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NEUROPSYCHOPHARMACOLOGY OF WORMS AND FLIES

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Pharmacologic agents often biochemically interact with multiple receptor or channel proteins, and induce multiple changes in cellular physiology and signal transduction. Thus, identifying the biologically relevant targets and effectors of a given neuroactive substance can be a challenging problem. This chapter describes how genetic analysis in simple model organisms, primarily worms or flies, has been used to identify molecules that mediate drug responses in the nervous system.

Essentially all the studies described here rely on the same general strategy. The drug of interest is tested for its ability to affect worm or fly behavior. Once a behavioral response is defined for wild-type animals, it is then used as a behavioral assay to identify mutant worms/flies that exhibit abnormal drug responses and thus define genes whose products are involved in the drug's mechanism of action. Once these genes are identified and cloned, human homologues can be identified based on sequence similarity, and tested for involvement in human drug responses. This sort of approach has a number of potential advantages. For one, phenotype-driven genetic screens essentially make no prior assumptions about the types of molecules involved in the process being studied; any gene that is not essential for life and affects the behavioral response to a drug is in principle equally likely to be identified in a mutant hunt. Thus, this approach is well suited for identifying previously unknown receptors or signal transduction molecules that participate in drug responses. Furthermore, modern molecular genetics provides the ability to manipulate specific gene products in an intact animal, often in a cell-type–specific manner. By making it possible to assess a particular protein function within the context of an intact nervous system, this approach can provide a most compelling demonstration of *in vivo* function.

Among organisms with nervous systems, two are particularly amenable to genetic analysis: the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*. These organisms share a number of advantages that make them especially well suited for classic and molecular genetics. For example, both have short generation times (2 weeks for *Drosophila*, 3 days for *C. elegans*), can be maintained easily and in large numbers in the laboratory, and are amenable to germline transformation. In addition, detailed genetic maps of both organisms are available, and the genome sequences of both organisms are now virtually complete. Although both organisms contain relatively simple nervous systems, they differ significantly in scale and level of characterization. The *C. elegans* nervous system consists of exactly 302 neurons, whose precise position, cell lineage, and anatomic connectivity are known (1–3). Consequently, it is possible to identify the roles of specific neurons and muscle cells in behavior using techniques such as single-cell laser ablation, and to thereby understand in a precise manner how the action of a particular gene product in a defined set of neurons influences the whole animal's behavior (4). *C. elegans* is particularly suitable for genetic analysis of basic intracellular processes in neurons because the worm's nervous system is nearly dispensable for growth in the laboratory. Thus, even mutants with defects in basic neuronal functions such as neurotransmitter release are often viable and fertile (5). The *Drosophila* nervous system is somewhat more complex, and contains approximately 10^5 neurons. Consequently, it is somewhat less well characterized at the cellular level than the *C. elegans* nervous system; however, the increased behavioral complexity afforded by this bigger nervous system also makes it perhaps better suited for investigating more complex forms of behavior and learning (6).

STUDIES OF DRUG MECHANISMS IN MODEL ORGANISMS

Genetic pharmacology has historically been a powerful approach for neurobiological studies in *C. elegans* and *Drosophila*. Many studies of drug-resistant flies or worms have made use of pesticides or antihelminthic drugs that target

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the insect or nematode nervous system. For example, screens for *C. elegans* mutants resistant to the pesticide (and cholinesterase inhibitor) aldicarb have been used with notable success to identify genes involved in synaptic function; molecules first studied in this way include the vesicular acetylcholine transporter and the synaptic proteins UNC-13 and UNC-18 (7,8). Likewise, studies of *C. elegans* mutants resistant to the anthelminthic ivermectin have provided insight into the functions of the invertebrate-specific family of glutamate-gated chloride channels (9). More recently, attention has turned to the possibility of using genetic pharmacology to study the mechanisms of action for psychotropic drugs, including therapeutic agents and drugs of abuse. The following sections describe some examples of drugs whose mechanism of action has been studied in worms and/or flies, and the information that these studies have provided so far.

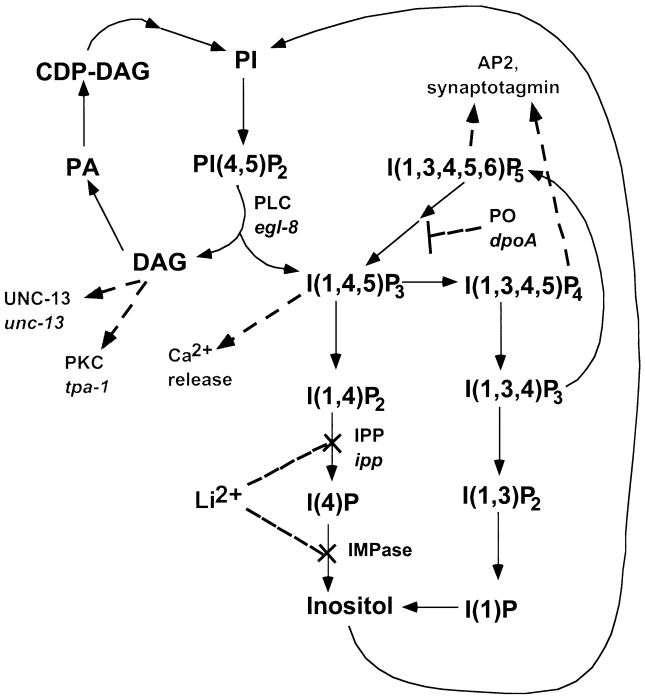

FIGURE 21.1. The phosphoinositide signaling pathway.

