Drug addiction: bad habits add up

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We know how many drugs of abuse — cocaine, heroin and nicotine — work, but less about how they lead to addiction. Studies of the brain-learning systems concerned are addressing the causes of addiction, with the intent of developing better treatments.

Drug addiction — which is increasingly seen as a neuropsychiatric disorder — places an enormous burden on society through its repercussions on crime rate and healthcare. The economic costs of addiction have been estimated at 80 billion dollars in the United States alone, and many Western countries have invested heavily in research towards understanding, treating and preventing addiction. Recently, through genetic and cell-biological approaches, many of the molecular targets for drugs of abuse have been identified and cloned. But the value of these powerful reductionist approaches depends, in turn, on an integrative framework of systems and cognitive neuroscience. Such a framework allows us to formulate new hypotheses that take into account the complex factors influencing addiction and its treatment.

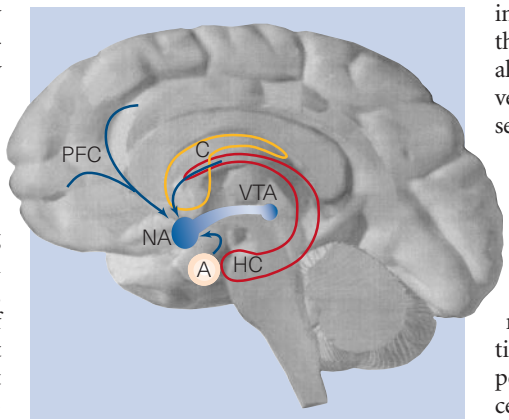

Figure 1 **Some of the brain structures affected by drugs of abuse. The mesolimbic dopamine system originates in the ventral tegmental area (VTA) of the midbrain, and projects to the nucleus accumbens (NA). The amygdala (A), hippocampus (HC) and medial prefrontal cortex (PFC) send excitatory projections to the nucleus accumbens. C, caudate nucleus (striatum).**

The dopamine hypothesis

By the early 1990s, converging evidence suggested that many (if not all) drugs of abuse act through mechanisms involving the brain neurotransmitter dopamine and the neural systems that it regulates[1]. Although these drugs — including 'stimulants' such as amphetamine and cocaine, opiates such as heroin, and even 'legal' drugs such as alcohol and nicotine (Box 1, overleaf) — can influence several different chemical neurotransmitter systems in the brain, many of these 'primary' responses lead to secondary effects involving dopamine. For example, morphine and heroin bind first to an opiate receptor, which then increases the activity of the so-called 'mesolimbic' dopamine neurons in the midbrain. These neurons send their projections to interconnected forebrain structures such as the prefrontal cortex and striatum (Fig. 1). A region at the base of the striatum, the nucleus accumbens, is the key zone that mediates the rewarding effects of drugs such as amphetamine and cocaine, which act directly by increasing the levels of dopamine at this site.

Evidence for this hypothesis came from several sources[1]. Rats will self-administer tiny injections of amphetamine to the nucleus accumbens (by pressing a lever to activate a microsyringe connected to a stainless-steel tube, or cannula, implanted there)[2]. The rats give themselves more of the amphetamine when their dopamine receptors are partly blocked pharmacologically, suggesting a drive to self-regulate the level of dopamine activity (possibly a form of homeostasis; see Box 2 on page 570 for terminology). This tendency to repeat behavioural acts that lead to drug effects we assume to be rewarding is an example of 'positive reinforcement' — a necessary feature of certain forms of memory or learning. If dopamine is massively depleted using a neurotoxin called 6-hydroxydopamine then, despite the recuperative capacity of the dopamine systems, the rats no longer self-administer amphetamine and cocaine. This is presumably because, in the absence of dopamine, these drugs lose their reinforcing properties[1].

