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Mechanisms of neurotransmitter release by amphetamines: A review

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Abstract

Amphetamine and substituted amphetamines, including methamphetamine, methylphenidate (Ritalin), methylenedioxymethamphetamine (ecstasy), and the herbs khat and ephedra, encompass the only widely administered class of drugs that predominantly release neurotransmitter, in this case principally catecholamines, by a non-exocytic mechanism. These drugs play important medicinal and social roles in many cultures, exert profound effects on mental function and behavior, and can produce neurodegeneration and addiction. Numerous questions remain regarding the unusual molecular mechanisms by which these compounds induce catecholamine release. We review current issues on the two apparent primary mechanisms — the redistribution of catecholamines from synaptic vesicles to the cytosol, and induction of reverse transport of transmitter through plasma membrane uptake carriers — and on additional drug effects that affect extracellular catecholamine levels, including uptake inhibition, effects on exocytosis, neurotransmitter synthesis, and metabolism.

Contents

Abbreviations: AMPH, amphetamine; CHO, Chinese hamster ovary; DAT, dopamine (plasma membrane) transporter; GABA, gamma-aminobutyric acid; hDAT, human DAT; MAO, monoamine oxidase; MDMA, 3,4-methylenedioxymethamphetamine; METH, methamphetamine; NET, norepinephrine transporter; PCA, parachloroamphetamine; rTARl, rat trace amine receptor; SERT, serotonin transporter; TMA, 3,4,5-trimethoxyamphetamine; VMAT, vesicular monoamine transporter

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I. Introduction: what is an amphetamine?

Amphetamine (AMPH) (1; see Fig. 1 for enumerated chemical structures) and its many derivatives are so varied in structure and effects that they could be considered the orchids of the psychoactive drugs. This review addresses the multiple mechanisms of action that underlie these compounds' most prominent and paradigmatic biological effect—to elevate extracellular levels of catecholamines and serotonin via a mechanism that is independent of the classical means of transmitter release by secretory vesicle fusion. We review current ideas within a historical context to help frame future research on this rewarding but addictive, popular but despised, beneficial and destructive class of drugs.

Alexander Shulgin (1978), a specialist in psychomimetic phenethylamines, states that "the name amphetamine designates one unique chemical and there can be no justification for its use in the plural"; nevertheless, the plural is destined to survive until the language evolves, as the generic names for many compounds that contain the AMPH structure also include the term. For the purposes of this review, we cautiously define "the amphetamines" to follow the rules implied by the generic name of the wr-compound as sharing an a-methyl-phenethyl-amine motif. The chemists J. H. Biel and B.A. Bopp (1978) state the definitive structural features of AMPH as (1) an unsubstituted phenyl ring, (2) a two-carbon side chain between the phenyl ring and nitrogen, (3) an a-methyl group, and (4) a primary amino group. The most studied such compounds, AMPH and methamphetamine (METH) **(2),** are addressed most specifically in this article (although METH does not obey the fourth rule, having a secondary amine). While both compounds are stellar examples of catecholamine releasers, they simplify our review by having little affinity for neuronal receptors (although see Section 6.4). This article is not intended to review data that compare how chemical substitutions to AMPH alter the drugs' efficacy in relation to each mechanism, an enormous subject [see an excellent review by David Nichols, 1994]. For purposes of comparison, we nevertheless sometimes discuss chain-substituted amphetamines, such as methylphenidate (3), and ring substituted amphetamines, such as 3,4-methylenedioxymethamphetamine (MDMA: ecstasy) (4), as well as non-a-methylated phenethylamines, such as tyramine **(5)** or mescaline **(6)** (both of which also have ring substitutions).

It is often asked if AMPH and METH differ in effect. The two drugs show no differences in terms of changes in dopamine release in the striatum, elimination rates, or other pharmacokinetic properties (Melega et al., 1995), and equal doses of the two drugs are not distinguished in human discrimination studies (Lamb and Henningfield, 1994). There is, however, a subtly greater dopamine release by AMPH than METH in the prefrontal cortex, and likely as a result, some subtle differences in effects of the drugs on working memory and behavioral tolerance (Shoblock et al., 2003a, 2003b). AMPH can elicit somewhat more locomotor activity in rodents than METH, perhaps due to indirect effects (Shoblock et al., 2003a). The occasional statement in the literature that METH is more addictive, favored by drug addicts, a more potent psychostimulant, or has diminished peripheral activity appears to be unfounded (Shoblock et al., 2003a). By tradition, studies on mechanisms of action are generally on AMPH, and studies of neurodegeneration on METH. This may be because METH is more readily available on the illicit market due to its easier synthesis, which uses either a one-step reduction of ephedrine (7) or pseudoephedrine, drugs that at this writing remain readily available, or a condensation of phenylacetone and methylamine (Cho, 1990). Because ephedrine is a stereochemically pure natural product, the first method of METH manufacture yields only the more active S(+)-enantiomer (Section 5.2).

2. A brief overview of AMPH use

2.7. Natural AMPH sources

As is the case for nicotine, cocaine, opiates, marijuana, and alcohol, amphetamines have been administered as plant

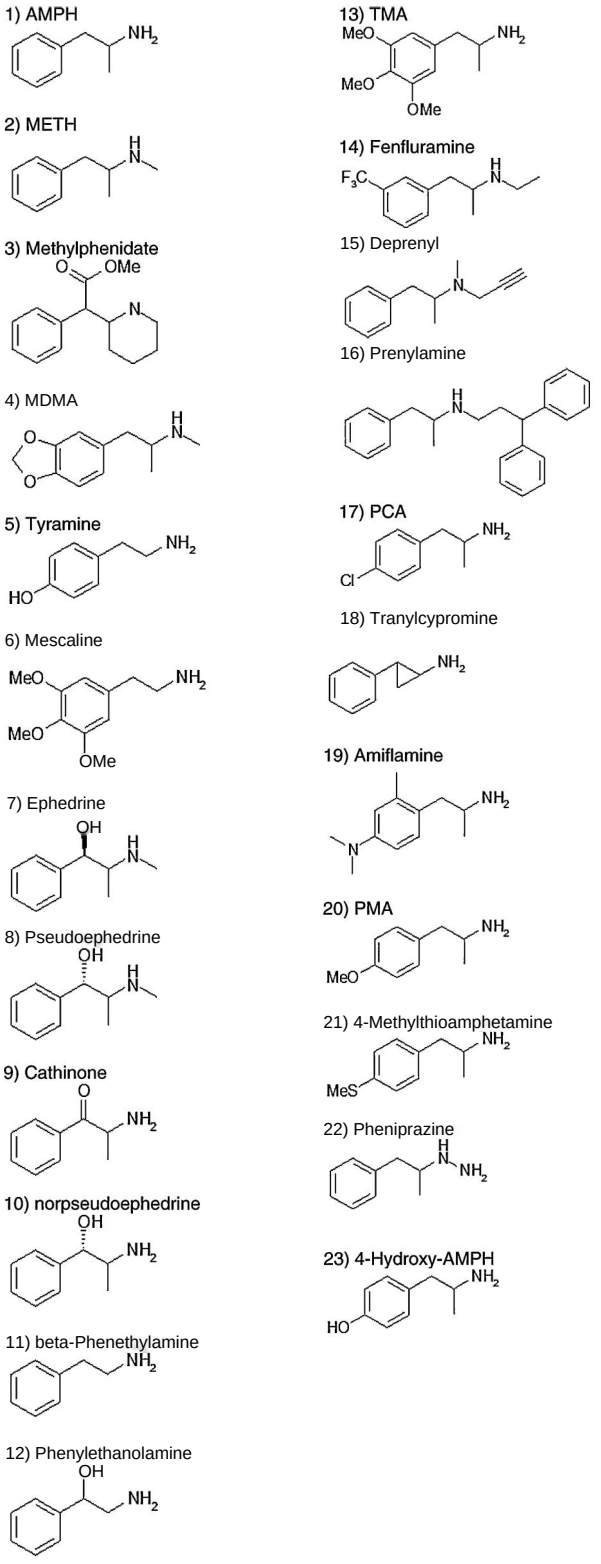

Fig. 1. Chemical structures of AMPH and related compounds enumerated in the text.

products for thousands of years. The principal species so used are members of the genus Ephedra and the tree Catha edulis, known in Arabic and Swahili as khat (qaf) and over much of East Africa as myrrha (miraa).

Ephedra sinica, often known simply as ephedra and in China as Ma huang ("looking for trouble"), has been recovered from Middle Eastern Neolithic gravesites and Vedic temples in India. The herb has been identified, although controversially, as soma, the food of the Vedic gods (Mahdihassan and Mehdi, 1989). A first century AD Chinese book of herbal medicine, Shen Nong Ben, mentions its use for treating asthma and upper respiratory infections. The major active component, ephedrine **(7),** was identified in 1887 by Nagajoshi Nagai, a founder of the Pharmaceutical Society of Japan. In the western United States, a stimulant tea was brewed from a different Ephedra species, the herb Mormon Tea (E. nevadensis), so called because it was not proscribed by the Church of Jesus Christ of Latter-day Saints, although a recent report suggests that New World Ephedra lacks phenethylamine-related alkaloids (Caveney et al., 2001). Over-the-counter ephedrine has been a popular appetite suppressant and widely used by athletes to boost performance. It was implicated in the death of Baltimore Orioles pitcher Steve Bechler in February 2003 and was banned from use as a dietary supplement on the American market by the FDA in April 2004. Today, ephedrine and pseudoephedrine **(8)** are the most common precursors for amateur laboratories that produce METH for the illicit market (Section 1).

The fresh leaves of the shrub or tree Catha edulis, native to Kenya and Somalia and widely cultivated in Yemen, contain the amphetamines cathinone (9) and norpseudoephedrine (cathine) **(10).** The leaves' clinical use was reported in the 11th century by Abu Al-Rihan Bin Ahmed Al-Baironi in Pharmacy and Therapeutic Art (Al-Motarreb et al., 2002). The fresh leaves or stems are chewed, as the stored product racemizes and loses activity. Khat is not proscribed by the Koran and is common from the Hom of Africa through the Middle East. The herb is used by an estimated 55% of the population of northern Somalia (Elmi, 1983) and as many as 85% of adult males in North Yemen (Pantelis etal., 1989), and represents 30% of Yemen's agricultural product (Al-Motarreb et al., 2002). It is associated with gregariousness, feelings of contentment, suppression of fatigue, and appetite loss. Khat is habit forming, induces paranoia and other behavioral disturbances (Jager and Sireling, 1994; Yousef et al., 1995), but is said to produce relatively benign withdrawal symptoms. Khat is incorporated into fascinating and complex social rituals, particularly in Yemen, where homes may maintain two separate rooms devoted to khat chewing, the mandher, a small upper story room with many windows, and a dewan for large social gatherings (Al-Motarreb et al., 2002). There is a suspicion expressed in Kenya that long-term khat addicts have motor deficits (personal communication), but this has not been studied systematically. (Myrrha, as the plant is known in Kenya, should not be confused with myrrh, one of the gifts to Jesus by Zoroastrian magi (Matthew 2:11). Myrrh

is derived from Commiphora myrrha, a different tree native to Kenya and Somalia, a region also native to the tree that provides frankincense, Boswellia sacra.)

While additional plant species contain natural amphetamines, including AMPH and METH in Acacia species (Clement et al., 1998) and AMPH in Egyptian jasmine (Nofal et al., 1982), we are not aware of other plants commonly used to administer these drugs. There are, however, plant products with components similar to amphetamines. Synephrine, a sympathomimetic AMPH congener lacking an a-methyl group, is found in citrus plants including Citrus aurantium, bitter orange (Pellati et al., 2002). The world's most widely used psychostimulant is said to be arecoline, the principal active compound in the betel nut palm Areca catechu, which is consumed by 10-20% of the world's population (Gupta and Wamakulasuriya, 2002). (N.B., caffeine and nicotine do not enhance locomotor behavior in rodents and are not considered psychostimulants.) In addition to arecoline, which is not a phenethylamine, some phenethylamine-like compounds are present in the betel nut and flower, the ingredients of the paste known as betel quid. While used experimentally as a muscarinic agonist, the psychostimulant mechanism of action of betel has been little studied, although it has been reported to produce AMPH-like catecholamine release (Wang and Hwang, 1997; Chu, 2002).

Another phenethylamine analog, mescaline **(6),** derived from dried tops of the peyote cactus Lophophora williamsii and related species, avoids classification as an amphetamine due to lack of an a-methyl group. This plant appears in Native American funerary art of 2000 years ago (Furst, 1972), and its use by the Aztecs for predicting the future and other practices was discussed by F. Hernandez, the Spanish king's personal physician, in Nova Plantarum, Animalium et Mineralium Mexicanorum Historia in 1576. Its ongoing use by Tarahumara and Huichol Indians in Mexico preceded the European invasion. Around 1880 it was incorporated by more northern tribes into the rituals of the Native American Church, a religion that includes about 200,000 adherents of mostly Plains Indians, and for whom religious use of peyote has been protected by Federal law since 1965, most specifically by the American Indian Religious Freedom Act Amendments of 1994. There are no reports of neurotoxicity, overdose, or drug dependence from peyote (McCann and Ricaurte, 1994), and the mechanism of action of this drug is quite different than that of amphetamines (Nichols, 1994; Schmidt, 1994), although mescaline has inspired the synthesis of numerous AMPH derivatives (Section 2.4).