Therapeutic Agents

Lithium

Lithium salts are widely used for the treatment of bipolar affective disorder (manic-depressive illness). Lithium remains among the most effective treatments for acute mania, and it is also an effective mood-stabilizing agent for the prevention of both manic and depressive episodes. However, lithium has a number of side effects; for example, it is a known teratogen in vertebrate embryos, and can mimic the action of insulin in inducing synthesis of glycogen (9a). However, although lithium has been shown to affect a number of molecular and cellular processes in neurons and other cells, the mechanisms through which it exerts its therapeutic effects on mood are not well understood.

One of the most prevalent theories for lithium's mechanism of action, first proposed by Berridge et al. (10), is the inositol depletion hypothesis. According to this model, the critical functional consequence of lithium treatment is to reduce the intracellular concentrations of inositol, a key component of the phosphoinositide signal transduction cycle that mediates the effects of many neuromodulators, including serotonin (11). Lithium ions are uncompetitive inhibitors of both inositol monophosphatase (IMPase), the enzyme that catalyzes the conversion of inositol monophosphates (IMPs) to inositol, and inositol polyphosphatase (IPP), the enzyme that converts inositol 1,4-bisphosphate to inositol 4-monophosphate (Fig. 21.1). Inositol is required for the generation of phosphatidyl 4,5-inositol bisphosphate (PIP_2), whose cleavage by phospholipase C yields the calcium mobilizing agent inositol 1,4,5-trisphosphate (IP_3) and the protein kinase C activator diacylglycerol (DAG). Since both of these phosphoinositide-derived second messengers are critical signal transduction molecules that mediate the effects of diverse neurotransmitters and neuromodulators, a severe depletion of intracellular inositol would be expected to dramatically alter neuronal function. Thus, it has been proposed that lithium exerts its psychoactive effects by depleting intracellular inositol pools and thereby attenuating phosphoinositide signaling in neurons and other cells. However, experiments in rats suggest that while clinically effective concentrations of lithium are sufficient to inhibit IMP activity in the brain, they result in only a modest decrease in inositol levels (12). Thus, it is not clear that inositol depletion can account for the psychotropic effects of lithium.

Recent genetic studies using simple eukaryotes has provided two plausible alternative hypotheses for lithium's mechanism of action. Interestingly, many of the key genetic findings on lithium response mechanisms have come from studies of a unicellular eukaryote that lacks a nervous system altogether, the slime mold *Dictyostelium discoideum*. Despite its considerable evolutionary divergence from the metazoa, many of the signal transduction mechanisms in *Dictyostelium* show remarkable conservation with those in human neurons. *Dictyostelium* usually exists as a free-living amoeba; however, during times of nutrient deprivation, these amoebae aggregate into a multicellular mass, or slug, which then develops into a fruiting body consisting of differentiated stalk and spore cells. Lithium has two effects on *Dictyostelium* development (13). At high concentrations, lithium blocks the aggregation of amoebae. In contrast, low concentrations of lithium permit aggregation, but block spore cell differentiation, causing cells that normally would form the spore head to instead form stalk cells. This latter effect of

lithium on spore differentiation is mimicked by a mutation in the gene *gskA* (14), which encodes a homologue of the signaling molecule glucogen synthase kinase 3 (GSK-3). GSK-3 molecules are conserved signaling molecules originally identified as negative regulators of glycogen synthesis, and subsequently implicated in the regulation of gene expression and cell movement. Since lithium's effects on both *Dictyostelium* development and glycogen synthesis were identical to those caused by inhibition of GSK-3, Klein and Melton (15) investigated whether lithium might affect GSK-3 signaling. They subsequently demonstrated that vertebrate GSK-3 is directly inhibited by lithium in *Xenopus* oocytes, and that GSK-3, but not IMPase, is responsible for the teratogenic effects of lithium on the embryo. Thus, at least some of the side effects of lithium, such as its teratogenic and insulin-mimetic effects, are almost certainly phosphoinositide-independent and instead mediated through the GSK-3 pathway. Because GSK-3 molecules are abundant in the brain, it is also possible that this pathway might also mediate some of lithium's therapeutic effects on mood.

However, other studies in both *Dictyostelium* and *Drosophila* support a link between the phosphoinositide pathway and the mood-altering effects of lithium. One such study concerned the mechanism of lithium's aggregation-inhibiting action in *Dictyostelium*, an effect that is independent of the *gskA* gene. A number of genes required for this high-concentration response to lithium were identified in genetic screens. One of these genes, *dpoA*, was shown to encode a proline oligopeptidase (PO), an enzyme involved in the degradation of bioactive peptides (16). Interestingly, *dpoA* appeared to act via the phosphoinositide signaling pathway, since both mutations in *dpoA* and treatment with PO inhibitors elevated the levels of intracellular IP_3, but had no effect on GSK-3 activity. The elevation of IP_3 in *dpoA* mutants was a consequence of increased dephosphorylation of IP_5 (inositol 1, 3, 4, 5, 6-pentaphosphate), an alternate source of IP_3 utilized by both *Dictyostelium* and animal cells. Thus, inhibition of PO compensated for the decrease in PIP_2 levels induced by lithium by activating an alternative pathway for the production of IP_3 (and by extension inositol). Interestingly, abnormalities in PO activity have been observed in patients with both bipolar and unipolar depression (17,18). Thus, these results in *Dictyostelium* raise the possibility that PO may be linked to depression and mania through its effect on inositol signaling, and that lithium's efficacy in the treatment of depression may result from its ability to exert compensatory effects elsewhere in the inositol pathway.

