of α-methyltyrosine with morphine (154), while abstinence symptoms were decreased (154, 155), as was self-adminstration of morphine in addicted monkeys (90). On the other hand, the administration of catecholamines or precursors, which increase the levels of brain catecholamines, potentiated the acute effects of morphine and other analgesics (156, 157). In tolerant animals, the administration of dopa increased abstinence signs (155). Catecholamine receptors are blocked specifically by butyrophenones such as haloperidol and phenothiazines such as chlorpromazine and fluphenazine (158). Acute responses to opiates were blocked by pretreatment with neuroleptic drugs (142, 151). In rats or monkeys made tolerant to morphine by self-administration, haloperidol blocked lever-pressing for morphine (90, 159). Abstinence signs were also blocked by haloperidol, in a dose-dependent way (155). Apomorphine and amantadine, which have a direct stimulatory effect on dopaminergic structures (160), antagonized the acute effects of morphine (45) and enhanced the aggressive response to withdrawal from morphine in tolerant rats (161).

Direct evidence that opiates alter the levels of brain catecholamines, obtained in early experiments of Vogt (162) and Quinn & Brodie (163), has been amply con-

firmed not only for morphine, but for codeine, methadone, etorphine, levorphanol, meperidine, ketobemidone, and pentazocine (39, 43). The depleting action of the opiates was blocked by the antagonists nalorphine and naloxone (39, 164), although these antagonists and related inactive compounds such as dextrorphan, thebaine, and *d*-methadone did not cause amine depletion in the CNS (39). The dopamine content of rat striatum was altered biphasically by morphine at doses of 5–60 mg/kg, and norepinephrine levels in many brain areas varied similarly (165). In tolerant animals, the amine-depleting effect no longer occurred (39, 164–167) and cross-tolerance was exhibited between morphine, levorphanol, methadone, and meperidine (39). The increased fluorescence in dopaminergic cell bodies evoked by a single injection of morphine was not seen after chronic morphine treatment (168).

The multiphasic nature of the responses in brain catecholamine levels after opiate administration suggested that the drugs might induce alterations in amine turnover as well as amine levels. After acute morphine treatment, dopamine synthesis in rat striatum was increased (169, 170). The opiate effect on turnover was dose-dependent and induced stereospecificially (164, 171, 172). Tolerance developed to the stimulatory effect of narcotic analgesic drugs on catecholamine turnover during chronic drug treatment (170). However, an acceleration of the rate of dopamine biosynthesis above that after the first injection was seen after the next few injections before tolerance obtained (169, 173, 174). Tolerance in brain amine turnover coincided with tolerance in several pharmacological parameters (170). Abstinence was accompanied by an increased rate of amine biosynthesis in the CNS (173).

The possibility that opiates influence the transport of amines into or out of the nerve ending was explored in brain slices and in isolated nerve-ending preparations. The uptake of dopamine into brain slices was inhibited by the addition of morphine (175). In striatal nerve ending also, the uptake of dopamine was inhibited by morphine (176). In both studies, kinetic analyses indicated that the inhibitory effect was nonspecific because only low affinity uptake was affected.

The postsynaptic function of neurotransmitters include a postjunctional element sensitive to the transmitter, the cAMP-adenyl cyclase system. Both dopamine and norepinephrine increase the activity of adenyl cyclase in appropriate areas of brain in a dose-dependent way (177, 178). The systemic or intracerebral injection of cAMP antagonized morphine effects (179) as did adenine, adenosine, AMP, and ATP (180). The basal activity of adenyl cyclase in homogenates of brain areas was not changed by acute morphine administration (181). However, in tolerant animals, basal cyclase activity was increased (182, 183). The sensitivity of striatal adenyl cyclase to dopamine was also increased in morphine-tolerant animals (184).