2.2. Endogenous AMPH-like compounds?

It has long been known that decarboxylated metabolites of the aromatic amino acids — P-phenethylamine **(11)** from phenylalanine, its metabolite phenylethanolamine **(12),** tyramine (5) from tyrosine, and tryptamine from tryptophan — are formed in the peripheral nervous system (Nakajima et al., 1964) and the brain (Inwang et al., 1973). These compounds have been suggested to modulate affective behaviors including excitement and alertness, are decreased in the urine of depressed patients (Sabelli and Mosnaim, 1974) and are elevated by marijuana (Cannabis sativa) (Sabelli et al., 1974). These "trace amines," however, do not appear to be stored in substantial quantity in either the central nervous system or periphery (Berry, 2004).

Trace amines share with AMPH several mechanisms of action and could possibly act as endogenous amphetamines. High levels of P-phenethylamine applied exogenously or reached during monoamine oxidase (MAO) inhibition elicit AMPH-like psychostimulant responses (B ergman et al., 2001). Eike AMPH, it releases dopamine in a manner dependent on the presence of an intact dopamine transporter (DAT) (Section 5.3) as assayed using cyclic voltammetry and microdialysis in wild-type and DAT knockout mice (Sotnikova et al., 2004), although a subset of behavioral responses to the compound were found to be independent of the presence of DAT.

It remains to be determined if P-phenethylamine is a neurotransmitter or released in a regulated fashion; at least when present in high levels, it would likely be transported into synaptic vesicles (Niddam et al., 1985) by the vesicular monoamine transporter. Other studies, however, variably implicate release by diffusion across membranes rather than reverse transport via catecholamine transporters and in some cases, insensitivity to reserpine (an inhibitor of vesicular catecholamine uptake: Section 3.1) (Berry, 2004).

As is the case with mescaline and in contrast to AMPH, the trace amines lack the a-methyl group that inhibits MAO (Section 6.1), and are thus thought to be rapidly metabolized in the brain. It remains unknown if local levels of p-phenethylamine normally reach concentrations that elicit endogenous AMPH-like effects, or even reach levels that significantly activate receptors, although some of the trace amine compounds have very high affinity for the recently identified trace amine receptors (Section 6.4).

2.3. Synthetic amphetamines

Synthetic AMPH was invented in 1887 by Lazar Edeleanu (1862-1941, a.k.a. Edeleano), a Rumanian chemist who described its synthesis in his doctoral dissertation under A.W. Hofmann at the University of Berlin (Edeleano, 1887). Edeleanu later became famous for also inventing the method to distill petroleum using sulphur dioxide, providing the tell-tale odor of gasoline distilleries.

AMPH was bequeathed its generic name from a contraction of a-methyl-phenethyl-amine. Some notion of the colorful history of this drug can be seen from the 1989 Merck Index listing, which lists 17 trade names, not even including such familiar trade names as Adderall, Benzedrine, and Dexedrine, or for that matter the myriad nicknames used by drug abusers. Of course, chemists have since developed an astonishing range of synthetic AMPH derivatives.

Following work discussed below by Barger and Dale that introduced the concept of sympathomimetic amines (Sec-

tion 3.1), researchers have examined a wide range of catecholamine-like derivatives for the ability to raise blood pressure and to relieve nasal and bronchial congestion from colds and hay fever. AMPH was independently resynthesized by Gordon Alles in 1927 in an effort toward developing synthetic sympathomimetics, and he and his coworkers are credited with the first report of its stimulant effects (Alles, 1933). Walter Hartung and James Munch from Sharp & Dohme laboratories identified AMPH (called in that study phenyl-2-amino-l-propane) as a particularly efficacious sympathomimetic, as well as being particularly active when administered orally (Hartung and Munch, 1931).

AMPH was introduced commercially in 1932 by the pharmaceutical firm Smith, Kline and French as Benzedrine, which was the free base administered in inhaler form. The first report of AMPH use as a clinical treatment, for narcolepsy, is credited to Myron Prinzmetal, a colleague of Alles (Prinzmetal and Bloomberg, 1935). In 1936, Smith Kline and French began to sell Benzedrine as 10 mg tablets without prescription. It is said that the first widespread use of AMPH stemmed from word-of-mouth reports spread from campus to campus following experiments by the Department of Psychology at the University of Minnesota on alertness in college students (Angrist and Sudilovsky, 1978). Over 50 million Benzedrine tablets were sold during the first 3 years of availability.

AMPH was made available by prescription only on January 1, 1939. Initially associated with students, and soon after with artists, musicians, the armed forces, and truck drivers, AMPH's popularity contributed directly to at least one deathless work of art, the boogie woogie "Who put the Benzedrine in Mrs. Murphy's Ovaltine?" by Harry "the Hipster" Gibson (1944).

Until 1946, the pharmaceutical industry promoted more than 30 uses for AMPH, including treatment of schizophrenia, opiate addiction, infantile cerebral palsy, seasickness, radiation sickness, and persistent hiccups (Bett, 1946; Miller and Hughes, 1994). By 1970, annual pharmaceutical production reached 10 billion tablets, with perhaps 50-90% diverted to the black market. The United States Justice Department imposed legal quotas on AMPH production in 1971.

AMPH, METH, and methylphenidate are still very widely prescribed for weight control, narcolepsy, and attention deficit disorder. The latter disorder is a particular concern, as the National Institute of Mental Health estimates that it is exhibited by 3-5% of the population, and the number of American children treated with these three AMPH congeners increased from 0.6 per 100 to 2.4 per 100 between 1987 and 1996 (Olfson et al., 2002). Adderall, a mixture of S(+)- and /?(-)-AMPH, is presently the most commonly prescribed treatment in the United States for juvenile attention deficit disorder. The mechanism underlying its efficacy remains obscure, although it has been suggested that it is due to a specific enhancement of "tonic" dopaminergic signalling (Knutson et al., 2004).

Beginning in the Spanish Civil War, and in all subsequent wars, AMPH has been used by the military to promote alertness. Millions of tablets and pills were supplied to American troops during World War II, and unknown quantities were administered to German, Japanese, and British military and support personnel, in particular to the air forces on long flights and bombing missions. This use remains current, especially for fighter pilots on long missions (Caldwell et al., 2003), although it has been suggested that it causes hallucinations, particularly in people with a common mutation in catecholamine-O-methyl transferase enzyme (Mattay et al., 2003).

An initial report on AMPH abuse and induction of psychosis in two cases soon followed its introduction (Young and Scoville, 1938), although this was thought to unmask a latent psychosis. The authoritative documentation of AMPH psychosis is credited to PH. Connell (Connell, 1958). The addictive potential of AMPH was mentioned as early as 1937 (Guttmann and Sargeant, 1937) but was a topic of much debate [see discussion in Angrist and Sudilovsky, 1978] and not fully recognized until the mid-1960s (Lemere, 1966). The initial reports of central nervous system neurotoxicity induced by amphetamines also appeared in the 1960s (Pletscher et al., 1963).

As with cocaine, AMPH use tends to occur in epidemic waves. A well-known example occurred in Japan from 1947 to 1957, and was associated with returning troops who were administered AMPH, partly under coercion, along with large-scale advertising of the drug to the general public from manufacturers. By 1954, 550,000 Japanese were using the drug illicitly (Fukui et al., 1994). The epidemic ended only after draconian legal intervention reduced the METH content of the illicit drug supply (Konuma, 1994). Another example occurred in Sweden in 1942, 4 years following its introduction, when an estimated 3% of the country's population used AMPH (Rylander, 1972).

As of 2000, the U.S. Drug Enforcement Agency states that METH is the most prevalent illicitly manufactured controlled substance, with 6394 reported clandestine laboratory seizures that year. The DEA estimates that 4% of the American adult population has used amphetamines at least once. Local epidemics are current in both rural pockets of the United States, including the western, mid-western, and southern states (F. Butterfield, New York Times, February 11, 2002) and large cities. One percent of Wyoming's population is now said to require treatment for METH addiction (T. Egan, New York Times, December 8, 2002). MDMA and METH are associated with dance parties and clubs, and METH use in gay dance clubs in New York may have reached so-called epidemic proportions (A. Jacobs, New York Times, January 29, 2002).

MDMA, originally developed by Merck in 1912 and patented in 1914 for use in synthesis of styptic drugs based on hydrastinine, was used in the United States by psychotherapists, particularly for couples therapy, from 1978 until 1985 when it was assigned Schedule I status.

There are nevertheless ongoing efforts by some to reintroduce MDMA for clinical use, as well as a significant and controversial body of literature suggesting neurotoxicity in serotonin neurons. Illicit MDMA is still widely self-administered, and 9.3 million tablets of MDMA were confiscated in the United States in 2000.

An early effort to synthesize AMPH derivatives on the basis of knowledge of structure-function relationships resulted in trimethoxyamphetamine (TMA) (Hey, 1947) **(13),** due to the addition of an a-methyl group to mescaline in order to inhibit its deamination (Section 6.1). In the words of David Nichols, the synthesis of TMA "began the journey that ultimately led to the synthesis and pharmacological evaluation of nearly 200 potentially hallucinogenic substituted amphetamines" (Nichols, 1994). Many other AMPH derivatives have been explored for clinical uses, including appetite suppression (e.g., fenfluramine; 14), and as MAO inhibitors (Section 6.1) for treatment of Parkinson's Disease (deprenyl; **15).**

3. Evidence for multiple sites of action

3.1. History of roles for plasma membrane transporters and secretory vesicles

The earliest scientific efforts into understanding AMPH action raised issues still debated today. This history is inextricably bound with the study of the adrenal medulla and its role as a secretor of catecholamines. The term catechol is derived from the plant product catechu (a.k.a. gambir), a cake of aqueous extract of leaves and twigs from the black catechu tree, Catechu nigrum, native to Burma and India, or from Uncaria gambier, a vine native to India and Malaysia. Catechu contains up to 30% catechol, and was traditionally used to dye fabric brown (although tannic acid, also present, contributes to this) and as an astringent in the treatment of a variety of medical disorders.

The British physician George Oliver discovered the ability of adrenal gland extract to increase blood pressure by injecting it into his own son, noting a contraction of his son's radial artery [see a fine review on the history of adrenal medulla research (Carmichael, 1989)]. By 1894, Oliver with Edward Albert Schafer confirmed that this extract increased blood pressure (in dogs). The compound responsible was independently identified by three laboratories; Stolz, Dakin, and John Jacob Abel in 1897, a founder of the Journal of Biological Chemistry, who named the compound epinephrine. The preparation from oxen and sheep adrenals was patented as Adrenaline by the pharmaceutical firm Parke Davis. Competing claims for priority provide one reason that this transmitter has 38 different names in the Merck Index. Ernst Joseph Friedman published epinephrine's chemical structure in 1906.

These findings inspired the hypothesis of secretory transmission that is the foundation of contemporary neuroscience. Thomas Renton Elliott, a student of John Newport Langley at Cambridge University, upon confirming Oliver and Shaefer's findings by injecting epinephrine into dogs, proposed that nerves communicate via release of a chemical (Elliot, 1904), writing that "adrenaline might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery" [see a fine review on the early history of neuro transmission (Valenstein, 2002)].

Not incidentally, Elliott also proposed as early as 1914 that nerves could accumulate epinephrine by an uptake system, suggesting that the adrenal gland might "depend on what could be picked up from the circulating blood and stored in its nerve endings", although demonstration of uptake mechanisms arrived more than 40 years later (Section 5.1).

Inquiry into pharmacological rather than endogenous stimulant mechanisms was essentially initiated by George Barger and Henry H. Dale (1910). Barger, a chemist studying compounds, he isolated from putrid meat, found that isoamylamine, a weak base that did not posses a catechol ring, could, like epinephrine, raise blood pressure. He and Dale, working together at the Wellcome Physiological Research Laboratories, then examined other non-catechol weak bases for epinephrine-like functions. Dale, the physiologist of the pair, initially assayed the compounds by observing uterine contraction. They suggested that active compounds be called sympathomimetic to indicate "the relation of the action to innervation by the sympathetic system, without involving any theoretical preconception as to the meaning of that relation or the precise mechanism of the action." Sympathomimetic potency was rated by the effect on blood pressure. Among the compounds they identified were |3-phenylethylamine, and the AMPH isomers, |3-methylphenethylamine and phenylpropylamine. In later work by J.H. Burn and coworkers, sympathomimetics that caused membrane contraction after sympathetic postganglionic denervation (and were not blocked by reserpine) were called directly acting, in contrast to indirectly acting amines that required innervation to produce contraction (and had reserpine-sensitive responses; see below) (Fleckenstein and Bum, 1953).

Another sympathiomimetic, cocaine, derived from the Andean Erythoxylon coca and related species, provided important insights into the mechanisms involved (cocaine is now understood to act primarily as an inhibitor of the plasma membrane monoamine uptake transporters: see below and Section 5.1). During the 1880s and until the Harrison Tax Act in 1914, which restricted coca products, there was a widespread vogue for cocaine use in the United States (Angrist and Sudilovsky, 1978), where it was associated with the introduction of Coca-Cola in 1886 and initially enthusiastic reports by Sigmund Freud (1884).

In the same year as Barger and Dale's study, Alfred Fröhlich and Otto Loewi at the Pharmacology Institute in Vienna initiated fundamental research into cocaine action, reporting that cocaine potentiated epinephrine's increase in

blood pressure (Fröhlich and Loewi, 1910). Surprisingly, a later study showed that cocaine did not potentiate but rather blocked the effect of the paradigmatic sympathomimetic drug, tyramine (Tainter and Chang, 1927) even though they each independently increased blood pressure; this was sometimes labeled the cocaine paradox.

Insight as to why two sympathomimetics respond in opposite manners to cocaine arrived with the introduction of reserpine to the west, derived from the Indian Snakeroot, Rauwolfia serpentina, known in Bihar and Uttar Pradesh as Pagal-ki-dawa ("medicine for the insane"). Snakeroot, a climbing shrub that grows from the Himalayas to Indonesia, was used in the subcontinent for treating mental illness, snakebite, and scorpion stings for thousands of years. During the 1940s, Rustom Jal Vakil of Bombay's KEM Hospital found it effective for lowering blood pressure, and it was prescribed to over 1 million hypertensive patients in India.