However, the mechanism by which lithium-induced changes in the inositol pathway affect neuronal function may not involve inositiol depletion per se. This conclusion rests in part on a study of mutant flies defective in the enzyme IPP, a lithium-sensitive enzyme in the inositol pathway involved in the conversion of IP_3 to inositol (19). ipp mutants were shown to be completely defective in the IPP activity, since they were unable to degrade $I(1,4)P_2$, the IPP substrate. However, contrary to the prediction of the inositol depletion model, the phosphoinositol signaling pathway (which is necessary for *Drosophila* phototransduction) remained fully functional in photoreceptor neurons of the ipp mutant. Similar effects were seen when photoreceptor neurons were treated with lithium; IPP activity was inhibited, yet the inositol-dependent phototransduction cascade was still functional. Thus, neither genetic nor pharmacologic inhibition of IPP resulted in a depletion of inositol pools sufficient to interfere with the phosphoinositide signaling cascade. The ability to maintain high levels of inositol in the absence of IPP was apparently due to an alternate pathway involving synthesis and dephosphorylation of inositol 1,3,4,5-tetrakisphosphate (Fig. 21.1). However, although ipp mutations and lithium treatment did not affect phosphoinositide signal transduction, they had unexpected and dramatic effects on synaptic function. Specifically, in ipp mutant and lithium-treated wild-type photoreceptor neurons, the probability of vesicular release was greatly increased, and affected neurons were unable to maintain a synaptic response to a prolonged tetanic stimulus. A variety of molecules involved in synaptic fusion and vesicular traffic, including synaptotagmin and adaptor protein 2 (AP2), are regulated through specific physical interactions with inositol polyphosphates (e.g., IP_4, IP_5, and IP_6) (20). Thus, the effects of lithium on neuronal function in *Drosophila* as well as in humans may stem not from defects in inositol signaling per se, but from defects in synaptic function and plasticity due to alterations in inositol polyphosphate pools.

In summary, lithium provides a good example of the power of genetic neuropsychopharmacology in simple model systems. Studies in *Dictyostelium* were instrumental in identifying the GSK-3 pathway as a possible mediator of lithium's deleterious side effects, and have also provided insight into a possible link between neuroactive peptides and depression. Work in *Drosophila* has provided an important lead into discovering how lithium's effects on phosphoinositide signaling affect neuronal function. Future studies in both organisms have the potential to provide further insight into lithium's mechanism of action, in particular to address more precisely how lithium-induced changes in inositol lipid content alter synaptic transmission and plasticity in neurons.

Fluoxetine and Other Antidepressants

Another group of drugs that have been the subject of research in simple eukaryotes are those used in the treatment of unipolar depression. Such drugs include the monoamine oxidase (MAO) inhibitors, the tricyclic antidepressants (e.g., imipramine and clomipramine), and the selective serotonin reuptake inhibitors (SSRIs; e.g., fluoxetine). A common property of many of these molecules is their ability

to potentiate serotoninergic neurotransmission, either by interfering with reuptake of serotonin from the synapse (tricyclics and SSRIs) or by blocking enzymatic degradation of serotonin (MAO inhibitors). Thus, the therapeutic actions of all of these molecules are usually explained in terms of a model for depression known as the serotonin hypothesis. According to this model in its simplest form, levels of serotoninergic neurotransmission in the forebrain are a key determinant of mood, with high activity leading to euphoria and low activity to dysphoria. Thus the chronic dysphoria experienced by depressed patients could be a consequence of chronically low serotoninergic transmission, which could be compensated for by interfering with serotonin degradation. This serotonin hypothesis, or variations thereof, represents the most widely accepted explanation for antidepressant action (21,22).

However, the serotonin hypothesis, at least in its simplest form, fails to account for a number of observations about antidepressants. For one, a direct correlation between the level of serotoninergic transmission and mood has not been demonstrated; normal individuals treated with serotonin reuptake blockers do not typically experience euphoria, nor does dietary serotonin depletion induce depression in individuals not already prone to depression (23). Moreover, the mood-altering effects of serotonin reuptake blockers in depressed patients occur on a different time scale from their effects on serotoninergic transmission; whereas SSRIs and most tricyclics elevate synaptic serotonin levels within hours, their effects on mood are not apparent for 2 to 6 weeks. Finally, a number of effective antidepressants appear to function independently from serotonin, including selective norepinephrine reuptake inhibitors (SNRIs) such as desipramine, MK869, which antagonizes substance P receptors, and bupropion, whose target is unknown (24,25). Because of these observations, many current models hypothesize that SSRIs are effective against depression not because of their acute effects on serotoninergic transmission, but because of long-term adaptive changes in monoamine neurotransmission that arise from chronic inhibition of serotonin reuptake (21). An appealing feature of this type of model is that long-term activation of different direct targets by different classes of antidepressants (the serotonin transporter by SSRIs, other targets by atypical antidepressants) could in principle lead to a common set of adaptive responses in the brain. Alternatively, it is possible that antidepressants might act, at least in part, at serotonin-independent direct targets.

Studies in *C. elegans* have provided insight into potential serotonin-dependent and -independent activities of antidepressants. Nearly all antidepressants have at least two clear effects on *C. elegans* behavior: stimulation of egg laying and hypercontraction of muscles in the nose. Whereas the stimulation of egg laying by antidepressants is primarily due to potentiation of serotoninergic transmission (see below), the effect of antidepressants on the nose muscles appears to be independent of serotonin, since serotonin itself does not cause nose contraction, whereas antidepressants still contract the noses of serotonin-deficient mutants. Mutations conferring resistance to the induction of nose contraction by fluoxetine have been identified in seven genes, designated *Nrf* genes, for *n*ose *r*esistant to *f*luoxetine (26). All the *Nrf* mutations are recessive and confer resistance to several chemically disparate antidepressants in addition to fluoxetine; thus, the products of the *Nrf* genes might potentially represent common, serotonin-independent antidepressant targets. So far, two *Nrf* genes have been cloned, *nrf-6* and *ndg-4*. These two genes define the first members of a novel gene family, and encode predicted multipass integral membrane proteins that are expressed in the nasal epidermis and the intestine. *nrf-6* and *ndg-4* have been shown to be defective in the transport of yolk proteins across the intestinal membrane, suggesting that NRF-6 and NDG-4 may be components of a complex that transports molecules across epithelial membranes. Based on this result, it is reasonable to suppose that the fluoxetine resistance of *nrf-6* and *ndg-4* mutants might reflect a defect in drug uptake rather than the absence of a functional drug target in the neuromuscular system. However, while NRF-6 and NDG-4 (and by extension their yet unidentified vertebrate homologues) may not represent antidepressant targets per se, they might represent molecules that function in transport of antidepressants across the blood–brain barrier.