Dopaminergic pathways have been implicated in the mechanism of opiate action because manifestations of opiate-induced activity (catalepsy, muscle rigidity, analgesia, mania, stereotypy, locomotor activity, self-administration of opiates, and crowding aggression) have been associated with striatal dopamine metabolism. The effects due to dopamine or to norepinephrine have been differentiated by the use of haloperidol (a dopaminergic blocker), adrenergic blocking agents, and catabolites [homovanillic acid (HVA) is derived from dopamine]. The involvement of both catecholamines was suggested by such studies (43, 142, 185). Regulatory influences

on the rates of catecholamine biosynthesis occur at the first biosynthetic step, the hydroxylation of tyrosine. The activity of tyrosine hydroxylase is controlled for short time periods by endproduct inhibition by catecholamines, and for longer periods, by transsynaptic induction of new enzyme synthesis. The induction of tyrosine hydroxylase in the caudate nucleus of rats treated 15 days with morphine was shown by Reis et al (186), and in rat striatum and hypothalamus after long-term treatment with morphine (165). It is possible that tyrosine hydroxylase is induced after a single injection but requires time and more drug exposure for the amount of additional enzyme to be measurable.

Catecholamines thus play a role in tolerance. In both tolerance and abstinence the levels and turnover of these neurotransmitters have been shown to be opiate-responsive. Some indications of an adaptation by induction of tyrosine hydroxylase and adenyl cyclase in brain areas have been obtained. It is possible that such inductions of biosynthetic enzyme systems are a homeostatic response to chronic opiate treatment.

ACETYLCHOLINE Acetylcholine has long been implicated in the mechanism of action of narcotic analgesic drugs. In 1939, Slaughter & Munsell suggested that cholinergic mechanisms in the CNS may be inhibited by morphine and other opiates (187). Acetylcholinesterase was shown to be inhibited in vitro by narcotic drugs (188). However, because narcotic antagonists also possessed this activity, and there was no alteration of esterase activity in brain after morphine treatment (189), it is unlikely that the inhibition of acetylcholinesterase is important in the mechanism of opiate action.

At low doses of morphine, there was no change in acetylcholine levels in rodent brain, but at higher doses there was a transient increase (189–192). In tolerant animals this increase was no longer seen, and in abstinent animals the levels again increased, peaking several days after the last injection of morphine (192). In whole brain of cats no change in acetylcholine release was found after acute treatment, although naloxone administration elicited increased release in tolerant animals (193).

The spontaneous release of acetylcholine from the rat cerebral cortex was lower in morphine-dependent rats (194). Injections of morphine produced a consistent decrease in the rate of spontaneous release of acetylcholine in control animals but not in morphine-dependent animals (194).

The activity of the biosynthetic enzyme, choline acetylase, in the caudate nucleus, reflected these changes in acetylcholine levels (195). The increases seen in brain levels of acetylcholine after acute opiate administration probably are due to a decreased liberation of the transmitter from nerve-endings, because opiates inhibited acetylcholine release in isolated preparations of superior cervical ganglion (196), in brain cortex slices (197–200), and in isolated nerve endings (176).

Earlier, in the isolated guinea pig ileum, which is sensitive to opiates, the blockade of electrically stimulated contraction by morphine was found to parallel the inhibition of acetylcholine release in the preparation (201–203). This inhibition of release

was dose-dependent and stereospecific and antagonized by narcotic antagonists (204). Ilei isolated from morphine-tolerant guinea pigs were tolerant to morphine (205, 206).

The use of drugs that alter cholinergic function has extended information on the role that acetylcholine plays in opiate action. Domino & Wilson used the method of blockade of acetylcholine synthesis to estimate turnover in rat brain by the intraventricular injection of hemicholinium (HC-3) (207). Morphine antagonized the depleting effect of HC-3 in these experiments, as did many other opiate agonists. In tolerant rats, the depleting effect of HC-3 was not antagonized by the same dose of morphine (207). While HC-3 increased the morphine AD_{50} in both tolerant and nontolerant mice, its administration decreased the naloxone ED_{50} for precipitating abstinence in tolerant animals (208). In general, atropine increased, and physostigmine decreased abstinence signs (209). Jhamandas et al have divided abstinence signs in morphine-tolerant rats into autonomic and nonautonomic signs and showed differences in the dose-dependent effects of atropine, physostigmine, and imipramine in the two categories, concluding that both muscarinic and nicotinic receptors are activitated in abstinence (210).