Subsequent reserpine trials by Nathan Kline of New York Rockland State Hospital for treatment of violent mentally ill patients received extraordinary attention in the West. Reserpine was considered a "miracle drug" and to some extent replaced electric convulsive therapies and lobotomies. The U.S. News and World Report (November 11, 1955) said, "Mentally retarded children improved in behavior, developed higher IQs. Patients with the excruciating heart pains of angina pectoris had both severity and frequency of attacks reduced. Dogs were cured of carsickness; mares that spumed foals were made normal. Skin diseases have been made less severe, and intolerable itching relieved. Prospects for recovery in mental disease have risen to 90% from 65." It can be considered the first antipsychotic drag. However, side effects including nightmare, parkinsonism and gastrointestinal disturbances, together with the development of other drags, have limited subsequent clinical use of reserpine.

Arvid Carlsson et al. (1957) soon showed that reserpine blocked tyramine's increase of blood pressure. Yet reserpine and tyramine did not appear to act at the same site. The authors suggested that "tyramine belongs to a group of sympathomimetic amines which are fully active only in the presence of an intact adrenergic system." In a variation of the cocaine paradox, it was not clear why intact neurons would be required for tyramine's action, and not for norepinephrine.

This puzzle was essentially solved by J.H. Burn and M.J. Rand at Oxford (Bum and Rand, 1958), who showed that exogenous epinephrine could still elevate blood pressure following reserpine, whereas exogenous tyramine, phenethylamine, ephedrine, and AMPH were no longer effective following reserpine. They concluded "the sympathomimetic amines may be divided into two classes, one consisting of substances like noradrenaline and adrenaline, which act on vessels in both normal and reserpine-treated animals, and the other consists of substances like tyramine which act only on vessels of normal animals and have little or no action on those treated with reserpine" and that tyramine and AMPH, which were in the second class, "act in the normal animal by releasing a noradrenaline-like substance."

The statement by Rand and Burn above is to our knowledge the first clear declaration that amphetamines act by releasing catecholamines. It also provided a mechanistic explanation for the difference between directly- and indirectly-acting sympathomimetics. Burn and Rand further provided an important clue toward solving the 30-year-old mystery of the cocaine paradox, why cocaine blocks the effects of tyramine, in that "the action of cocaine may be to arrest the release of the noradrenaline-like substance from the store." In other words, their study also introduced the oft-confirmed finding that uptake blockers also block reverse transport, although the discovery of an uptake transporter system occurred only that year in independent work by Barbara Hughes and Bernard Brodie (Section 5.1).

Thus, many of the current concepts, controversies, and approaches used to elucidate AMPH action were introduced by 1958. Note, however, that while these studies used a plasma membrane uptake blocker (cocaine) to determine if a drag was a releaser and a vesicular monoamine transporter (VMAT) inhibitor (reserpine) to determine involvement of intracellular stores, the identification of reserpine as a blocker of vesicular catecholamine uptake per se did not occur until 1962 (Carlsson et al., 1962; Kirshner, 1962).

From the mid-1960s through the 1970s, a variety of studies by numerous groups showed that AMPH also released catecholamines in the CNS, as reviewed by David Segal and Roland Kuczenski (1994), following the earlier work in the periphery. A role for AMPH in inducing central dopamine release was suggested, again by using cocaine or other uptake blockers as AMPH antagonists (Heikkila et al., 1975b; Raiteri et al., 1979; Liang and Rutledge, 1982; Parker and Cubeddu, 1988), as had Bum and Rand earlier. In comparing AMPH with cocaine, Mary Ritz and Michael Kuhar pointed out that while self-administration of cocaine-like blockers correlated with their binding efficacy to DAT, AMPH was far more potent than would be expected from its binding. The investigators interpreted this finding to mean that, as found long before in the periphery, release rather than reuptake blockade may be most important for AMPH action in the brain (Ritz et al., 1987). A role for plasma membrane transport in AMPH-mediated release was directly confirmed much later by measuring AMPH-mediated dopamine flux through DAT and its inhibition by uptake blockers (Sulzer et al., 1995), as well as electrical currents associated with dopamine and AMPH transport (Sonders et al., 1997; Sitte et al., 1998) (Section 5.4).

3.2. Plasma membrane versus synaptic vesicles?

While classical pharmacological studies related above implicate both plasma membrane uptake transporters and monoamine secretory/synaptic vesicles as playing roles in AMPH action, and all subsequent studies concerned agreed

on a role for catecholamine transporters, the role of synaptic vesicle pools has been often doubted, mostly due to results from reserpine experiments. As discussed, this investigative direction was initiated by a study demonstrating reserpine blockade of tyramine action (Bum and Rand, 1958). Thus, the first such report indicated a role for secretory vesicles in AMPH action. The conclusions differed greatly in later literature due to differences in experimental design. An extremely brief review follows.

Most studies, both on dopamine and norepinephrine systems, were conducted with in vivo reserpine injections generally given 24 h prior to AMPH administration. In nearly all examples of which we are aware, reserpine blocked AMPH release of norepinephrine (Kalisker et al., 1975; Fitzgerald and Reid, 1993; Florin et al., 1995), consistent with a role for vesicular catecholamine. However, since norepinephrine is predominantly synthesized from dopamine within vesicles by lumenal dopamine p-hydroxylase, the fraction of total catecholamine concentration represented by norepinephrine would be expected to be lower in the cytoplasm than in vesicles. Thus, the effect of VMAT inhibition on AMPH-mediated release could be greater for norepinephrine than dopamine.

Within the literature on combinatorial effects of reserpine and AMPH on dopamine release, some in vivo experiments found little or no effect of reserpine on AMPH (Niddam et al., 1985; Callaway et al., 1989; Arbuthnott et al., 1990), others reported reserpine blockade (Parker and Cubeddu, 1986, 1988; Sabol et al., 1993), and still others found both responses depending on conditions (Florin et al., 1995). Attempts to resolve the issue by examining dopamine release from synaptosomes yielded contradictory results (Bagchi et al., 1980; Masuoka et al., 1982).

One likely explanation for the conflicting findings is that reserpine causes a delayed upregulation of tyrosine hydroxylase activity via enhanced transcription (Pasinetti et al., 1990), thus producing higher levels of cytosolic dopamine. In ventral midbrain neuronal culture, where it is straightforward to measure all of the dopamine in the system, shorter-term exposure to reserpine (90 min) depleted exocytic dopamine release but did not increase tyrosine hydroxylase activity (Larsen et al., 2002), and also inhibited AMPH-mediated dopamine release by 75% (Sulzer et al., 1996). In conclusion, the contribution of vesicular catecholamines to AMPH-mediated efflux appears to be greatest when vesicle stores are intact, and may thus be considerable under typical conditions in which the vast majority of catecholamines, perhaps >99% (Fon et al., 1997; Mosharov et al., 2003), are stored in the vesicles. Under conditions that enhance tyrosine hydroxylase activity (Section 6.2), the role for non-vesicular dopamine may be much greater.

New approaches to this long-standing question have arisen over the past decade as genetic manipulations have allowed the issue of a role for synaptic vesicles in AMPH action to be addressed without reliance on reserpine. Such an approach to understanding AMPH action provides a paradigmatic example of the application of gene knockout technology when the drug in question has two targets with functions that are closely intertwined. Christian Pill and collaborators transformed COS cells to express DAT, the central vesicular monoamine transporter (VMAT2), or both (Pill et al., 1995). While this cell line lacks synaptic vesicles, VMAT2 expression in other acidic organelles, possibly including endosomes and lysosomes as well as secretory organelles, apparently provides a means to accumulate dopamine. AMPH released dopamine only in cells that expressed DAT. The release was greater, however, for cells that also coexpressed VMAT, and whereas dopamine efflux from DAT cells reached a peak and quickly returned to baseline in the continued presence of the drug, release was sustained in cells that coexpressed DAT and VMAT2. The authors concluded that there was both a plasmalemmal component and vesicular component in the dopamine-releasing action of AMPH.

Consistent with the results from transfected cell lines, results from studies using mouse mutants indicate participation of both DAT and VMAT in AMPH action. Cyclic voltammetry studies by Sara Jones and coworkers demonstrated that knockout mice that do not express DAT did not exhibit AMPH-mediated dopamine release (Giros et al., 1996; Jones et al., 1998). Unfortunately, VMAT2 knockout mice do not feed and die soon after birth (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997), and so could not be assayed in the same manner. Neuronal cultures derived from VMAT2 knockout mice survive, however, and AMPH-mediated dopamine release from VMAT knockout ventral midbrain neurons was depressed by 65% (Fon et al., 1997). As was the case for neuronal cultures and reserpine, this preparation allowed the system's entire neurotransmitter pool to be measured. A rapid (30 min) effect of AMPH was to increase tyrosine hydroxylase activity in these mutant neurons (Section 6.2), and along with the drug's inhibition of MAO (Section 6.1), this provided most of the source for the remaining dopamine release (Larsen et al., 2002). The enhancement of tyrosine hydroxylase activity by AMPH was far greater in ventral midbrain dopamine cultures that lacked VMAT2 expression or wild-type cultures exposed to reserpine than in untreated wild-type neuronal cultures. Strikingly, far more dopamine was released into the extracellular media by AMPH than was present in the entire VMAT2 knockout cultures at the time that the drug was added.

The basis for the greater activation by AMPH of tyrosine hydroxylase in neurons that do not accumulate vesicular dopamine remains mysterious. The ability of AMPH to alter tyrosine hydroxylase activity may underlie much of the controversy in the literature of reserpine and AMPH interaction, prominently including findings using labeled dopamine that suggest that AMPH preferentially releases a "newly synthesized pool" of dopamine [as reviewed and explored by Kuczenski, 1975]. Interestingly, AMPH

promoted locomotion and survival in VMAT2 knockout mice incapable of synaptic vesicle-mediated dopamine neurotransmission (Fon et al., 1997) — while the basis for this observation remains unknown, it suggests the possibility of physiological roles for non-vesicular dopamine release.

Finally, perhaps the most satisfying evidence that both synaptic vesicle and cytosolic dopamine pools both contribute to AMPH action was provided by elegant cyclic voltammetry studies in striatal brain slice by Sara Jones et al. (1998). Using this approach, dopamine released by electrical stimulation of midbrain terminals or AMPH perfusion was detected at 100-ms resolution. Jones stimulated the terminals at regular intervals until the amount of dopamine released per pulse was stable. She then continued stimulating throughout the experiment and perfused AMPH. AMPH provided a rapid, relatively small amount of baseline, i.e., unstimulated, dopamine release. Minutes later, the amount of dopamine released per electrical pulse decreased while the baseline stimulation-independent release increased. Thus, it appeared that the greatest amount of dopamine released was redistributed from vesicles into the cytosol. A later study showed that a portion of the decrease in electrically-evoked dopamine release was due to a D2 dopamine receptor feedback mechanism (Schmitz et al., 2001), but also confirmed that the dopamine that previously resided in synaptic vesicles and was redistributed to the cytosol was responsible for most of the AMPH-mediated release.

A variety of reports suggest the notion that low concentrations of AMPH preferentially release catecholamine already resident in the cytosol, whereas higher concentrations are required to redistribute vesicular catecholamine to the cytosol. If true, with lower drug concentrations the release is from the cytosolic pool, and with higher concentrations mostly from the vesicular pool (Seiden et al., 1993). The first such claim we are aware of is from a study in which “bound” norepinephrine,i.e., that leftoverin the tissue after AMPH exposure, was more efficiently released at higher AMPH or tyramine levels (Langeloh and Trendelenburg, 1987). The “bound” transmitter was assumed to reflect the vesicular pool.

It has, however, until recently been impossible to carefully distinguish dopamine in one pool versus the other, as discussed (Parker and Cubeddu, 1988), except in the case of knockout mouse mutants as discussed above. One prediction that could test a role for the vesicle pool would be that if AMPH simply releases cytosolic catecholamine, then the free catecholamine levels in the cytosol should decrease. On the other hand, if AMPH redistributes catecholamine from vesicles to the cytosol, the free cytosolic levels might increase.

Testing these predictions has required a means to measure cytosolic catecholamines. An initial approach used an intracellular carbon fiber electrode to measure free cytosolic dopamine in a giant dopamine neuron in the pond snail Planorbis comeus (Sulzer et al., 1995); exposure to AMPH increased cytosolic dopamine, consistent with redistribution of vesicular dopamine to the cytosol. Moreover, when AMPH accumulation by plasma membrane transporters was skirted by an intracellular injection of ~100 |±M AMPH, there was a rapid (within 5 s) increase in cytosolic DA, indicating that the effects on vesicles can be quite rapid. In a more detailed approach, Eugene Mosharov et al. (2003) developed intracellular patch electrochemistry, using a carbon fiber electrode placed inside a patch electrode used in whole cell mode, to measure cytosolic catecholamine levels in chromaffin cells and neurons (Mosharov and Sulzer, unpublished). They found that 10 pM AMPH induced a 15-fold increase in cytosolic dopamine within 10-15 min of exposure, strongly supporting redistribution of vesicular catecholamines. Cytosolic catecholamines returned to near control levels by 40 min, but cytosolic catechol metabolites remained elevated as long as AMPH was present. The complete relationship between AMPH concentration and resulting cytosolic catecholamine and vesicular levels remains to be elucidated, as do the interacting roles of catecholamine synthesis and metabolism (Section 6), but to date all results indicate that AMPH redistributes vesicular catecholamine to the cytosol.