Another *C. elegans* molecule that clearly represents a serotonin-independent antidepressant target is encoded by the gene *egl-2*. *egl-2* was originally defined by the dominant gain of function mutations that impaired the activity of the vulval muscles (which mediate egg laying) and enteric muscles (which mediate defecation) (27,28). Both of these defects in muscle activation could be relieved by treatment with the tricyclic antidepressant imipramine, though not by serotonin or fluoxetine. Thus, imipramine appeared to act through a serotonin-independent target to suppress the *egl-2* muscle activation phenotype (29). The nature of this target was revealed when *egl-2* was cloned and shown to encode a potassium channel homologous to the *Drosophila* ether-a-go-go (eag) channel (30). Studies on EGL-2 channels expressed in *Xenopus* oocytes demonstrated that the imipramine-suppressible dominant alleles of *egl-2* encoded mutant channels that opened inappropriately at low voltages. Remarkably, imipramine was shown to function as a specific antagonist of both the *EGL-2* channel and its mammalian homologue MEAG. Thus, this class of calcium channels appears to represent a conserved target of tricyclic antidepressants in both worms and humans. Interestingly, an important side effect of tricyclic antidepressants is a type of cardiac arrhythmia called long QT syndrome, a disorder which has also been linked to mutations in potassium channel genes (29a,29b). Thus, the blockade of eag-related potassium channels by tricyclics provides a likely explanation for this clinically important side effect of tricyclics.

Studies in *C. elegans* may also provide insight into the serotonin-dependent mechanisms of antidepressant action.

The ability of antidepressants (other than tricyclics) to stimulate egg laying in *C. elegans* depends on their ability to potentiate serotoninergic neurotransmission (29), and can be mimicked by exogenous serotonin itself (31). Serotonin is released from egg-laying motor neurons called HSNs (27), and appears to function as a neuromodulator that modifies the functional state of the egg-laying muscles to potentiate contraction (32). Serotonin also inhibits locomotion, apparently by inhibiting neurotransmitter release from excitatory motor neurons (32,33). The signal transduction mechanisms that mediate both of these actions of serotonin have been analyzed genetically, and in both cases the phospholipase C (PLC) homologue *egl-8* is required for serotonin response. In the egg-laying muscles, the effects of PLC appear to be mediated through the protein kinase C homologue tpa-1, whereas in the motor neurons the most important mediator appears to be the diacylglycerol-binding synaptic protein UNC-13. The involvement of the phosphoinositide signaling pathway in serotonin signal transduction in both the egg-laying muscles and the motor neurons of *C. elegans* has an interesting parallel in mammals, since a number of mammalian serotonin receptor subtypes also signal through activation of PLC.

The apparent conservation between the signaling pathways mediating serotonin response in *C. elegans* and humans raises the possibility that the long-term effects of elevated serotoninergic transmission might also be accessible to genetic analysis in *C. elegans*. As noted previously, the alleviation of depression by serotonin-potentiating antidepressants is thought to involve adaptive signaling pathways that are activated by prolonged elevation of serotoninergic neurotransmission. In *C. elegans*, prolonged exposure to serotonin has been shown to lead to adaptive down-regulation of egg-laying behavior and recovery from serotonin-induced paralysis (34). Genes encoding possible components of serotonin adaptation pathways have been identified on the basis of serotonin hypersensitive or adaptation-defective phenotypes (35); however, at present little is known about how these or other genes affect long-term responses to serotonin. Future analysis of serotonin adaptation genes may provide insight into the molecular mechanisms underlying long-term responses to elevated serotonin transmission that may be important for the therapeutic action of antidepressants.

Volatile Anesthetics

A variety of volatile molecules, including diethyl ether, halothane, and isoflurane, are capable of inducing general anesthesia, a behavioral state involving loss of consciousness, analgesia, amnesia, and loss of motor activity. Although these agents have been widely used in surgery for over a century, their mechanism of action remains poorly understood. General anesthesia appears to result from defects in synaptic transmission rather than axonal firing; however, it is not clear whether anesthesia results from potentiation of inhibitory synapses, inhibition of excitatory synapses, or both. The potency of a given volatile anesthetic shows a very strong correlation to its lipid solubility; this observation, known as the Meyer–Overton rule, has led to the hypothesis that volatile anesthetics act by disrupting hydrophobic interactions between proteins and/or lipids in neurons. However, the biologically relevant targets for volatile anesthetics have not been conclusively identified. In principle, this problem appears ideally suited to attack by a phenotype-driven genetic approach; by identifying mutants that are resistant or hypersensitive to anesthetics and cloning and sequencing the mutant genes, it should be possible to identify anesthetic targets that are essential for anesthesia *in vivo*. In fact, such screens have been conducted in both *Drosophila* and *C. elegans*, and a variety of genes affecting sensitivity have been identified (36). At present, none of the *Drosophila* anesthetic response genes have been cloned; thus, molecular information about their gene products is not available. However, the recent cloning of several *C. elegans* genes with quantitatively large effects on anesthetic sensitivity raises the possibility that they might define conserved molecular targets important for anesthetic action.