The rewarding effects of drugs other than stimulants may also depend on the mesolimbic dopamine system. For example, rats will also self-administer morphine to the area of the midbrain from which the dopamine neurons project[3]. Using a microdialysis technique, which allows extracellular dopamine concentrations to be sampled directly from living brain tissue, withdrawal from several drugs — including alcohol and nicotine, as well as opiates and stimulants — has been shown to be associated with reduced levels of dopamine in the nucleus accumbens[4]. From these observations comes the 'modal hypothesis', which states that the reinforcing effects of all drugs of abuse partly depend on the mesolimbic dopamine system. These effects could, perhaps, stem from the more general role of this system in mediating the motivating properties of natural stimuli such as food or sex. In some sense, then, drugs 'short-circuit' or 'usurp' normal behavioural and motivational processes mediated by this region of the brain[1]. But although there is compelling evidence that amphetamine-like stimulants interact with this circuitry, the 'strong' form of the dopamine hypothesis — which embraces all other drugs of abuse — is certainly not universally accepted. For example, opiates also seem to have reinforcing effects mediated by dopamine-independent mechanisms in the nucleus accumbens[5].

Other experimental approaches

The dopamine hypothesis has been a starting point for other investigations, ranging from molecular genetics to functional neuroimaging. For example, using positron-emission tomography, regional cerebral blood flow can be measured in humans after administering amphetamine-like drugs such as methylphenidate. Consistent with the results of animal studies, challenge with the drug leads to significant changes in the striatum, which correlate with subjective responses (such as euphoria and craving) in cocaine addicts[6]. Moreover, dopamine receptors have been investigated as products of possible 'candidate' genes to help explain why drug abuse tends to run in families[7]. One idea is that inherited variability in function of the dopamine D2 receptor explains why people differ in their responses to drugs of abuse, and why some run a greater risk of drug abuse or addiction.

Another big focus of interest has been the dopamine transporter. During transmission of a nerve impulse, dopamine neurons release this neurotransmitter into the synapse, which is a tiny gap between two neurons. Dopamine diffuses across the synapse and binds to receptors on the other side. Having done its job, dopamine is then recycled by the transporter, which facilitates re-uptake by the presynaptic neuron. But cocaine, amphetamine and methylphenidate all block such re-uptake by binding to the dopamine transporter, leading to increased levels of dopamine in the synapse (Fig. 2 on page 569). So, the transporter, as well as various dopamine receptors in the nucleus accumbens, has been a target for gene deletion or disruption ('knockouts') and other forms of genetic modification.

A graphic demonstration of the knockout approach is the finding that mice lacking the dopamine D2 receptor consume less alcohol than normal mice[8]. This result impli-

cates the dopamine system in the effects of ethanol, although ethanol affects many other neurotransmitter systems, including serotonin (also known as 5-hydroxytryptamine; 5-HT), the amino-acid transmitters GABA (γ-aminobutyric acid) and glutamate, and also neuropeptide Y. The knockout strategy has also produced results that do not readily harmonize with previous pharmacological evidence. For example, experiments on mice lacking the D2 dopamine receptor indicate that this receptor mediates some of the positive effects of morphine, but not the negative physical symptoms produced after withdrawal from the drug[9]. Yet this selectivity is contradicted by pharmacological evidence that the D2 receptor contributes to the physical dependence induced by withdrawal of morphine in rats, and that the symptoms can be reduced by treatment with drugs that stimulate the D2 receptor[10]. Paradoxically, in mice lacking the dopamine transporter, the rewarding effects of cocaine are reduced — but not abolished — as measured by their tendency to self-administer the drug[11]. This observation fits with the fact that, in human brain-imaging studies[12], subjective responses to cocaine do not correlate with its action at the dopamine transporter. And it is a challenge for the primacy of the dopamine hypothesis.