In summary, experiments using electrochemical detection techniques, mutated transporters, and reserpine in neuronal cultures are all consistent with the ability of AMPH to act on both vesicular and plasma membrane transporters, and accordingly, to affect monoamine pools both in synaptic vesicles and the cytosol. These findings also indicate a generally unappreciated role for AMPH-induced de novo dopamine synthesis (Section 6.2) in providing much of that dopamine still released after reserpine as well as additional effects on catecholamine metabolism that contribute to AMPH effects. It may be that AMPH, but not reserpine, causes extensive catecholamine release in parts because it blocks MAO (Section 6.1); strikingly, while either MAO inhibition or reserpine alone does not increase cytosolic catecholamine, the combination does (Mosharov et al., 2003). There are additional potential actions of AMPH on extracellular catecholamine levels, such as induction of small excitatory currents at DAT (Section 6.5), the ability to directly bind receptors (Section 6.4), and effects on DAT and VMAT expression (Section 6.3). The multiple interplay of these actions easily provides us the opportunity for a verbose review.

4. AMPH actions on synaptic vesicles

4.1. Early studies on vesicular uptake

Given the very controversial role for synaptic vesicles in AMPH action, it is interesting that effects of these drugs on isolated vesicles have long been noted. The first secretory vesicles studied were, not surprisingly, the so-called chromaffin vesicles of the adrenal gland. The term

chromaffin was introduced in 1902 by Alfred Kohn (1867-1959) (Kohn, 1902) of the Prague German Medical Faculty, who later survived imprisonment in the Nazi's Theresienstadt (Terezin) concentration camp (Carmichael, 1989). He used the term to describe both the reaction of chromate salts to produce a brown color and the cells that reacted. In 1918, William Cramer showed that reaction with osmic acid vapor demonstrated "adrenalin granules" with the "appearances of fine coal dust scattered over the medulla" and suggested that adrenaline secretion to the blood was mediated by these organelles, effectively extending Eliot's secretory hypothesis, but now suggesting structures that might be involved. The above terms were combined by Fritiof Stif Sjostrand to coin the phrase chromaffin granules (Carmichael, 1989) although they are genuine secretory vesicles, and the terms chromaffin granules and chromaffin vesicles are interchangeable.

The first report that AMPH-like compounds redistribute catecholamines from isolated vesicles preceded the demonstration of catecholamine uptake by vesicles. In 1953, Hermann Blaschko with Arnold Welch and Nils-Åke Hillarp and coworkers independently published means to isolate chromaffin vesicles by centrifugation. In 1960-1962, HJ. Schümann and coworkers showed that tyramine, |3-phenethylamine, AMPH, METH, and ephedrine each induced the release of catecholamines, but not ATP, from suspended chromaffin vesicles (Schümann and Weigmann, 1960; Schümann and Philippu, 1961, 1962). They also showed that tyramine and |3-phenethylamine were accumulated into chromaffin vesicles stoichiometrically with catecholamine release. They concluded that AMPH (and similar compounds) "acts by displacement of the catechol amines without releasing equivalent amounts of ATP."

These results were confirmed by Carlsson and coworkers for a variety of amines, including ephedrine, mescaline, and prenylamine (Segontin: n-diphenylpropyl-amphetamine) **(16)** (Carlsson and Hillarp, 1961; Carlsson et al., 1963). They noted that, except for reserpine, prenylamine was the most potent amine inhibitor of vesicular catecholamine uptake. Moreover, prenylamine was far more efficacious than reserpine at promoting efflux from the vesicles, and at "higher [10 |J.M], but still very low concentrations it caused complete release of the granule amines," making it "the most active releasing agent known to date, apart from mercuric chloride." Uptake of norepinephrine into isolated small synaptic vesicles was later shown by Charles Rutledge and coworkers to be inhibited by AMPH and less potently by eight AMPH analogs (Knepper et al., 1988).

A closely related concept to the uptake of AMPH in secretory vesicles is that of the "false transmitter" (Kopin, 1968). In this scenario, a molecule other than the native biogenic amine transmitter is accumulated within a secretory vesicle and is then available for release during exocytic fusion. In the process of being loaded into secretory vesicles, the false transmitters promote non-exocytic efflux of the native catecholamines by displacing them from storage vesicles into the cytoplasm and ultimately into the extracellular milieu. Acutely, the false transmitters indirectly promote sympathomimetic activity whereas they diminish it over longer periods by reducing vesicular stores. The paradigmatic false transmitter is tyramine, and it is thought that dietary tyramine of microbial origin found in red wine, beer, cheese, and sausage promotes hypertension in patients taking MAO inhibitors by a false transmitter action.

One prediction stemming from the hypotheses above is that if drugs such as AMPH and tyramine redistribute dopamine from synaptic vesicles, the drug should decrease the amount of transmitter released per secretory vesicle fusion event, i.e., the "quantal size." This prediction became testable with the development of carbon fiber electrodes capable of recording quantal exocytosis by amperometry, initially (as usual) from adrenal chromaffin cells (Wightman et al., 1991). Using this technology, AMPH provided the first instance of a manipulation that affected the quantal size of catecholamine release, as amperometric recordings in PC 12 cells, an adrenal chromaffin cell-derived cell line, demonstrated that 10 |±M of AMPH for 10 min decreased quantal size by 50% (Sulzer et al., 1995).

Decreased quantal size by AMPH and other weak bases was later confirmed in chromaffin cells (Mundorf et al., 1999) and the giant dopamine neuron of Planorbis comeus. Using the latter system, Andrew Ewing and coworkers demonstrated the existence of two classes of dopamine vesicles that were differentially depleted by AMPH (Anderson et al., 1998): at low concentrations, AMPH preferentially depleted the large vesicles, while at higher concentrations, AMPH depleted small vesicles more than large vesicles. Interestingly, biphasic effects of amphetamines also occur in isolated chromaffin vesicles (Slotkin and Kirshner, 1971), possibly due to lipophilic diffusion vs. VMAT-mediated uptake, although the basis for this observation remains enigmatic.

4.2. Vesicular pH and catecholamine accumulation

AMPH is a lipophilic weak base with a pK of 9.9 (Mack and Bönisch, 1979) and is thus protonated in acidic organelles including catecholamine vesicles (Sulzer and Rayport, 1990): once charged, it is less membrane permeable and accumulates in the acidic structure.

Not surprisingly given the preceding discussion, chromaffin vesicles were the first subcellular organelle shown to acidify, as well as to use the resulting electrochemical gradient to provide the energy for accumulation of intravesicular transmitter. Following the elucidation of the vacuolar proton pump (known as the V-ATPase), again in chromaffin vesicles (Cidon and Nelson, 1983), it became clear that all components of the secretory system, including endosomes, lysosomes, and the Golgi apparatus, use this system to regulate internal acidity.

The acidic pH gradient in secretory vesicles provides the energy to accumulate transmitter against its concentration

gradient. Literature on vesicular uptake of catecholamines and its relationship to vesicular pH gradients is featured in an extensive review (Johnson, 1988). The vesicular monoamine transporters used to harness this energy to selectively accumulate catecholamines, histamine, and serotonin were initially cloned by Robert Edwards and coworkers from a chromaffin cell tumor line (PC12 cells) (Liu et al., 1992), and later termed the vesicular monoamine transporter 1 (VMAT1). Soon to follow was the description of a closely related gene expressed preferentially in the CNS now known as VMAT2 (Erickson et al., 1992; Liu et al., 1992).

There is extensive evidence that AMPH is a substrate for both VMATs. By measuring inhibition of serotonin uptake into CHO cells transfected to express either VMAT1 or VMAT2, Doris Peter et al. (1994) found a nearly 10-fold higher affinity for METH at VMAT2, with a preference for the S(+)-isomer. They also demonstrated competition for binding between METH and reserpine, suggesting they might bind to the same site on VMAT. George Uhl's laboratory similarly reported that AMPH displaced the VMAT2 blocker tetrabenazine (Gonzalez et al., 1994).

It should be noted that tetrabenazine and reserpine are thought to bind to different sites on VMAT (Schuldiner et al., 1993a), and that the means by which AMPH apparently displaces both ligands is still not understood. Nevertheless, the antagonism of VMAT ligand binding by AMPH supports the possibility of its transport.

In fibroblasts transfected to express either VMAT1 or VMAT2, Jeffrey Erickson et al. (1996) found that |3-phenethylamine, AMPH, and MDMA exhibited 10- to 20-fold higher affinity for VMAT2 than VMAT1, whereas fenfluramine had similar affinity for both transporters. For both VMATs, S(+)-AMPH was about 5-fold more potent than the R(—)-isomer. It should be noted that, as has also been true with the plasma membrane transporters, it is difficult to design experiments to prove that AMPH is an actual substrate, in part due to its lipophilicity and retention in acidic compartments due to its charge.

There are at least two non-exclusive hypotheses that may explain the mechanism by which AMPH redistributes vesicular monoamines to the cytosol, the weak base hypothesis and VMAT competition.

4.3. The weak base hypothesis

All sympathomimetics are weak bases with amine moieties that are capable of accepting protons with pk'"s in the range of ~8 to 10 (Sulzer and Rayport, 1990). Secretory vesicles are acidic; chromaffin vesicles, which are the best characterized, maintain a pH of 5.0-5.6, depending on conditions (Johnson, 1988; Pothos et al., 2002; Markov et al., submitted for publication) that as above (Section 4.2) provide the energy to accumulate monoamine transmitters. The final catecholamine concentration gradient at equilibrium is impressive; given sufficient synthesis (in dopamine neurons and chromaffin cells, following administration of the precursor ı-DOPA), vesicles can achieve levels that, if they were free in solution, would be close to a molar (Staal et al., 2004). As cytosolic catecholamine levels in chromaffin cells are ~10 |jıM (Mosharov et al., 2003), acidification provides the energy to maintain an accumulation of about 100,000-fold.

Whether used as drugs or not, weak base compounds that are sufficiently membrane permeable to enter secretory vesicles bind free protons, alkalinize the existing acidic pH gradient and thus decrease the energy that provides accumulation of neurotransmitter. The alkalinization of vesicle interiors by AMPH was initially demonstrated on isolated chromaffin vesicles and in organelles (likely a combination of several secretory organelles) of cultured midbrain dopamine neurons (Sulzer and Rayport, 1990) labeled with the weak base vital dye acridine orange to provide real time observation of vesicular pH gradients. The concentration required for AMPH to collapse 50% of the chromaffin vesicle proton gradient was ~50 |±M for isolated vesicles. The effect of AMPH on chromaffin vesicle pH gradients was neither stereo-specific nor blocked by reserpine, suggesting that much of its entry into the isolated vesicle preparation was due to lipophilic diffusion rather than via VMAT1.

Similar results were later reported for MDMA, fenfluramine, and parachloroamphetamine (PCA) **(17)** on isolated chromaffin granules (Rudnick and Wall, 1992; Schuldiner et al., 1993b). Those authors suggested that the effect of PCA on vesicles was solely due to effects on pH, whereas MDMA and fenfluramine exerted effects both by altering both pH gradients and vesicular transport.

The hypothesis of vesicular pH gradient collapse by AMPH has recently been confirmed in cultured chromaffin cells with improved estimates of pH using fluorescent ratiometric methods. This approach showed that 100 |±M METH at 30 min can almost completely collapse the pH gradient in chromaffin vesicles; surprisingly, longer-term METH (24 H) can lead to a rebound acidification with an accompanying delayed increase in quantal size (Markov et al., submitted for publication); the mechanism underlying this apparent compensation is unknown.

The weak base hypothesis provides a straightforward explanation for the alkalinization of vesicular pH gradients observed with AMPH, and at least a partial explanation of AMPH's decrease of quantal size (Section 4.1) and its increase of cytosolic dopamine (Section 3.2). Even compounds such as ammonium chloride and chloroquine, agents long used to disrupt pH gradients in the laboratory, release dopamine from cultured dopamine neurons (Sulzer et al., 1993) and intact striatum as measured by microdialysis (Sulzer et al., 1992) via reverse transport following vesicle alkalinization. To date, all compounds that collapse vesicular pH gradients, including the weak bases chloroquine and ammonium chloride (Mundorf et al., 1999; Pothos et al., 2002), and vesicular chloride channel blockers and the H^+-ATPase inhibitor bafilomycin (Pothos et al.,

2002), lower the number of catecholamine molecules in vesicles in situ, i.e., decrease quantal size (Sulzer and Pothos, 2000), presumably by redistribution to the extravesicular milieu following pH gradient collapse.

There are, however, phenomena that occur at vesicles that are not explained by this action. First, there is no straightforward relationship between effects on pH gradients and monoamine accumulation (Reith and Coffey, 1994). Second, the effect of pH gradient collapse on monoamine release from isolated vesicles is comparatively less efficient than that on monoamine uptake (Sulzer and Rayport, 1990); this contrasts with findings of more effective AMPH-mediated release than uptake blockade at plasma membrane transporters (Section 5). The lower efficiency of release with vesicles could be due to the presence of intravesicular monoamine binding sites, such as chromogranins and additional glycosaminoglycan groups, both soluble and resident in the vesicle membrane, that continue to bind catecholamines in alkalinized states (and decrease the genuine free concentration of transmitter in vesicles).

Several studies have tested the weak base hypothesis by comparing effects on vesicular pH and catecholamine redistribution. In isolated synaptic vesicles from whole rat brain, 3 pM AMPH depleted at least 70% of previously accumulated labeled dopamine, but only collapsed the proton gradient by 12% (Floor and Meng, 1996), although the level of alkalinization from higher levels of AMPH (~100 p,M) appeared to correlate with release. In chromaffin granule ghosts, AMPH inhibited dopamine uptake more effectively than expected from its collapse of pH (Reith et al., 1993). Another indication that alkalinization may not be sufficient to fully explain redistribution of vesicular dopamine is that bafilomycin, a proton pump inhibitor that is not a VMAT substrate, decreased the pH gradient 2-fold more than AMPH (Floor and Meng, 1996), but released dopamine at only half the rate. [It should be noted, nevertheless, that bafilomycin, like AMPH and control weak bases like chloroquine, is quite effective at decreasing quantal size from vesicles that are resident in neurons and secretory cells (Pothos et al., 2002).]