C. elegans has two distinct responses to volatile anesthetics. At lower concentrations (similar to the alveolar concentrations used in human anesthesia), volatile anesthetics rapidly induce abnormalities in the pattern of locomotion (37). Although this effect is behaviorally quite dissimilar from anesthesia, it is similar to the effect of many mutations that affect synaptic transmission in *C. elegans*. In fact, treatment with volatile anesthetics confers resistance to the behavioral effects of cholinesterase inhibitors (38), a hallmark of defective neurotransmitter release (7). Thus, at these concentrations, volatile anesthetics appear to act presynaptically to interfere with synaptic transmission in *C. elegans*. A number of mutants with altered sensitivity to these low-concentration effects of volatile anesthetics have been identified. Potentially the most informative with respect to anesthetic mechanisms contain mutations in genes encoding components of the SNARE complex, the presynaptic machinery that mediates synaptic vesicle fusion. Recessive mutations in at least three SNARE genes, *unc-64* [encoding *C. elegans* syntaxin (39)], *snb-1* [encoding VAMP/synaptobrevin (40)], and *ric-4* (encoding SNAP-25), confer significant hypersensitivity on the effects of both halothane and isoflurane on coordinated movement. Furthermore, a novel mutation in *unc-64*, which affects a spice receptor site and consequently leads to the production of truncated syntaxin peptides, confers strong resistance to the effects of volatile anesthetics on both coordinated movement and cholinesterase sensitivity (38). These results suggest that volatile anesthetics interfere with synaptic transmission through direct interaction with one or more members of the SNARE complex.

At approximately 10-fold higher concentrations, volatile anesthetics induce reversible paralysis in *C. elegans*, a behav-

ioral effect qualitatively reminiscent of anesthesia. Interestingly, none of the synaptic mutations affecting the low-concentration effects on coordinated movements affect this high-concentration paralytic response. However, a different, nonoverlapping group of genes has been identified that confers resistance or hypersensitivity to paralysis by anesthetics in *C. elegans*. Several of these genes have been cloned, including *unc-1*, which encodes a homologue of stomatin (41), and *unc-8*, which encodes a subunit of the degenerin/ENaC family of passive sodium channels (42,43). Both *unc-1* and *unc-8* are expressed in neurons, and both genes can be mutated to confer either resistance or hypersensitivity to halothane (44). Allele-specific genetic interactions between *unc-1*, *unc-8*, and the yet uncloned *unc-79* and *unc-80* genes suggest that their products may physically interact in a multimeric channel complex specifically involved in anesthetic responses. Since stomatin has been shown to function as a negative regulator of cation channels in erythrocytes, a reasonable hypothesis is that UNC-1/stomatin may modulate influx through UNC-8 degenerin channels in neurons that respond to anesthetics. Homologues of both stomatins and ENaC channels have been identified in mammals, and are known to be expressed in the central nervous system; thus, in principle stomatin-regulated ENaC channels could also affect anesthetic responses in humans.

In summary, there are two distinct sets of genes that affect responses to volatile anesthetics in *C. elegans*, which affect different behavioral responses to different concentrations of anesthetics. At present, it is not clear which of the two (or whether both) might encode homologues of biologically relevant human anesthetic targets. Although the genes involved in synaptic function alter anesthetic responses at clinically relevant concentrations, the behavioral responses they affect are qualitatively quite different from general anesthesia. Conversely, although the stomatin/degenerin genes affect a paralytic response that closely resembles anesthesia, the response also has a relatively long time delay and occurs at concentrations well above those clinically relevant in humans. Given the effective drug concentrations for these two behavioral responses, it is possible that the synaptic genes might encode targets relevant to anesthesia, while the stomatin/degenerin genes might encode targets relevant for side effects of anesthetics. Alternatively, it is possible that genes affecting high-concentration anesthetic responses do define molecules involved in anesthesia, especially since the nematode cuticle is relatively impermeant and presents a significant barrier for the entry of many drugs. Since well-defined mammalian homologues exist for both classes of anesthetic response genes, it should be possible in the future to examine these issues directly in mammalian systems.

Drugs of Abuse

Ethanol

Unlike many neuroactive substances, ethanol is not believed to have a single molecular target in neurons; rather, a number of receptors and channels, including the *N*-methyl-D-aspartate (NMDA), serotonin, and γ-aminobutyric acid (GABA) receptors and various voltage-gated ion channels, appear to be modulated by the presence of ethanol (45). Very little information exists concerning the relative importance of each of these putative direct targets for the psychoactive effects of ethanol; however, a variety of experiments in cultured cells suggest that a critical short-term effect of ethanol is to enhance receptor-mediated synthesis of the second messenger 3′,5′-cyclic adenosine monophosphate (cAMP). Conversely, long-term ethanol exposure appears to decrease intracellular cAMP levels. Both the acute and chronic effects of ethanol have also been linked to changes in dopaminergic neurotransmission (46). In particular, ethanol has been shown to promote release of dopamine in the mesolimbic pathways of the brain, in particular the so-called reward pathway synapses between the ventral tegmental area (VTA) and the nucleus accumbens (NAc). At present, the *in vivo* significance of these findings with respect to the psychoactive effects of ethanol in mammals remains to be determined. Moreover, although sensitivity to both the acute and chronic effects of ethanol are clearly affected by genetic factors, the nature of the genes affecting human ethanol sensitivity are not known.

Recent work in *Drosophila* has provided support for both the dopamine and cAMP hypotheses of ethanol action. Ethanol vapor has a number of effects on *Drosophila* behavior, including hyperactivity, disorientation, uncoordination, and ultimately immobilization. Using an instrument called an inebriometer (47), lines of mutant flies have been identified that exhibit abnormal sensitivity to volatilized ethanol. Among the mutants showing significant hypersensitivity to ethanol were those containing a mutation in the learning gene amnesiac, which encodes a homologue of the mammalian pituitary adenylyl cyclase activating peptide (PACAP) (48,49). Consistent with the implications of this homology, the effects of amnesiac on ethanol response appeared to involve the adenylyl cyclase pathway, since the adenylyl cyclase activator foskolin blocks the ethanol sensitivity associated with amnesiac loss-of-function mutations. Moreover, several other loss-of-function mutations affecting cAMP pathway components, including the adenylyl cyclase gene rutabaga and the cAMP-dependent protein kinase gene *DCO*, also conferred ethanol sensitivity. Although one might suppose based on these results that the response to ethanol is simply a function of the level of cAMP signaling in the relevant neuronal targets (with increased ethanol response corresponding to low cAMP signaling), a variety of data are inconsistent with this simple model. For example, genetic or pharmacologic activation of the cAMP pathway does not lead to ethanol resistance. Nonetheless, these genetic data provide the first conclusive link between the activity of the cAMP pathway and the behavioral effects of ethanol in an intact organism; the precise nature of that link remains to be determined, but should be accessible to further genetic analysis.