SEROTONIN The role of serotonin in opiate tolerance and dependence was reviewed by Way in 1971 (211) and reassessed by him in 1973 (212). The administration of an inhibitor of serotonin biosynthesis, *p*-chlorophenylalanine (PCPA) modified the acute effect of morphine on analgesia (213), hypothermia (214), and hyperactivity (16, 153). In tolerant animals serotonin turnover was increased in brain more than twice that in nontolerant animals (215). When serotonin biosynthesis was inhibited by PCPA, tolerance and physical dependence were prevented in part (215). That morphine enhances serotonin turnover in brain has been both denied (216, 217) and affirmed (150, 218, 219). The rate-limiting biosynthetic enzyme, tryptophan hydroxylase, was increased in midbrain in morphine-dependent animals (220, 221), but not in whole mouse brain (222).

It is interesting that stimulation-evoked analgesia produced via electrodes implanted in the dorsal raphe was blocked by PCPA, which suggests that analgesia is associated with this serotoninergic pathway (223).

Methadone has been shown to inhibit the uptake of serotonin into isolated nerve-ending preparations from rabbit brain at drug concentrations of $10^{-9}M$ (224). Other opiates were inhibitory only at much higher concentrations. The uptake of serotonin into slices of rat hypothalamus was also inhibited by $10^{-9}M$ methadone, but not by morphine (225). In the studies of serotonin uptake into nerve-ending preparations, *d*-methadone was not inhibitory. However, in uptake into hypothalamic slices, the *d*-isomer was as active as the *l*-isomer, suggesting that the inhibition of serotonin uptake by methadone is not a specific opiate effect (225).

Morphine antagonized the stimulatory properties of serotonin in the guinea pig ileum (226). In preparations from morphine-tolerant guinea pigs, the sensitivity to serotonin was enhanced tenfold (227). In other mammalian species, however, the intestinal stimulatory effect

Thus the serotonin-blocking action of morphine on intestine seems to be unique to the guinea pig.

A UNIQUE ROLE FOR A SINGLE NEUROTRANSMITTER Evidence for the involvement of catecholamines, acetylcholine, and serotonin in the mechanism of opiate action and/or the development of tolerance and dependence are described in the preceding sections. It is impossible to ascribe acute or chronic effects of opiate use to a single transmitter system in the CNS, although it is occasionally possible to define the neuronal pathway for a single effect of an opiate. The interaction between neurotransmitter systems must be considered when assessing the role played by a single neurotransmitter. For example, the interaction between acetylcholine and dopamine in striatum has been demonstrated in experiments in which haloperidol-induced increases in HVA were blocked by anticholinergic drugs (229), and, in which an increase in acetylcholine output followed the blockade of the dopamine receptor by haloperidol (230). The importance of serotonin:catecholamine ratios has been indicated in morphine-induced analgesia (231), temperature regulation (232), and oxytremorine-induced analgesia (233). In these studies, norepinephrine antagonized morphine effects while serotonin potentiated them. No role has been ascribed to γ-aminobutyric acid (GABA) in opiate action, as yet. However, GABA neurons in the substantia nigra modulate the nigro-striatal pathway which has been involved in the catatonic-stereotypic effects of opiates (234).

The preceding discussion forces the conclusion that no known neurotransmitter is uniquely involved in tolerance and dependence, although the expression of pharmacological effects of opiates, including tolerance and dependence must be mediated via neurotransmitter systems.