The arguments above should also be tempered by considering that the suspension buffer greatly affects the results in these experiments, particularly the chloride concentration, which provides a counterion that decreases the vesicular voltage gradient and allows a larger pH gradient (Johnson, 1988; Pothos et al., 2002). The Floor and Meng study, however, found a similar discrepancy between proton gradient collapse and DA release at both 8 and 90 mM chloride (Floor and Meng, 1996).

Another important consideration is the relationship between the proton gradient and vesicular monoamine accumulation is not linear, but rather

$$\log\frac{[A]_{in}}{[A]_{out}} = \frac{MP}{Z} + 2\Delta pH \quad \text{(Johnson, 1988)}$$

where [A] is the concentration of monoamine, MP is the voltage gradient, and Z is RTIF = 50 mV. If only ApH changes, one monoamine molecule would be redistributed for two protons. Assuming a typical MP = 80 mV, alkalinization of vesicular pH gradients from pH 5.6 to 5.9, a seemingly unimpressive change, would in theory lead to a loss of 75% of vesicular transmitter. Thus, an apparently small change in pH that may be difficult or impossible to measure, particularly in small vesicles, may greatly affect vesicular transmitter.

Another objection to the weak base hypothesis based on the relationship between the pH gradient and redistribution is that in wild-type CHO cells, a 2 order-of-magnitude higher level of METH was required to abolish acridine orange staining than to inhibit serotonin uptake in VMAT-transfected CHO lines (Peter et al., 1994). A caveat particular to that experiment, however, is that CHO cells do not contain synaptic vesicles, and the effects of METH were likely measured on other acidic organelles that accumulated serotonin, such as lysosomes.

Perhaps most damning to the completeness of the weak base action for explaining effects at vesicles is that the S(+)-AMPH stereoisomer is several fold more effective than the R(—)-isomer (Peter et al., 1994). As the S(+)-isomer exhibits preferential binding to the transporter (Peter et al., 1994; Erickson et al., 1996), these results endorse a complementary role for VMAT competition.

As an interesting addendum to the weak base effects of AMPH, rapid perfusion of the drug as well as non-psychostimulant weak bases induced quantal exocytosis from chromaffin cells via a weak base action (see Section 6.6).

4.4. VMAT competition

A recurring theme is that while amphetamines compete with reserpine binding to VMAT (Peter et al., 1994) and with ligands for plasma membrane transporters (Section 5), it is not straightforward to prove that they are actually transported substrates. If it is transported by VMAT, it is likely that AMPH translocation from the cytoplasm to lumen will promote the reverse transport of monoamines from the lumen since such ‘exchange diffusion’ is a general phenomenon of carrier proteins (Section 5.5). Regardless of whether AMPH is a VMAT substrate or merely binds without being transported, there must be a competition between monoamines and AMPH for the uptake site. While uptake blockade would not itself deplete vesicular transmitter, there is an ongoing leak of vesicular transmitter, particularly with synaptic vesicles (Floor et al., 1995; Pothos et al., 2000; Schonn et al., 2003). The combination of these effects would increase cytosolic monoamines providing that AMPH in binding VMAT does not act like cocaine at the plasma membrane transporter and block reverse transport to the cytosol. Such blockade appears unlikely as there is a leak of vesicular transmitter even with reserpine, which binds

strongly to the cytosolic face of the transporter: in a manner still not explained, reserpine apparently blocks uptake but not release through the VMAT. It should be noted that, while sheer conjecture, the release of monoamines by AMPH or reserpine could occur via a different vesicular transporter or channel.

Differential binding of AMPH and its derivatives to VMAT has been measured in isolated vesicles (Reith et al., 1993) and digitionin-permeabilized cells (Erickson et al., 1996), but competition for binding per se is not a simple parameter to measure since a vesicular/cytosolic pH gradient is also required for uptake and can be altered by the protocols used. This point was made explicit by Shimon Schuldiner et al. (1993b), who showed that genuine ApH dissipation can yield an artifactual apparent competitive inhibition in translocation assays. This would lead to an overestimation of the effects due to VMAT binding and an underestimate of effects due to weak base action.

Attempts have been made to integrate both hypotheses. For example, a study on isolated synaptic vesicles from whole brains and adrenal chromaffin vesicles (Reith et al., 1993) suggested that AMPH binding to VMAT is too weak to provide the underlying mechanism for vesicular monoamine uptake inhibition, although the authors agree that low concentrations of AMPH might inhibit uptake with little effect on pH.

In summary, there are numerous unanswered questions about the effects of AMPH on vesicles. A role for AMPH uptake competition with catecholamines can be inferred, particularly at low levels of AMPH, but remains to be clearly differentiated from the drug's weak base effects on transvesicular proton and voltage gradients, particularly for synaptic vesicles. The mechanism of how reverse transport occurs is unknown, and a very old issue of how reserpine can inhibit uptake but not halt reverse transport remains opaque. The intravesicular milieu including catecholamine and AMPH binding sites and vesicular ion channels, particularly the anion conductances of Cl^-, may play important roles that remain uncharacterized. The rebound acidification of vesicles may have a significant effect on neurotransmitter release, but this remains to be explored. As will shortly be apparent, many similar concerns pertain to effects of AMPH at the plasma membrane, even where study of many of these issues is easier to conduct.

5. Actions at plasma membrane transporters

5.1. Early studies of cellular monoamine uptake

While the sine qua non property of AMPH at monoamine transporters is the promotion of monoamine release via reverse transport, there are yet profound mysteries in understanding how this works. It is additionally clear that AMPH is an uptake blocker as well as a releaser, and differentiating between elevating extracellular monoamines by reverse transport or uptake blockade can be difficult. Of course, the many AMPH derivatives and different transporters maintain different combinatorial properties, an important topic beyond the range of this article.

Although a specific uptake system for epinephrine was theorized by T.R. Eliott around 1914 (Section 3.1), the actual identification of a specific transmitter uptake system occurred more than forty years later by Bernard Brodie and his fellow, F. Barbara Hughes, with many further insights provided by Julius Axelrod. Axelrod received a bachelor's degree in chemistry in 1933 and wrote many of his celebrated papers as a technician in Brodie's lab before entering graduate school and receiving a Ph.D. 21 years later (Axelrod, 2003). His co-discovery of neuronal norepinephrine uptake along with discovery of catechol-O-methyl transferase was acknowledged by the 1970 Nobel Prize in medicine.

In their initial reports, Hughes and Brodie examined serotonin and catecholamine uptake in guinea pig blood platelets (Hughes et al., 1958; Hughes and Brodie, 1959). Their argument for a specific uptake transport system was mostly based on inhibition of accumulation by reserpine; this was prior to the identification of reserpine with vesicle uptake sites (Section 3.1), and they assumed that reserpine inhibited the plasma membrane uptake site. They concluded that there was "an endergonic mechanism that rapidly extracts serotonin from the surrounding medium against a concentration gradient".

Similar findings were soon published for neuronal catecholamine uptake by two groups. Georg Hertting and Axelrod (1961) injected tritiated norepinephrine (provided by Seymour Kety's laboratory, which had recently developed the technology) into cats, comparing its presence in peripheral tissues following removal of superior cervical ganglia. During the same year, Hans Dengler et al. (1961) first demonstrated CNS uptake of norepinephrine by examining cortical brain slices. As had Barbara Hughes, they identified inhibition by reserpine but suggested that the uptake system worked to deliver catecholamines to "an intracellular pool distinct from the particulate sites," or in contemporary terms, to the cytosol rather than to vesicles.

Leslie Iversen introduced the terms "uptake 1" for cocaine-sensitive neuronal uptake of norepinephrine and "uptake 2" for the less avid, non-neuronal uptake, and these terms were widely adopted in the literature. Many years later, uptake 2 was shown to likely be due to the action of an organic cation transporters on astrocytes (Jonker and Schinkel, 2004).

Axelrod's group re-addressed the classical issue of how and which psychostimulants block reuptake and which induce release. They initially reported that cocaine blocked norepinephrine uptake into the nerves innervating heart, spleen, and adrenal gland (Whitby et al., 1960). While Bum and Rand had shown in 1958 that cocaine blocked what later was confirmed to be reverse transport (Section 3.1), to our knowledge, Whitby's study is the first to show that cocaine

blocks catecholamine uptake, which is now acknowledged to be its primary means of elevating extracellular catecholamine levels.

In a reformulation of Bum and Rand's earlier categories of directly and indirectly acting sympathomimetics, Axelrod divided drags that elevated norepinephrine levels in the blood into compounds that (a) prevent norepinephrine uptake, i.e., lower tissue norepinephrine levels when administered before the labeled norepinephrine, or (b) release norepinephrine, i.e., lower tissue norepinephrine when administered after labeled norepinephrine (Axelrod et al., 1961). The tricyclic antidepressants imipramine and chloropromazine were labeled uptake blockers, while reserpine, AMPH, and tyramine were considered releasers. While Axelrod confirmed Burn and Rand's prior hypothesis that AMPH releases catecholamine, he eventually reported that AMPH could block both uptake and release of labeled norepinephrine in the brain (Axelrod, 1971), underlining the reoccurring theme that both aspects at the transporter may be important.

Most of the neurotransmitter transporters were cloned over a very short period. Two families of plasma membrane neurotransmitter transporters were identified on the basis of sequence homology: one family includes the norepinephrine (Pacholczyk et al., 1991), dopamine (Kilty et al., 1991; Shimada et al., 1991; Usdin et al., 1991), gamma-aminobutyric acid (GABA) (Clark et al., 1992), and serotonin (5HT) (Blakely et al., 1991; Hoffman et al., 1991) transporters and the other includes transporters for glutamate (Pines et al., 1992; Storck et al., 1992; Tanaka, 1993; Arriza et al., 1994). The transfection of these transporters into cells provided new means to identify pharmacological effects on uptake and reverse transport.

5.2. How is catecholamine uptake powered?

Plasma membrane and vesicular neurotransmitter transporters are referred to as secondary active transporters or concentrative transporters because they utilize electrochemical energy derived from preexisting ion gradients, particularly sodium, and the transmembrane electrical potential. The coupling coefficients of the transporter for organic substrate and the pertinent ions determine the magnitude of the substrate concentration gradient at equilibrium. The monoamine plasma membrane transporters and vesicular transporters are thus analogous in depending on electrochemical gradients built by other pumps, but differ in that the plasma membrane transporters mostly rely on co-transport of driving co-substrate ions with substrates whereas vesicular carriers counter-transport monoamines and protons (Sections 4.1 and 4.2).

In principle, concentrative transporters must possess at least one conformation that prevents substrates from simply diffusing down their concentration gradient. This property is often labeled a gating mechanism. Literature on the traversal of a substrate or ion co-substrate binding site between internal and external faces often invokes an alternating access model (Jardetzky, 1966), meaning that binding sites for substrates and co-substrates are alternately exposed to extracellular and cytoplasmic environments via conformational changes in the transporter protein.

Studies by Leslie Iversen's laboratory and others indicated that catecholamine uptake by membrane transporters followed Michaelis-Menten type kinetics and required an ion gradient that would presumably act as a co-substrate, principally consisting of sodium (Iversen, 1963). The sodium concentration gradient could then be invoked as a means to provide energy for the accumulation of cytosolic catecholamine against its concentration gradient.

The precise stoichiometry of ion exchange for catecholamines has been the subject of many studies. Perhaps the most notable in neurons was of DAT activity in striatal synaptosomes, indicating that dopamine accumulation against a concentration gradient required the cotransport of 2 Na^+ and 1 Cl^- ions (Krueger, 1990), resulting in net import of two cations per transport cycle, and these values are now widely assumed under "physiological" conditions for DAT. The stoichiometry of ion gradients have nevertheless continued to be controversial, in part because this coupling may be altered under different conditions (Pill and Singer, 1999), and there may well be different values for the other catecholamine transporters. Isotopic studies on neurotransmitter transporter-expressing cells and membrane vesicles indicated a stoichiometry of one Na^+ and one Cl^- for each molecule of norepinephrine for NET (Galli et al., 1996), while mammalian SERT was proposed to be electroneutral due to additional counter ion flow (Rudnick, 1998). For all of the monoamine transporters, the role of Cl^- may be particularly variable, even between paralogs (species variants of a single transporter). The electrical gradient (i.e., membrane potential) also contributes to the driving force for substrate uptake as recently pointed out for DAT (Section 5.5), but this factor is also likely to vary between different transporters.

5.3. AMPH uptake

It might seem that AMPH ought to be subject to the same rales for accumulation as catecholamines, but this remains uncertain, and indeed, as is the case for VMAT (Section 4.4), it has historically been difficult to determine if AMPH is a genuine substrate of DAT and other plasma membrane transporters, i.e., whether it is transported. This is in part due to the drag's lipophilicity and accumulation as a weak base in lysosomes and other acidic intracellular organelles (Mack and Bőnisch, 1979). This is an important issue, as if AMPH is not a substrate, it may simply block uptake or even cause release via a channel-like mechanism (Section 5.5).