Some of the behavioral effects of ethanol on *Drosophila* have also been shown to be dependent on dopamine (50). Ethanol has varying effects on fly locomotion depending on the duration of exposure. During the first 7 to 10 minutes of ethanol treatment, animals become hyperactive and move at a greatly increased rate; subsequently, they become increasingly uncoordinated and eventually become completely immobile. When flies are depleted of dopamine through ingestion of a tyrosine hydroxylase inhibitor, they become significantly less susceptible to this stimulation of motor activity by ethanol. However, these dopamine-depleted flies exhibited no abnormalities in their sensitivities to ethanol-induced uncoordination or immobilization. Thus, the stimulation of motor activity by ethanol may involve ethanol-induced enhancement of dopaminergic transmission in brain areas controlling locomotion, whereas the other behavioral effects of ethanol are likely to involve other neurotransmitter systems.

The genetic analysis of ethanol response mechanisms in *Drosophila* is still in its early stages. However, it is already clear that mutants with altered responses to ethanol can be identified in straightforward genetic screens, and at least in some cases analyzed in the context of well-defined neuronal signaling cascades. Perhaps the greatest promise for future studies is the possibility that novel ethanol response genes, possibly including the direct molecular targets of ethanol, can be identified in ethanol-resistant or ethanol-hypersensitive screens.

Nicotine

Tobacco has been implicated in more deaths than any other addictive substance (51), yet the biochemical basis for compulsive tobacco use remains poorly understood. The substance most responsible for the addictive properties of tobacco is nicotine, a potent stimulant and cholinergic agonist. Long-term exposure to nicotine is known to cause adaptive changes in the activity and number of nicotinic receptors in the brain, which are thought to be important for nicotine addiction (52). For example, nicotinic receptors exist in multiple functional states, some of which are relatively refractory to channel opening though they retain affinity for agonists. Chronic exposure to nicotine or other agonists results in an increased fraction of receptors adopting the lower activity states, leading to an attenuation of the overall nicotine response (53). Long-term nicotine treatment also causes a long-lasting functional inactivation of some nicotinic receptors (54), which has a slower time course and is much longer lasting than the rapid, receptor-intrinsic desensitization induced by acute agonist exposure. Depending on the receptor and cell type, long-term nicotine treatment can also either increase or decrease the number of nicotinic receptors on the cell surface, effects that appear to be mediated at the level of protein turnover (55,56). The cellular pathways that promote these changes are not well understood; for example, little is known about the cellular pathways that regulate receptor turnover, or the molecular mechanisms that regulate the switching between different nicotinic acetylcholine receptor (nAChR) states.

Genetic analysis in *C. elegans* may provide insight into the mechanisms underlying long-term responses to nicotine. Both acute and chronic nicotine treatment have striking effects on the behavior of *C. elegans*, including hypercontraction of body wall muscles, stimulation of egg laying, and increased pharyngeal pumping. The effects of nicotine on the body and egg-laying muscles are mediated through a nicotinic receptor known as the levamisole receptor (57, 58). The antihelminthic drug [and ganglionic nAChR agonist (59)] levamisole is a potent agonist of this receptor; like nicotine, levamisole causes body muscle hypercontraction and (at high doses) spastic paralysis. Although the levamisole receptor is found on nematode muscle, its pharmacologic profile generally resembles that of ganglionic nicotinic receptors of vertebrates. By screening for levamisole-resistant mutants, it has been possible to identify genes affecting the function of the levamisole receptor (60). Mutations conferring strong resistance to levamisole have been identified in six genes. Three of these genes, *unc-38*, *unc-29*, and *lev-1*, encode nicotinic receptor subunits (61,62). The UNC-38 protein is most similar to the insect α-like subunits ALS and SAD (49% amino acid identity); among vertebrate receptor subunits, the closest similarity is to neuronal α subunits (61). UNC-29 and LEV-1 are closely related proteins whose closest homologues in vertebrates are neuronal non-α subunits (approximately 55% sequence similarity). Three additional genes conferring strong levamisole resistance, *unc-50*, *unc-74*, and *unc-63*, have not been cloned, but have been shown to be required for assembly of a functional levamisole receptor as assayed *in vitro* (63). In addition to conferring resistance to levamisole (and other nicotinic agonists), mutations in these genes cause defects in the coordination of body movement (60). Mutations in three additional genes (*lev-8*, *lev-9*, and *lev-10*) confer weaker resistance to levamisole, do not cause defects in locomotion, and have no detectable effect on the biochemical properties of the receptor as assayed *in vitro* (60,63). Thus, the proteins encoded by these genes have been hypothesized to regulate the activity of the receptor indirectly.