The serotonin challenge

The challenge to the dopamine hypothesis has recently been highlighted by the role of the serotonin neurotransmitter system in cocaine abuse. This is particularly relevant given the often mutually inhibitory interactions between the serotonin and dopamine systems. Anti-depressant drugs such as Prozac share with cocaine a high affinity for the serotonin transporter molecule. Commonalities and co-morbidity between many forms of drug dependence and depression strengthen this link[13]. But again, the pharmacological and genetic evidence does not always seem to gel. For example, mice lacking the 5-HT_{1B} serotonin receptor are more likely than normal mice to self-administer cocaine[14]. Yet ostensibly the same behaviour can be achieved by the opposite action — treating rats with drugs that stimulate the 5-HT_{1B} receptor[15].

Such controversies can be put down to the shortcomings of both approaches. For the pharmacological approach, the problem is one of selectivity — drugs are rarely selective for just one receptor. In the knockout mice, it's a case of functional compensation. The time lag between the genetic intervention and exposure of an adult mouse to cocaine means that, during development, there could be massive changes to compensate for the deletion of a particular receptor. The resulting 're-wired' brain may not function in the same way as normal, so invalidating — or, at least, greatly complicating — the use of knockout or transgenic animals to model adult drug dependence. We need not only more selective drugs, but also mice in which the affected genes can be regionally knocked out or induced; that is, synthesis of the protein in question can be turned off or on at a precise time in adulthood, preferably in specific regions of the brain.

Gene expression

Other molecular strategies may provide less ambiguous pointers to possible therapies. A complementary approach to that of deleting genes is to examine what happens to gene expression when particular drugs are repeatedly administered. This differs from the pharmacological and transgenic approaches in that it provides essentially correlative information; nonetheless, expressed gene products can be the subsequent targets of drug probes, with possible therapeutic dividends. There is growing information on the molecular maladaptations that occur once dopamine has bound to its receptors[16]. The two main classes of dopamine receptor (D1- and D2-like) can have opposite effects from one another on signalling pathways and gene transcription in postsynaptic neurons. In addition, chronic exposure to opiates, cocaine or ethanol decreases levels of some intracellular signalling molecules in the nucleus accumbens (inhibitory guanine-nucleotide-binding proteins, for example), but increases the activity of others (adenylyl cyclase and cyclic-AMP-dependent protein kinase), ultimately affecting gene transcription (Fig. 2).

Two of the best-studied families of tran-

Box 1 Common drugs of abuse

Psychomotor stimulant drugs This class includes amphetamines, cocaine and methylphenidate (which is used to treat hyperactive children). These drugs work directly on the monoamine (especially dopamine) neurotransmitter systems. They produce euphoria when taken intravenously, and the effects of withdrawal include dysphoria (mild depression), fatigue, sleep disturbances, increased appetite and anxiety. MDMA (methylenedioxymethamphetamine) or 'ecstasy' also falls into this class, although its main effects are probably on serotonin systems.

Opiates These analgesics, which include morphine and heroin (diacetylated morphine), are usually injected. Many of their subjective effects, including a sense of well-being and euphoria, act through opiate receptors of the mu type, to which naturally occurring chemical messengers such as β-endorphin and the enkephalins also bind. Symptoms of withdrawal include dysphoria, nausea, muscle cramps, tear production, diarrhoea, sweating, anxiety and fever ('cold turkey').

Alcohol Acts in many ways, some of which are analogous to those of anxiety-relieving drugs such as the benzodiazepines (abuse of which can also lead to dependence). Withdrawal symptoms include autonomic hyperreactivity, nausea, hand tremor, anxiety and hallucinations. Alcohol has striking sedative effects and can lead to memory loss.

Nicotine Works at receptors for the neurotransmitter acetylcholine, found, among other sites, in the neocortex, hippocampus and midbrain (Fig. 1). Withdrawal symptoms include dysphoria, insomnia, anxiety, restlessness, decreased heart rate and weight gain.