An initial case for specific uptake of AMPH by transporters was made in studies of stereospecificity, although it certainly is possible that binding has stereospecificity even if no transport occurs. A variety of studies in

the 1970s examined differential effects of AMPH stereoisomers on plasma membrane uptake transport of tritiated catecholamines by synaptosomes. In an initial study by Joseph Coyle and Solomon Snyder (1969), the S(+)-isomer preferentially blocked catecholamine uptake in cortical but not striatal synaptosomes. As there is more dopamine in the striatum and more norepinephrine in the cortex, it was suggested that the S(+)-isomer was more effective than the R(—)-isomer at the norepinephrine than the dopamine uptake system. Most follow-up studies, as reviewed by Kenneth Moore (1978), however, found the opposite response, with the S(+)-isomer more potent at blocking dopamine uptake by DAT, and less stereoselectivity for NET. Presently, selectivity for the S(+)-isomer by DAT is widely accepted.

While there was evidence of cocaine inhibition of AMPH uptake into heart tissue in the 1960s (Ross and Renyi, 1966), the classic demonstration of genuine AMPH uptake in neuronal-like cells did not occur until 1984 (Bönisch, 1984), mostly due to the aforementioned background uptake due to the drug's lipophilic nature. For instance, although Cho et al. (1975) demonstrated that the more hydrophilic metabolite 4-hydroxyamphetamine (23) could be accumulated by way of the dopamine carrier in rat striatal synaptosomes, they were unable to demonstrate temperature-dependent uptake of AMPH itself. H. Bönisch used PC12 cells, a cancer (phaeochromocytoma) cell line of rat chromaffin cells with neuronal characteristics (Greene and Tischler, 1976). This study finally confirmed that cellular accumulation of radiolabeled AMPH was indeed similar to that of labeled norepinephrine in that it required Na^+ and Cl^-, and was fully blocked by cocaine and desipramine. PC12 cells were later shown to endogenously express the NET but not the DAT (Lorang et al., 1994).

The first convincing data we are aware of that confirmed AMPH as a transporter substrate in true neurons were from careful experiments using low concentrations (5 nM) of radiolabeled AMPH and striatal synaptosomes by Robert Zaczek et al. (1991a). They showed that AMPH accumulation was saturable, ouabain-sensitive (ouabain blocks Na^+/K^+ exchange, and thus runs down the Na^+ gradient required for uptake) and temperature-dependent, consistent with active transport. They estimated an apparent kj_{TI} of 97 nM and V_{max} of 3.0 fmol/mg tissue/min for S(+)-AMPH uptake into striatal tissue. The DAT blockers GBR12909, methylphenidate, and cocaine, as well as METH, were potent inhibitors of AMPH accumulation while serotonin was relatively weak, presumably because striatal AMPH uptake relied on DAT, which is much more highly expressed in the striatum than SERT.

The same study also confirmed as discussed above that S(+)-AMPH was an 8-fold more potent inhibitor of the transport than R(—)-AMPH at blocking dopamine uptake (see also preferential binding of the S(+)-isomer at VMAT, Section 4.1, and at MAO, Section 6.1). Interestingly, the lab's accompanying study showed evidence that the AMPH once accumulated into the cytoplasm may not be free but rather bound to small soluble acidic peptides in the cytosol (Zaczek et al., 1991b).

More recently, whole cell electrophysiological recordings of the transporter-mediated currents have convincingly shown that AMPH is a genuine substrate for the transporters, as related in Section 5.4. Given that, it may be surprising that the ionic stoichiometry for AMPH uptake remains uncharacterized. It may be that binding and transport have different requirements. It appears that AMPH binds even in the absence of extracellular Na^+, whereas high affinity binding of cocaine analogs appears more strongly affected by Na^+ removal (Reith et al., 1980; Sonders et al., 1997; Li et al., 2002; Wang et al., 2003).

5.4. Differentiating uptake blockade from release

Very early work found that cocaine and other uptake blockers inhibit tyramine-induced increase of extracellular catecholamines (Tainter and Chang, 1927) (Section 3.1), and much later, a similar inhibition by cocaine was reported for AMPH-mediated release (Fischer and Cho, 1979; Liang and Rutledge, 1982; Parker and Cubeddu, 1988). Those findings, however, only demonstrated a role for plasma membrane transporters. Cocaine sitting at the entry site of the transporter should in principle block both uptake and reverse transport. While one might imagine that uptake blockade precedes reverse transport, until quite recently, available approaches were too slow to differentiate whether cocaine blocked AMPH action due to its inhibition of AMPH uptake as a substrate or its blockade of dopamine reverse transport (Heikkila et al., 1975a).

Thus, analogous to the problems related above in proving that AMPH was a transported substrate, it was difficult to prove directly that catecholamines could undergo reverse transport, and it was argued that transporters might be designed so as to not allow reversal. A real time demonstration of this function via DAT was finally provided from studies using the giant dopamine neuron of the pond snail Planorbis corneus. Dopamine was injected directly into the cytosol by a pipette and its efflux from the neuron measured by amperometric recording, thus avoiding the problem with concurrent uptake blockade. The resulting dopamine efflux was completely and reversibly blocked by the uptake inhibitor nomifensine, thus indicating that all of the detectable dopamine was released by reverse transport (Sulzer et al., 1995). Since the released dopamine was never taken up by the transporter, this further suggested that reverse transport occurred when an equilibrium across the transporter was disrupted. Similarly, when AMPH was directly injected into the cytosol, dopamine was released in a nomifensine-inhibitable manner even though AMPH had not been accumulated by the transporter. These findings indicated not only AMPH-induced reverse transport in the absence of uptake blockade, but also that uptake and reverse transport can be dissociated under some circumstances. (As

well as strongly suggesting a role for AMPH-mediated redistribution and subsequent release of synaptic vesicle dopamine.) Later studies of labeled NE flux in transfected cells confirmed that reverse transport and uptake blockade both occur, dependent in part on AMPH concentration (Pill et al., 1999).

A rapid method that can characterize compounds as uptake blockers or transported substrates is to measure the transporter-associated ion currents (Section 5.1) that accompany substrate uptake. This approach was first approached by recording a heterologous system of cloned DAT in the oocyte of the African clawed frog, Xenopus laevis (Sonders et al., 1997). Known substrates of DAT induce Na^+-dependent currents mediated by DAT, whereas non-substrate inhibitors of the transporter such as cocaine block these currents. Because AMPH elicited such "transport-associated currents," this electrophysiological approach thus clearly identified AMPH as a DAT substrate regardless of its ability to lipophilically traverse the membrane. This approach further indicated that the AMPH derivative methylphenidate (3), for instance, was not a substrate but rather a DAT blocker. Given Ritalin's widespread use as a pharmacotherapy for ADHD in children and adults and the neurotoxicity associated with the class of releasers, this might be considered welcome news (Section 2.3).

The extent of contributions of both reverse transport and uptake blockade to AMPH action in striatum were finally measured using rapid electrochemical recordings in brain striatal slice preparations and a "random walk/finite difference" analysis that incorporated Michaelis-Menten kinetics. (A tutorial on this approach is available at http://cumc.columbia.edu/dept/neurology/sulzer/download.html.) The results showed that AMPH's effect on dopamine overflow was due primarily to reverse transport but that uptake inhibition also contributed a substantial component of the total effect (Schmitz et al., 2001).

5.5. Models of how AMPH activates reverse transport

If neurotransmitter transporters had no gating mechanism they could not maintain a high level of cytosolic substrate. Their obvious ability to concentrate substrate could arise from either of two non-exclusive mechanisms: (1) the transporter could function asymmetrically, operating more efficiently for uptake, or (2) the prevailing gradient of pertinent ions, substrate, and membrane potential might determine substrate flux to favor uptake.

Current research suggests that both mechanisms are important. For instance, for the GABA transporter GAT1, GABA elicits "transport currents" (a surrogate measure of GABA flux) with clear asymmetries (Hilgemann and Lu, 1999). In contrast, some mutants of the bacterial lactose permease transporter affect both uptake and reverse transport equally (Abramson et al., 2003), while glycine transporters appear to transport substrate according to the imposed electrochemical gradient (Roux and Supplisson, 2000).

For reverse transport to be effective, one or both of these functions needs to be countervailed, and accordingly, there are at least two non-exclusive hypotheses that may explain the mechanism by which AMPH enhances reverse transport. We label these as:

(1) facilitated exchange diffusion: this implies a net asymmetric substrate flux due to intrinsic structural properties, and in its classical formulation, an upper limit of one-for-one molecular exchange of AMPH for catecholamine.
(2) channel-like transport modes: this implies a reliance on transmembrane gradients for net flux and an exchange ratio that can exceed one-for-one catecholamine exchange of AMPH.

As related below, there are also hypotheses that integrate both mechanisms.

5.5.1. Facilitated exchange diffusion

The most prominent model in the literature used to explain how AMPH induces monoamine release at plasma membrane uptake transporters is the facilitated exchange diffusion model. This is based on concepts introduced by Wilfred Stein and coworkers at Hebrew University to describe glucose transport (Stein, 1967). The model relies on a binding site for substrate that can crisscross the plasma membrane. To accumulate cellular glucose, the binding site would take up a molecule of glucose extracellularly and then translocate the molecule across the membrane to release the glucose in the cytosol. Reverse transport would occur when the binding site faces the cytosol after release of the substrate, where it could bind another molecule of cytosolic glucose and later release it extracellularly following its retransversal to the external site. An important aspect of this classical model is that the traversal of the binding site is driven by the substrate; thus, there would be a limit of no more than one molecule of glucose released from the cell for each molecule taken up.

An important facet of this model is the question of why there would be relatively little reverse transport of substrate, since as above, there would otherwise be no net cytosolic accumulation. In Stein's classical formulation of the model, transport in the reverse direction would be relatively infrequent due to sodium binding to the binding site, which would be much more frequent at the extracellular face and would favor a confirmation that preferentially elicits uptake. This idea was specifically extended to catecholamine transporters by Donald Bogdanski and Brodie, who suggested that sodium ions, present at higher levels on the extracellular surface, would favor an outward facing orientation (Bogdanski and Brodie, 1969). This hypothesis essentially partakes of both the idea of transporter asymmetry and that a transporter can be driven by transmembrane gradients. Thus, while there is still an upper limit of one molecule of glucose released for each molecule

taken up, uptake under normal sodium gradients would exceed efflux.

In its adaptation to explaining AMPH action (Paton, 1973), facilitated exchange diffusion states that AMPH-induced dopamine release resulted from translocation of AMPH as a substrate of DAT, thus increasing the probability that the DAT binding sites face the cytosol. Then dopamine, which is at higher concentration in the cytosol, could bind the internalized binding site, thereby increasing the rate of reverse transport of dopamine. A molecule of dopamine released by reverse transport would follow the uptake of an AMPH molecule, and in this model's classical form, there would be at most one molecule of dopamine released for each molecule of AMPH taken up. Since there is less intracellular sodium to immobilize the inward face, most of the reverse traversals of the binding sites would return empty. Thus, AMPH would work by increasing the rate of outward-to-inward traversals and increasing the opportunity for intracellular dopamine to bind and be carried outwards. Also by this model, any external substrate, including dopamine itself, would be predicted to stimulate counterflux of internal substrate molecules.

In 1969, Hans Thoenen et al. showed that tyramine-mediated norepinephrine release from cat iris required the presence of external sodium. This supported the idea that AMPH-like releasers must be accumulated by plasma membrane transporters to induce release. The first suggestion that facilitated exchange diffusion per se underlies sympathomimetic-mediated catecholamine release was by D.M. Paton in 1973 in a study that showed that tyramine and metaraminol (3',alpha-dihydroxyamphetamine) increased efflux of norepinephrine from rabbit atria in a manner inhibited by cocaine or the norepinephrine uptake blocker desipramine. While essentially a restatement of the old "cocaine paradox", and not substantially different than much earlier experiments by Bums and others (Section 2), Paton interpreted the results to indicate that "The ability of noradrenaline, metaraminol and tyramine to increase efflux is in keeping with an accelerative exchange diffusion process". The idea that the cotransport of sodium with a substrate, which would increase the affinity for substrate for the inward face, could favor reverse transport was suggested by Sammet and Graefe (1979). Thus, all of the ingredients for an AMPH facilitated exchange diffusion mechanism were in place by 1979.

It has not, however, been an easy task to prove the existence of classical facilitated exchange diffusion driven by AMPH. First, some of the presumed requirements for this model, such as the ability to saturate transport, temperature dependence, inhibition by uptake blockers, sensitivity to structural modification or stereospecificity, could equally well apply to other modes of release via the transporter that do not require a mobile carrier to traverse the membrane (in the case of the standard version of the model) or to have a binding site(s) with alternating access to either side (a more common version of the model at the present time); moreover, these same properties could be consistent with release by a channel-like mode.

One prediction that might specifically endorse facilitated exchange diffusion is that for compounds that elicit release via exchange, those that are better substrates for uptake should also be better releasers, i.e., a linear regression between the two properties. The first study we are aware of designed to test Paton's theory of facilitated exchange diffusion as a means of AMPH action was by Charles Rutledge and coworkers (Arnold et al., 1977), in which they showed that the S(+)-AMPH was a more potent releaser of dopamine and norepinephrine from chopped cortical brain tissue than its stereoisomer. They wrote that the temperature dependence and stereospecificity of AMPH-mediated release "suggest that a carrier-mediated, facilitated diffusion is involved in amphetamine-induced transport of norepinephrine and dopamine."

A more specific prediction of a linear relationship between uptake and release was tested by J.K. Fischer and Arthur Cho (1979), who reported a nearly linear relationship between the inhibition of dopamine uptake and release for both AMPH optical isomers, and three other sympathomimetics. For instance, S(+)-AMPH was about 3-fold more efficacious than the R(—)-isomer at both uptake and release. They concluded that while AMPH enhances cytoplasmic dopamine levels by "stimulation of granular release," i.e., redistribution of vesicular dopamine, and by MAO inhibition, there was no release of dopamine unless AMPH was taken up the transporter.