Long treatments with nicotine and other nicotinic receptor agonists lead to adaptation (57). Animals treated with exogenous nicotine initially hypercontract to the point of spastic paralysis; however, after several hours in the presence of nicotine, they recover their ability to move and regain much of their body length. In some *C. elegans* strains (for example, strains with weakly crippled nAChRs), long-term nicotine treatment eventually leads to almost complete inactivation of the response to nicotine. Moreover, when nicotine-adapted animals are removed from nicotine, their locomotive behavior becomes uncoordinated and resembles that of mutants with strong defects in the levamisole receptor (i.e. an *unc-29* or *unc-38* null mutant). Thus, long treatments with nicotine cause nicotine dependence in addition

to nicotine tolerance in the *C. elegans* body muscle. Long-term nicotine treatment also down-regulates levamisole receptors in the egg-laying muscles. Overnight treatment with nicotine leads to an almost complete attenuation of levamisole sensitivity with respect to egg laying, and this attenuation of levamisole response persists for up to 24 hours after removal from nicotine. This loss of levamisole responsiveness is accompanied by a corresponding decrease in the abundance of UNC-29–containing receptors in the vulval muscles, an effect that may be mediated at the level of protein turnover (64). Interestingly, the nicotine-dependent decrease in UNC-29 receptor abundance requires the activity of TPA-1, a vulval muscle-expressed PKC isoform. Since UNC-29 and other nicotinic receptor subunits contain consensus sequences for PKC phosphorylation, this raises the possibility that direct phosphorylation of nicotinic receptors might represent a signal for increased turnover. In the future, it should be possible to test this hypothesis, as well as identify other genes required for long-term responses to nicotine in *C. elegans*.

Another set of genes, the weak levamisole-resistance genes *lev-8* and *lev-9*, appear to represent positive regulators of nicotinic receptor activity. Mutations in these genes confer partial resistance to levamisole and nicotine with respect to body muscle contraction and strong resistance with respect to egg laying (65). However, *lev-8* and *lev-9* mutations do not affect the assembly of levamisole-binding nicotinic receptors as assayed *in vitro* (58), and the abundance of UNC-29 receptors in the vulval muscles is not significantly reduced by mutations in these genes (65). *lev-8* and *lev-9* may therefore encode regulatory proteins that stimulate the activity of nicotinic receptors *in vivo*, but are not subunits or essential accessory proteins. In principle, the inhibition of the *lev-8* or *lev-9* gene products might represent a plausible mechanism for functional inactivation of nicotinic receptors. Once *lev-8* and *lev-9* are cloned, it will be interesting to determine whether mammalian homologues exist for these molecules, and if so, whether they are involved in regulating the functional activity of nicotinic receptors in human neurons.

Cocaine

Cocaine is a potent psychostimulant, and among the most widespread addictive drugs of abuse. The psychoactive effects of cocaine are thought to result largely from its ability to potentiate aminergic neurotransmission in the limbic pathways of the brain. Cocaine inhibits the reuptake transporters for dopamine, serotonin, and norepinephrine, which leads to accumulation of monoamine transmitters at the synapse. The dopaminergic synapses of the nucleus accumbens are thought to be particularly important for cocaine addiction, since pharmacologic inhibition or surgical lesioning of these areas confers significant resistance to both the short-term and long-term effects of cocaine in rodents (46). However, dopamine is probably not the only neurotransmitter involved in cocaine addiction, since mice lacking the vesicular dopamine transporter will still self-administer cocaine after repeated administration of the drug (66, 67). Although dopaminergic transmission in the limbic reward pathways has been implicated in the reinforcing properties of a wide range of addictive substances in addition to cocaine, the molecular and cellular mechanisms that lead to addiction in these neurons are not well understood.

Recent work in *Drosophila* suggests that the mechanisms of cocaine action may be accessible to genetic analysis. When flies are exposed to volatized free-base cocaine, they exhibit dose-dependent stereotypical behaviors that are surprisingly reminiscent of cocaine's psychostimulant effects in mammals (68). For example, at low doses treated flies become hyperactive and exhibit compulsive, continuous grooming behavior. At intermediate doses animals move more slowly and display stereotyped locomotive behaviors such as circling. Finally, at high doses animals undergo tremors, spastic paralysis, and finally death. Repeated treatment of flies with low doses of cocaine results in an increased behavioral response, a phenomenon known as sensitization; cocaine sensitization also occurs in mammals and is thought to underlie some aspects of addiction in humans. Interestingly, male flies are more sensitive to cocaine than females, a sexual dimorphism that also holds true in mammals (69). Thus, cocaine has both short-term and long-term effects on fly behavior that are remarkably analogous to its effects on mammals.

These behavioral similarities between cocaine's action on flies and mammals raise the possibility that they might share a common functional basis as well. In fact, recent evidence indicates that cocaine's actions on fly behavior also involve effects on aminergic neurotransmission. Insects contain cocaine sensitive reuptake transporters for dopamine, serotonin, and octopamine (an invertebrate neurotransmitter chemically similar to norepinephrine); thus, cocaine at least in principle could increase synaptic levels of multiple monoamine neurotransmitters in the fly brain (70–72). The monoamine most convincingly implicated in cocaine's acute effects on flies is dopamine. Dopamine receptor antagonists have effects on grooming and locomotive behaviors that are the converse of the effects of cocaine, and these antagonists can also block the effects of cocaine and cocaethylene on these behaviors in decapitated *Drosophila* preparations (Fig. 21.2) (69,73). Moreover, when flies are depleted of endogenous dopamine using tyrosine hydroxylase inhibitors, they acquire resistance to the acute effects of cocaine treatment (50). Paradoxically, however, transgenic animals in which dopamine and serotonin release is blocked by ectopic tetanus toxin expression are actually hypersensitive to cocaine (74). Thus, although dopaminergic neurotransmission is clearly involved in behavioral responses to cocaine in *Drosophila*, the specific role that it plays in these responses is not completely clear.