Cannabis Also known as hashish or marijuana, this drug is generally inhaled. It can produce a dependence syndrome, as well as mild cognitive impairment. The main active constituent is Δ^{-9}, tetrahydrocannabinol, which works at cannabinoid receptors in the hippocampal formation, striatum and globus pallidus. A naturally occurring cannabinoid, anandamide, may be the normal occupant for these receptors.

LSD (lysergic acid diethylamide) Produces vivid hallucinations. Abuse occurs in a different pattern to that of other drugs. For example (with the possible exception of cannabis), LSD is the only drug that animals other than humans do not reliably self-administer.

Phencyclidine (PCP) Described as a 'dissociative anaesthetic', its effects include altered body image, feelings of isolation, cognitive disorganization and drowsiness, hostility and negativism, as well as euphoria and inebriation. It is more noted now as a model of human psychosis than as a major drug of abuse. High levels of the PCP receptor, which is part of the glutamate–*N*-methyl-D-aspartate receptor complex, are found in the hippocampus and neocortex, and intermediate levels occur in the amygdala, nucleus accumbens and caudate nucleus (striatum).

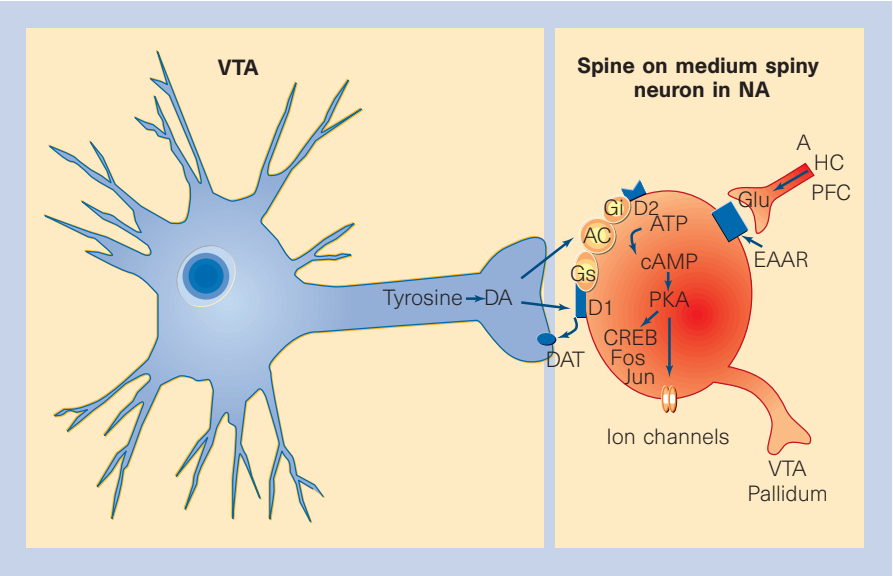

Figure 2 **Neural systems of addiction. A dopamine-releasing neuron from the ventral tegmental area (VTA) is shown innervating a medium spiny neuron dendritic spine in the nucleus accumbens (NA). The dopamine transporter (DAT) is a main site for cocaine and amphetamine action. These drugs inhibit the re-uptake of dopamine by the VTA neuron, where it is initially produced from the amino acid tyrosine. Dopamine is shown acting at the two main families of dopamine receptor (D1 and D2). These are coupled to guanine-nucleotide-binding proteins (G_s and G_i), components of the intracellular cyclic AMP system, which also includes adenylyl cyclase (AC) and cAMP-dependent protein kinase (PKA). Possible substrates for this kinase include ion channels and the nuclear transcription factors CREB, Fos and Jun. A, amygdala; HC, hippocampus; PFC, prefrontal cortex; EAAR, excitatory amino-acid receptor; Glu, glutamate. (Adapted from ref. 16.)**