In contrast, a follow-up study by U. Trendelenburg and collaborators showed a non-linear relationship between uptake and release of various AMPH-like compounds (Langeloh et al., 1987). They compared the effects of a variety of sympathomimetics using rat vas deferens, tubes that transport sperm from the epididymis to the ejaculatory ducts and are surrounded by smooth muscle innervated by norepinephrinergic terminals. They did not confirm the predicted straightforward relationship between uptake and release reported by Fischer and Cho, but rather that some compounds, including AMPH, were substantially better releasers than substrates for uptake. The curve of release versus uptake was related to uptake to the 4th power, and they used that relationship to surmise that there might be four different contributors to efflux. They suggested that these were (in the absence of vesicular stores and MAO activity, which were pharmacologically blocked in that study): (1) facilitated exchange diffusion, (2) cotransport of sodium that would increase the affinity of catecholamines to the inward facing binding site, (3) cotransport of chloride, which would also increase affinity, and (4) competitive inhibition of catecholamine reuptake.

A study by Sarah Jones et al. (1999) to test facilitated exchange diffusion in the striatum found that dopamine displacement from vesicles by Ro4-1248 and reserpine-like compounds, which would be expected to increase cytosolic dopamine levels, did not cause dopamine efflux. This was

interpreted to mean that simply increasing the intracellular dopamine concentration was not enough to cause dopamine efflux and that actual uptake of AMPH by DAT was essential. Later results, however, contradicted the assumption that reserpine elevates cytosolic dopamine levels (Mosharov et al., 2003), as cytosolic catecholamines were rapidly metabolized by MAO to DOPAC and similar derivatives that are not DAT substrates (Section 6.1). Note also that a requirement of AMPH to elicit reverse transport can be consistent with a gated channel-like model.

An electrochemical study of norepinephrine reverse transport in transfected cells by Nianhang Chen and Joseph Justice (1998) used tyramine and cocaine to test predictions of facilitated exchange diffusion. After allowing the cells to accumulate dopamine and then challenging them with tyramine, they found that more tyramine was taken up than dopamine released. As they increased dopamine accumulation, tyramine-induced dopamine release became comparatively more efficient (but still less than one-for-one exchange). The authors concluded that "theoretically, with the intracellular dopamine approaching infinitely high concentration, the efflux rate of internal dopamine would equal the uptake rate of external m-tyramine," which would be consistent with facilitated exchange diffusion.

There are, however, conflicting results in the tradition of the Langeloh study indicating that uptake and release can be uncoupled. In the case of DAT, applying zinc (10 pM) to DAT-transfected cells stimulates efflux of intracellular [^{3}H]dopamine despite its concomitant inhibition of substrate uptake (Scholze et al., 2002; Meinild et al., 2004). This dissociation contravenes the expectation that efflux velocity principally depends on the rate of outward-to-inward facing binding sites. Recent efforts to produce concatemers of SERT and GABA transporters suggest that the quaternary arrangement of the proteins may regulate AMPH driven reverse transport (Seidel et al., 2005).

Despite the difficulty in proving classical facilitated exchange diffusion, there is also little reason to state that it does not occur. There are, nevertheless, instances of AMPH-driven reverse transport that facilitated exchange diffusion cannot explain. For instance, AMPH injected directly into giant dopamine neurons of the Planorbis corneus pond snail induces reverse transport of dopamine (Sulzer et al., 1995), although in that case, the AMPH was never transported by DAT. Similarly, membranophilic weak bases that are not DAT substrates, such as chloroquine and methylamine, collapse synaptic vesicle pH gradients and induce reverse transport that is halted by DAT blockers (Sulzer et al., 1993). Moreover, simply increasing intracellular sodium can drive dopamine efflux in the absence of extracellular AMPH (Khoshbouei et al., 2003), and release of synaptosomal dopamine in Na^+-free medium or after inhibition Na^+/K^+ exchange by ouabain is well established (Raiteri et al., 1979). Each of these examples points out that classical facilitated exchange diffusion cannot be sufficient to explain all of the release of catecholamines by AMPH.

5.5.2. Channel-like transporter modes

Direct evidence for channel-like release events mediated by AMPH is recent, as very rapid recording technology is required to observe such events. In the initial reports, patch clamp recordings of cell lines expressing catecholamine transporters displayed transient very large events that appeared to indicate ion channel-like mode of conduction of catecholamine (Galli et al., 1996, 1998). Such events resemble uncoupled ion conductances similar to those recorded in classical ion channels. These uncoupled ion conductances have been associated with DAT (Sonders et al., 1997), serotonin transporter (SERT) (Mager et al., 1994) and glutamate transporters (Wadiche et al., 1995; Otis and Jahr, 1998).

The existence of ion-channel like conductions of catecholamines themselves was confirmed by simultaneously measuring both large current and catecholamine release events by combining patch-clamp with amperometric recordings, which demonstrated that the channel-like activity of the NET transporter was temporally associated with transmitter flux (Galli et al., 1998). The authors thus concluded that the transmitter can cross the cell membrane through an aqueous NET pore.

AMPH-triggered channel-like dopamine release events mediated by DAT were recently recorded from cultures of both midbrain dopamine neurons and a DAT-transfected cell line (Kahlig et al., 2005). The channel-like events were surprisingly large, consisting of ~10,000 molecules released over at most a few milliseconds, startlingly similar to quantal release events measured from synaptic vesicle exocytosis from axons of the same neurons (Pothos et al., 1998; Staal et al., 2004); unlike quantal events, however, AMPH-triggered events were completely blocked by cocaine.

Using simultaneous patch-clamp and amperometric recordings as they had for NET, the investigators confirmed that AMPH-mediated reverse transport at DAT required the presence of intracellular sodium, but found that the number of released dopamine molecules per ion transported was far higher during the brief episodes of channel-like release than the much slower, lower level of release assumed to be due to exchange diffusion. They estimated that about 10% of AMPH-mediated dopamine released was due to the channel-like events; although due to the difficulty in measuring slow release with amperometry (Schmitz et al., 2001), the genuine contribution by channel-like events may be higher. More strikingly, extracellular AMPH increased the frequency of channel-like release events by ~8-fold, while extracellular dopamine had no effect on the frequency of the channel-like events. These results suggest an unexpected very different response by DAT to AMPH or dopamine, the basis of which is entirely unknown, although the authors speculate that it may have to do with additional

proteins associated with DAT that are regulated selectively by the neurotransmitter.

5.5.3. A grand unified theory? A role for second messengers?

As above, Stein's introduction of a facilitated exchange diffusion model underlying reverse transport already had a role for transmembrane gradients. Under most circumstances, its adaptation by Paton, Sammet and Graefe, and Fischer and Cho as an exchange mechanism of AMPH for cytosolic catecholamine via increasing outward-to-inward facing conformations is consistent with AMPH action, so long as a sufficient pool of cytosolic substrate is available. Nevertheless, the ability of AMPH, membranophilic weak bases, Na^+, or Zn^{2+} to release transmitter even when not taken up by DAT, as well as the recent demonstration of the existence of AMPH-induced channel-like release events mediated by DAT that release very large quantities of substrates via reverse flow through the transporter, indicates that a more detailed explanation of the structural changes that occur during reverse transport and its regulation by the various transmembrane gradients is required.

An important aspect may be due to AMPH's ability to stimulate the cotransport of ions, as originally suggested by Thonen's study (Thoenen et al., 1969), and the consequent rearrangement of electrochemical gradients (Sitte et al., 1998; Khoshbouei et al., 2003). The recent finding that AMPH's increase of intracellular sodium concentration may be sufficient to stimulate AMPH-induced DAT-mediated dopamine efflux (Khoshbouei et al., 2003) supports the notion that this action is essential for its stimulation of dopamine efflux (Pill and Singer, 1999; Pill et al., 2004).

How might this work at the level of transporter structure? A "unified" model that encompasses roles for both substrate and ion gradients, as well as channel-like properties in which multiple substrates could be transported without a shuttling binding site, could be consistent with reports of the structure of the first secondary active transporters to be crystallized, the plasma membrane E. coli bacterial lactose permease and glycerol-3-phosphatase transporters (Abramson et al., 2003; Huang et al., 2003) as well as a putative prokaryotic glutamate transporter from the thermophilic archaean Pyrococcus horikoshii (Yemool et al., 2004); unfortunately, only a single conformation is currently available for each transporter, and so these hypotheses are quite conjectural. These first two structures were suggested to be consistent with a rocker-switch alternating access model, in which tilting of transporter domains would close a "cavity" on the cytoplasmic side (the inward-facing conformation) to thereby open a new "cavity" on the extracellular (or for bacteria, periplasmic) side (the outward-facing conformation) (Huang et al., 2003; Locher et al., 2003). The structure of the putative glutamate transporter (Yemool et al., 2004) suggested an alternate model in which coordinated "flipper" movements of two sets of two hairpin loops might allow alternating access by occluding the transporter, perhaps in a series of three conformations. As pointed out in a short review (Kavanaugh, 2004), this differs from the rocker-switch model in that it provides an "open state" on either side of the membrane without an "open channel".

Both models would provide variations on the classical facilitated exchange diffusion, but without the requirement for a binding site that traverses the membrane, and so they can seemingly provide for channel-like events. There would then be no limit of a one-for-one molecule exchange of cytosolic substrate in reverse transport. Rather, reverse transport may follow electrochemical/ionic/substrate gradients that act to lower the energy barrier for favorable conformations that favor translocation. If in the case of AMPH reverse transport, this would be provided by AMPH acting as a substrate itself or its accompanying cotransported ion(s). This would be neither classical facilitated exchange diffusion with a one-to-one molecule limit nor a channel that simply follows a substrate gradient once it has been opened, but rather a gating mechanism that exhibits aspects of both classical hypotheses.

New work (Khoshbouei et al., 2004) seems consistent with such a unified model, invoking an asymmetric transporter that suggests a conformational property of DAT that typically favors influx over efflux, but with net flux controlled by transmembrane substrate gradients, and also introduces a potential second messenger system that may provide the basis for these observations. Evidence for an asymmetric conformation of the transporter is that when the first 22 amino acids of the N-terminal region of DAT were truncated or if serine residues in that region were mutated to alanine (which cannot be phosphorylated), AMPH-mediated dopamine efflux was selectively reduced by ~80%. As mutating the same serine residues to aspartate in order to simulate serine phosphorylation resulted in normal AMPH-mediated efflux, the authors suggested that phosphorylation of some serines may shift DAT from a "reluctant" state to a "willing" state that favors AMPH-induced dopamine efflux, without affecting normal dopamine uptake.

Consistent with a regulation of transport direction by second messenger systems, numerous putative phosphorylation sites for various protein kinases were identified in the intracellular domains of the DAT (Giros et al., 1996; Granås et al., 2003). In addition, multiple protein kinases have been shown to regulate DAT function (Melikian and Buckley, 1999; Carvelli et al., 2002; Granås et al., 2003; Loder and Melikian, 2003). Interestingly, AMPH has been shown to increase striatal particulate protein kinase C (PKC) activity (Giambalvo, 1992). Accordingly, PKC activation has been shown to stimulate DAT-mediated release of dopamine (Giambalvo, 1992; Kantor and Gnegy, 1998), and AMPH-mediated DA release is inhibited by PKC inhibitors and by downregulation of PKC (Kantor and Gnegy, 1998), whereas [^{3}H]DA uptake is unaffected by these manipulations. Moreover, phosphorylation of NET by protein kinase C is reported to be required for AMPH-mediated efflux (Kantor et al., 2001).

It may be that the combination of AMPH and phosphorylation produces a DAT conformation that favors dopamine efflux. This would also be a new non-classical variant of facilitated exchange diffusion (if one could still call it that), as there would no longer be a requirement of a one-for-one exchange of dopamine and AMPH molecules. It may be that some such DAT states underlie the channel-like release events.

In summary, despite very strong confirmation of the notion that AMPH elicits reverse transport, there are many unanswered questions on how it does so. How do changes in ion and substrate concentrations and gradients affect facilitated exchange diffusion or channel-like properties? How important is the putative channel-like mode for the efflux of neurotransmitter, and why is it selectively activated by AMPH but not dopamine? How do second messenger systems and associated proteins alter these states? What are the relevant structural conformations, and are the neurotransmitter and ion substrate binding and translocation sites identical on the cytosolic and extracellular sides? While the classical transport models have contributed important ideas, is it time to develop new names for the revised models that avoid confusing semantic traps? Or, as in Friedrich Nietchze's version of the wisdom of Zarathustra, shall we researchers "muddy their water that it may seem deep" ?

6. Other mechanisms that affect extracellular catecholamine levels

There are many other properties of AMPH that contribute to drug effects, but we have attempted to limit this review to those that affect extracellular catecholamines. Here we review additional mechanisms that may be important in regulating extracellular catecholamines, in particular for AMPH and METH. For some mechanisms, such as MAO inhibition, the effects are clearly germane, while for others a genuine role is yet to be elucidated.

6.1. Amphetamines as monoamine oxidase inhibitors

A means by which amphetamines can influence neurotransmitter release apart from effects on plasma membrane and vesicular transporters is to increase the quantity of biogenic amine available for release by inhibiting MAOs — key mediators of amine catabolism located on the outer mitochondrial membrane. AMPH derivatives are competitive inhibitors of these enzymes, and recognition of this fact far predates the isolation of the enzymes. Hermann Blaschko et al. (1937) used a range of substrates and inhibitors to characterize amine oxidizing activities in various tissue, animal, and plant preparations. They demonstrated that many isopropylamines, including AMPH and ephedrine, are not themselves subject to degradation by the enzyme preparations but can nevertheless block the consumption of oxygen otherwise used to oxidize substrates including epinephrine and tyramine. It is worth noting Merton Sandler's opinion that these studies of amine oxidation by Blaschko "were probably responsible for triggering off the psychopharmacology revolution" (Sandler, 2004) as they presaged the introduction of MAO inhibitors for treatment of depression: such use of the drugs was initiated in the early 1950s after mood elevation was noted following administration of iproniazid to tuberculosis patients.