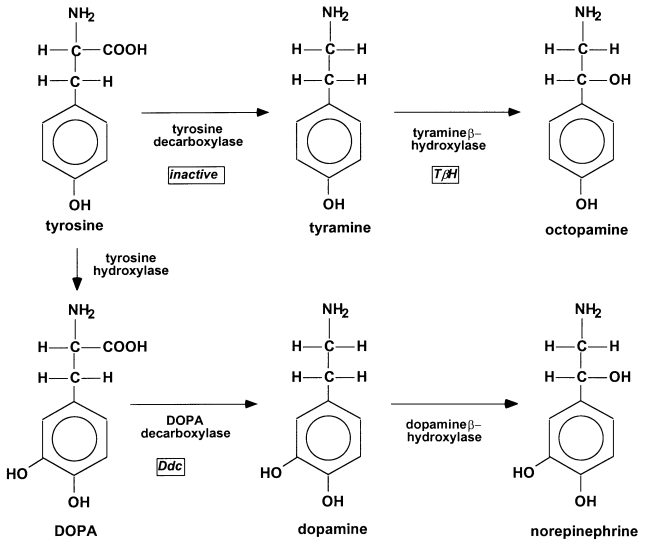

FIGURE 21.2. Biogenic amines and their biosynthesis.

Surprisingly, cocaine sensitization in *Drosophila* has been linked to a different biogenic amine—tyramine. Tyramine is present only in trace quantities in mammalian nervous systems; however, in insects it is a somewhat more abundant molecule and also serves as a precursor for the important neuromodulator octopamine (Fig. 21.3). Mutants with defects in this biosynthetic pathway have been identified in *Drosophila* behavioral screens. For example, inactive mutants have low levels of the enzyme tyrosine decarboxylase, and consequently fail to efficiently synthesize both tyramine and octopamine; in contrast, TβH mutants are defective in the tyramine β-hydroxylase enzyme, and thus synthesize tyramine but not octopamine. Interestingly, while inactive mutants display an essentially normal acute response to cocaine, they are strongly defective in sensitization (75). This sensitization defect can be rescued by feeding the mutant flies tyramine but not octopamine; moreover, TβH mutants (which lack octopamine but not tyramine) and Ddc mutants (which fail to synthesize dopamine) show normal cocaine sensitization. Furthermore, cocaine actually increases the levels of tyrosine decarboxylase activity in treated flies, suggesting that cocaine sensitization may actually occur at least in part through induction of tyramine synthesis. Remarkably, both the induction of tyrosine hydroxylase activity by cocaine and cocaine sensitization itself require the activities of the period, clock, and double-time genes, three members of the conserved signal transduction pathway that controls circadian rhythms in animals and fungi (76).

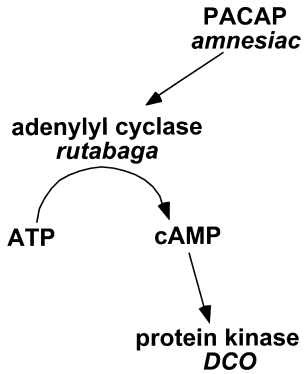

FIGURE 21.3. The adenylyl cyclase signaling pathway.

How might tyramine mediate cocaine sensitization in flies, and does it play a similar role in mammals? At present, these questions are difficult to answer. Although the function of tyramine in insect nervous systems has not been clearly established, putative tyramine receptors have recently been identified in both *Drosophila* and the honeybee (77). Possibly cocaine might act in a period-dependent manner to facilitate tyramine release from nerve terminals, which could then induce plasticity in other monoamine pathways in the brain. Future studies will be needed to identify the specific tyramine receptors that might mediate such responses and to understand the neural basis for their effects on behavior. In vertebrates, tyramine receptors have not been identified; thus, it remains an open question whether tyramine plays a role in human sensitization to cocaine that parallels its role in *Drosophila*. However, the involvement in *Drosophila* of the circadian clock pathway, which is highly conserved between insects and humans, suggests that at least some components of the molecular mechanisms underlying this process may be shared between these widely divergent organisms.

QUESTIONS AND FUTURE PROSPECTS

Perhaps the major potential pitfall of using worm or fly genetics to investigate drug mechanisms is that there is no guarantee that those mechanisms will be conserved across the evolutionary gulf separating these disparate animals. Certainly at the anatomic level, the brains of humans, flies, and worms are vastly different organs. Nonetheless, for most pharmacologic studies, the critical issue is conservation at the molecular level, and with the worm and fly genomes essentially complete, it is clear that at the molecular level the *C. elegans* and *Drosophila* nervous systems are quite similar to their human counterpart. For example, the *C. elegans* and *Drosophila* genomes contain homologues of each of the basic types of potassium channels, calcium channels, and G proteins, as well as putative receptors for most human neurotransmitters (78,79). To be sure, there are a small number of nervous system molecules found in vertebrates and flies but not nematodes (e.g., voltage-gated Na channels), as well as molecules found in nematodes and flies but not vertebrates (e.g., the ivermectin-sensitive glutamate-gated Cl channel). However, on the whole the nematode,

fly, and vertebrate nervous systems appear to be remarkably similar at the molecular level given their vast differences in scale and functionality.

What are the prospects for model organism neuropsychopharmacology in the postgenomic future? The availability of substantial portions of the worm and fly genomes has already made the rate-limiting step of classic forward genetics—cloning a mutant gene—significantly easier and more straightforward. This cloning process will become easier still as high-resolution, single-nucleotide polymorphism maps of the worm and fly genomes become available. The imminent completion of the human genome will also provide great benefits to model organism studies, since it will allow rapid identification of human homologues for worm or fly genes and more reliable distinction of genuine mammalian orthologues from other members of a gene family. The great advantage of worm and fly studies for the elucidation of drug mechanisms is the ability to conduct unbiased, phenotype-driven mutant screens to identify unknown gene products involved in drug response. Since ethical considerations will always preclude such approaches in humans, and since time, space, and cost considerations make them inefficient even in simpler vertebrates, *C. elegans* and *Drosophila* are likely to serve as workhorses for basic neuroscience research for many years to come.

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