HYPOTHALAMIC-PITUITARY NEUROHORMONES Many of the responses to the administration of narcotic analgesic drugs are mediated by the hypothalamic-pituitary hormone system. The acute administration of morphine or other opiates to animals and man produced an increased release of the pituitary trophic hormones: luteinizing hormone (LH), adrenocorticotropic hormone (ACTH), growth hormone (GH), thyroid-stimulating hormone (TSH), melanocyte-stimulating hormone, and the gonadotropins (235–237). During chronic treatment, there was no longer an increased release of most of the trophic hormones, with GH an apparent exception (236). The localization of active sites in the hypothalamus which activate the hypothalamic-releasing factors that control the pituitary, by lesioning or microinjection techniques, has led to the identification of sites at which opiates act on releasing factors. The medial mammillary nuclei was the site for morphine effects on thyrotropin releasing factor (TRF) release, although lesions in the median eminence also blocked the effect of morphine on TSH secretion (235). Direct injection of morphine into the area of the mammillary nuclei has added to the evidence that this area is a site of opiate action on TSH release (238). The injection of morphine into midhypothalamic areas also activitated ACTH secretion (239). Electromicrographic examination of the hypothalamus of morphine-tolerant rats showed a prolif-

eration of whorls of smooth endoplasmic reticulum in the median eminence and arcuate and preoptic nuclei of the type generally related to increased protein synthesis (240).

The hormones of the posterior pituitary are also affected by the administration of narcotics. The antidiuretic hormone was excreted in excess amounts after a single injection of morphine, an effect to which tolerance developed, in rats, chicken, and man (241).

It is evident that opiates influence pituitary function by their action on the hypothalamus, at the final neural integration of anterior pituitary function. Whether opiates act directly on hypothalamic-releasing factors, or via neurotransmitters, is not certain at the present time.

Opiate Receptors in the CNS

After the administration of radio-labeled opiates to rats in pharmacologically active doses, the labeled drugs were found throughout the brain in a temporal and regional pattern related to the lipid solubility of the opiates (242). The drugs were localized in the nerve-ending fractions in amounts as high as one nmol/g brain, without any stereospecificity in binding. A similar lack of stereospecificity was reported by Seeman et al, who found that the *d*- and *l*-isomers of methadone were taken up in equal amounts in perfused brain, and were equipotent in blocking the nerve impulse in rat phrenic nerve (243). Herz & Teschemacher reviewed the kinetics of distribution of opiates in the CNS and concluded that narcotic agonists have an intrinsic receptor activity quite apart from the lipid-related penetrability into brain (244). Following the suggestion of Goldstein et al that stereospecific binding can be measured as the difference between the binding of radio-labeled opiate in the presence of unlabeled excess active and inactive isomers (245), three groups of investigators have described stereospecific binding of opiate agonists and antagonists to brain tissue in vitro (246–248). Some of the chemical and pharmacological characteristics of opiate binding to brain receptors have been summarized by Goldstein (249). No differences have been found between the binding capacity of brains from tolerant or from nontolerant animals, although the administration of either an agonist or antagonist to mice rapidly elevated the stereospecific binding capacity of mouse brain extracts in vitro, with antagonists more potent in this effect (250).

This rapidly developing field should contribute much in the future to an understanding of the initial drug:tissue interactions and, ultimately, of the complex phenomena of tolerance and dependence.

Adaptations Outside the CNS

The distribution and metabolism of narcotic analgesics have not been related causally to opiate action or tolerance and dependence, aside from the obvious effects on drug availability that rates of absorption, catabolism, excretion, drug:tissue binding, and the conversion of an inactive drug to an active metabolite have (251). Opiates have been known to depress drug metabolism by the mixed oxidase system in liver, but only in mature male rats (252–254). Glucuronide formation, possibly not a

function of the liver mixed oxidase system, also was depressed during chronic morphine treatment (255). The sex differences in Type I drug metabolism found in the rat have suggested that morphine impaired an androgen-induced stimulation of hepatic mixed oxidase system in this species (253, 254). In other species, opiate administration did not alter liver drug metabolism (252, 256), except that the daily oral administration of methadone to mice has been reported to increase liver N-demethylase activity in male mice (257).

Tolerance develops in the target tissue for trophic hormones, e.g. the stress response to acute morphine, shown by an elevated plasma corticosterone level (258), decreased rapidly when morphine was administered twice daily to rats (236). However, morphine-tolerant animals still responded normally to stressful stimuli. This type of tolerance probably plays a very minor role in opiate dependence.

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