Mann and Quastel (1940) reported an analogous finding on MAO inhibition motivated by an entirely different rationale. AMPH has long been used for treating narcolepsy (Section 2.3). The authors were interested in testing a hypothesis that narcolepsy resulted from amine-induced inhibition of brain metabolism by determining whether AMPH prevented a gradual decline in respiration observed in tissue treated with amine oxidase substrates such as tyramine. Their findings suggested that aldehydes formed by amine oxidation depressed respiration and that AMPH inhibited their formation, albeit with modest potency.

In 1971, Leitz and Stefano published a study of norepinephrine metabolism and release in which they drew the bold conclusion that the principal actions of AMPH, tyramine, and metaraminol on neurotransmitter release is mediated through MAO inhibition. Using perfused rat hearts preloaded with [^{3}H]norepinephrine, they observed that AMPH and tyramine increased [^{3}H]norepinephrine and concomitantly decreased its tritiated deaminated metabolites in the perfusate. To bolster their argument, the authors also showed that tranylcypromine **(18),** an irreversible MAO inhibitor which is an AMPH analog, had effects comparable to those of AMPH on releasing tritiated species. These results, however, were likely the conflated outcome of at least three distinct mechanisms: (1) effects on MAO activity; (2) efflux through plasma membrane NET; and (3) the efflux of the acidic and glycol metabolites. Furthermore, tranylcypromine is a substrate of the plasma membrane transporters and therefore could promote efflux, while the timescale tested may have been too short to observe irreversible effects of tranylcypromine on MAO. Rutledge (1970) made a concerted attempt to disentangle such factors using rabbit brain slices and synaptosomal preparations and reached a different conclusion: that AMPH prevents deamination most potently by acting at plasma membrane transporters to block reuptake and facilitate efflux of amines from the cytoplasm which thus escape MAO, although AMPH additionally acts to directly inhibit MAO.

The development of relatively specific irreversible inhibitors for MAO A (clorgyline) and B (deprenyl) **(13)** subtypes in the 1960s (Knoll et al., 1965; Johnston, 1968; Knoll and Magyar, 1972) facilitated the study of inhibitor selectivity. AMPH and many of its analogs show 5-fold or greater selectivity for MAO A over MAO B (Mantle et al., 1976; Robinson, 1985; Scorzaetal., 1997), and most behave as reversible and competitive non-substrate inhibitors. The affinity of AMPH for MAO A is typically in the range of 10 |±M for the S(+)-enantiomer, a few fold higher in affinity

than its optical antipode (Mantle et al., 1976; Robinson, 1985). Thus, the stereoselectivity of MAO A for S(+)-AMPH is similar to those of DAT and VMAT. METH, which shows comparable affinity and stereoselectivity to AMPH at the plasma membrane transporters, in contrast, displays about five-fold lower affinity (~100 p-M) than (±)-AMPH for MAO A and essentially no stereoselectivity (Robinson, 1985). The affinities of AMPH and METH for MAOs compare unfavorably with their potency at NET and DAT, which are usually reported in the 100-200 nM range (references in Sections 5.1 and 5.2).

Considering the poor affinity and stereoselectivity of MAO B for AMPH and METH, it is curious that the prototypic substrate for MAO B is p-phenethylamine, which differs from AMPH only in lacking the a-methyl group, and that the first and still widely-used irreversible selective MAO B inhibitor is (—)-deprenyl, an A-2-propynyl analog of METH. [Note that deprenyl is metabolized to AMPH and METH (Reynolds et al., 1978).] AMPH-like structures have served as a useful scaffold for the development of much more potent competitive and irreversible inhibitors including amiflamine (Ask et al., 1989) **(19),** 4-methoxy-AMPH (PMA) **(20),** 4-methylthio-AMPH (Scorza et al., 1997) **(21),** pheniprazine **(22),** and tranylcypromine **(18).**

In summary, while there is a relatively low potency of AMPH at MAO, one cannot discount a contribution from MAO inhibition to its spectrum of physiological activity. Notably, while neither reserpine nor MAO inhibitors alone increase cytosolic dopamine, intracellular patch electrochemistry shows that the combination of the two causes a profound increase similar to that of AMPH itself (Mosharov et al., 2003). As AMPH is a substrate of the plasma membrane transporters, it could be concentrated in the cytoplasm to a level that inhibits MAOs on the outer mitochondrial membranes. Numerous factors already mentioned make it difficult to determine the cytoplasmic AMPH concentrations attained after physiological doses of the drug, including the ability of AMPH to lipophilically diffuse across membranes and its uptake into vesicles by VMAT and due to its accumulation as a weak base in acidic compartments.

Finally, although little data is available, a major metabolite of AMPH (Cho and Kumagai, 1994) 4-OH-AMPH (a-methyl-p-tyramine) (23) is likely to serve as a competitive inhibitor of MAO A. As a substrate of the plasma membrane transporters (Cho et al., 1975, 1977) with lower membrane permeability than AMPH, the 4-OH-AMPH metabolite might be more likely than AMPH to reach high levels in the cytoplasm to serve as an effective MAO inhibitor.

6.2. Promotion of dopamine synthesis

AMPH has long been noted to enhance dopamine synthesis, and this provides an important role in its action under some conditions, e.g., following reserpine treatment (Section 3.2). This effect can be so profound, as measured in dopamine neuronal culture, that AMPH releases far more dopamine in the culture medium than was initially present in the entire culture prior to AMPH exposure (Larsen et al., 2002).

There were initial indications that AMPH enhanced dopamine synthesis in in vivo experiments (Costa et al., 1972), but a confirmation that this was due to tyrosine hydroxylase activity awaited measurement of the conversion of radiolabeled tyrosine to dopamine in rat striatal synaptosomes (Kuczenski, 1975), where AMPH at concentrations up to 15 |±M enhanced dopamine synthesis by as much as 70%, while higher levels sharply decreased conversion, reaching control levels of activity at ~500 |j iM. This enhancement of tyrosine hydroxylase requires extracellular calcium (Fung and Uretsky, 1982).

The mechanism by which AMPH enhances tyrosine hydroxylase activity is unknown. While calcium-dependent second messenger phosphorylation of serine residues on tyrosine hydroxylase can regulate enzymatic activity (Griffiths and Marley, 2001), the relationship to AMPH remains obscure. An alternate possibility is that AMPH may substitute for dopamine at its tyrosine hydroxylase-binding site from where it exerts feedback inhibition (Goldstein and Greene, 1987; Haycock, 1993). Similarly the mechanism of AMPH's tyrosine hydroxylase inhibition at higher levels remains unknown, although it has long been suggested to be due to feedback inhibition from increased levels of cytosolic dopamine (Harris et al., 1975) (Section 6.1).

6.3. Effects on DAT and VMAT trafficking

This hypothesis that AMPH acutely regulates DAT cell surface expression originated from a study by Annette Fleckenstein and coworkers in which a single, high dose of AMPH administered to rats decreased DAT function 1 h later (Fleckenstein et al., 1997). Further work confirmed that dopamine regulates transporter cell surface expression. For example, in Xenopus laevis oocytes expressing DAT, application of dopamine reduced DAT cell surface expression as measured by binding by the DAT ligand [^{3}H]WIN 35,428 (Gulley et al., 2002) and similar dopamine regulation of DAT membrane expression was observed in rat striatal synaptosomes (Chi and Reith, 2003).

Like dopamine, AMPH acutely reduced cell surface expression of human DAT (hDAT) in cell lines, leading to a concomitant loss of DAT activity (Saunders et al., 2000). Kahlig et al. (2004) using confocal imaging and electrophysiology, demonstrated that AMPH application causes hDAT to redistribute from the plasma membrane as an active carrier. Sorkina et al. (2003) extended these observations by showing that AMPH-induced intracellular accumulation of hDAT on endosomal vesicles.

In addition to mediating hDAT cell surface redistribution, AMPH and its derivatives appear to regulate VMAT2 function. In particular, a single METH injection rapidly (1 h) and reversibly decreased dopamine uptake in a vesicle-enriched fraction obtained from rat striatum (Brown et al.,

2000, 2002) [for review, see Fleckenstein and Hanson, 2003]. The METH action was likely largely associated with the dopaminergic system, as the destruction of the serotonergic projections did not alter the effects on vesicular dopamine uptake. It is possible that the response to AMPH is indirect and that the apparent inhibition of VMAT2 is due to D2 autoreceptor activation following dopamine release (Brown et al., 2002). If VMAT2 is indeed redistributed to endosomes or other compartments, it will be important to determine the signaling and mechanics involved.

6.4. Trace amine receptors and other potential receptors for AMPH

As discussed, there has long been speculation that endogenous AMPH-like biogenic amines, prominently including p-phenethylamine, act as endogenous amphetamines (Section 2.2). There has been a resurgence in interest in this topic due to the discovery of a family of 15 mammalian G protein-coupled trace amine receptors (Borowsky et al., 2001; Bunzow et al., 2001). The rat trace amine receptor, rTARl, which has been the most thoroughly characterized of these receptors (Bunzow et al., 2001), acts to stimulate cAMP production; additional downstream second messenger responses are likely to be involved. Little is now known about the physiological actions of these receptors, and there is as yet no data indicating that they are involved in AMPH's role in enhancing extracellular catecholamines.

rTARl is activated by a broad array of amines, including P-phenethylamine and tyramine. AMPH and METH are both potent activators, with nearly identical responses to both isomers. MDMA and hydroxylated AMPH derivatives are likewise effective, while fenfluramine has no action. Interestingly, the meta-O-methyl derivatives of the catecholamines, namely 3-methoxytyramine, normetanephrine, and metanephrine, are more efficacious activators of rTARl than their respective precursors dopamine, NE, and epinephrine.

There are other receptors that may be activated by AMPH. AMPH binds to a-2 adrenergic receptors (Ritz and Kuhar, 1989), and while these are presynaptic receptors, we are unaware of a demonstration that they are located on dopamine terminals. It is nevertheless possible that this action underlies effects of AMPH on anxiety and other effects of central norepinephrine neurons, where the presynaptic receptor is found, and it is interesting that the anxiogenic and psychotomimetic drug yohimbine (from the West African tree Corynanthe yohimbe), is an a-2 blocker.

It should be noted that some AMPH analogs, prominently including "hallucinogenic" derivatives directly activate serotonin receptors and moreover that some are more specific for SERT than DAT (e.g., fenfluramine). Many derivatives have been developed by medicinal chemists through extensive studies on structure-function relationships and are beyond the scope of this review, but are discussed in excellent reviews by others (Nichols, 1994; Schmidt, 1994).

One study claims that AMPH releases catecholamines by activating nicotinic acetylcholine receptors (Liu et al., 2003), primarily based on measuring Ca^{2+} transients in chromaffin cells (see also Section 6.6). In apparent contrast, single channel electrophysiological studies indicate that AMPH (Spitzmaul et al., 1999) and ephedrine (Bouzat, 1996) act as nicotinic receptor channel blockers. It will be interesting to see how this issue develops and whether there is any pharmacological significance.

6.5. Depolarization at the DAT

As detailed above (Section 6.4), catecholamine transporters transport Na^+ and Cl^- ions. Susan Ingram et al. (2002) reported that DAT activation by AMPH can induce sufficient excitatory current to potentiate neuronal firing. Surprisingly, this excitation follows the inward flux of Cl^-, which normally hyperpolarizes these neurons. If borne out, the finding could explain reports of AMPH-induced neuronal excitation (Shi et al., 2000) and exocytic catecholamine release (Pierce and Kalivas, 1997; Darracq et al., 2001). It may be that relatively low exposures to AMPH stimulate dopamine neuronal firing or enhance the releasable vesicular pool, although we are not aware of direct evidence at this time beyond Ingram's study to support these possibilities. Rather, experiments with striatal slices demonstrate that AMPH-induced dopamine release inhibits dopamine release by activating presynaptic terminal D2 autoreceptors (Schmitz et al., 2001), and there is a significant literature on D2 autoreceptor inhibition of firing.

6.6. Intracellular calcium triggered via weak base effects

Mark Wightman and coworkers reported that rapid perfusion of AMPH as well as non-psychostimulant weak bases induced quantal exocytosis from chromaffin cells via a weak base action (Mundorf et al., 1999), as the accumulation of intravesicular calcium by chromaffin vesicles can may be indirectly dependent on H^+ gradients (Haigh and Phillips, 1993). The level of calcium redistributed from the vesicles by AMPH may be sufficient to induce vesicular exocytosis, and this could underlie a portion of AMPH action in secretory cells under some conditions. AMPH has not yet been observed to induce exocytosis via effects on cytosolic calcium levels from vesicles in central neurons, and from the data discussed in Sections 2-5, there remains little evidence suggesting a substantial direct effect on exocytosis in central acute AMPH-mediated catecholamine release, although this has by no means been ruled out. It should, however, be pointed out that a prominent indirect effect of AMPH is to activate presynaptic D2 or adrenergic receptors that inhibit subsequent evoked vesicular catecholamine release (Schmitz et al., 2001).

1. **Epilogue**

Despite the importance of self-administered drugs of reward and addiction, a thorough understanding of the mechanisms of action of some, including ethanol and AMPH, continues to elude us. Perhaps our deepest impression from perusing over a century of literature on AMPH is that while new technologies allow some long-standing hypotheses to be revised, refined, or discarded, variations on the earliest questions on mechanisms of action continue to re-arise. We hope that the historical approach we have adopted here underlines the profound insights gleaned from relatively restricted experimental tools by the talented pioneers of this research.

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