scription factors are the so-called cAMP response element binding protein (CREB) family, and products of some immediate-early genes (referring to the time they are expressed after the ligand has bound to its receptor) such as *c-fos* and *c-jun*. Chronic exposure to morphine reduces levels of CREB. But chronic exposure to amphetamines may activate CREB in the nucleus accumbens, possibly by acting on the cAMP signalling pathway[16]. Certain Fos-like proteins (the Fos-related antigens) are also induced by chronic exposure to cocaine, amphetamine, morphine and nicotine, whereas mice that lack the *c-fos* gene show altered responses to cocaine[16]. Moreover, mice lacking the 5-HT_{1B} receptor show changes in their Fos-related antigens, even when never previously exposed to drugs[14]. This result indicates that the genetic make-up and subsequent development of these mice accelerates the molecular adaptation to drugs, correlating with a greater reinforcing effect.

Addiction as aberrant learning

All these discoveries support the consensus that drug dependence and addiction can be partly understood as gradual adaptations of the brain to chronic drug exposure. These adaptations may be triggered by a drive to regulate activity of the various brain systems within certain defined activity limits[5], and they are the underlying processes for both the decreasing ('tolerance')[17] and increasing ('sensitization')[18] effects of repeatedly administered drugs. They are consistent with the rebound consequences, following drug withdrawal, of chronic drug administration, which may further modulate the addiction process[5]. But how do the neurochemical and molecular changes produced by drug exposure relate to the clinical reality of human drug addiction?

First, we need to understand how addiction works at the cognitive, behavioural and neuropsychological levels. Several factors are increasingly seen as important for understanding variation in genetic and environmental vulnerability to drug abuse, and the treatment of addiction as a chronic, relapsing disorder. These include distinctions between the psychological, as well as neural, processes implicated in why people start to take drugs; 'consolidation' of these effects by the reinforcing action of the drug; subsequent maintenance of drug taking; and the eventual progression to addiction as a form of habit-based learning[19]. In the addicted stage, "the 'drug user' loses the voluntary ability to control its use" (ref. 19, page 237).

In the real world, drugs are not freely available, and the drug abuser has to forage for them. Drug-seeking behaviour can become powerfully associated with environmental cues, which, as 'conditioned stimuli', predict not only the availability of drugs (and their associated hedonic effects), but also aversive withdrawal states. The addict may seek to avoid such states by 'self-medication', through prophylactic or reactive drug taking.

The process of associative learning — by which the drug abuser connects specific cues such as a particular place with drug-induced states — probably includes structures in the brain that have strong anatomical links to the nucleus accumbens. These include the amygdala, hippocampus and orbitofrontal cortex, as well as related regions such as the pallidum and other sectors of the striatum (Fig. 1). Rats with damage to the amygdala readily self-administer cocaine, but cannot learn long sequences of behaviour to gain access to it[20]. When human addicts are allowed to see the paraphernalia associated with giving themselves cocaine, several interconnected areas of the brain are activated — including the amygdala [21,22]. As a result of such learning, behaviour is often sustained long after the original goal (in this case, cocaine) has been withdrawn. This may even lead to people developing a drug-seeking habit just as the subjective effects (for example, the 'rush' and euphoria) that initially encouraged it are reduced[23]. If the addictive behaviour is, to some extent, ultimately divorced from the original drug effect that generated its development, 'surrogate' treatments, which mimic effects of the abused drug[19] (such as D2-receptor agonists for cocaine abuse, methadone for opiate addicts, and nicotine patches for smokers), may have limited use.

The hypothesis that drug addiction is an aberrant form of learning, perhaps mediated by maladaptive recruitment of certain memory systems in the brain[24], is supported by several lines of evidence. First, receptors for both dopamine[25] and another neurotransmitter glutamate[26] are involved in normal learning in striatal and limbic structures. And second, transcription factors such as CREB are implicated in neuronal models of learning. Just as in other examples of learning, a conditioned stimulus alone (syringes, for example, or even a person associated with the drug) can activate the specific neural network that consolidated the original memory, through a series of plastic neuronal changes. In this way, the behaviour patterns associated with the drug and its attendant stimuli are evoked.

Relapse

Relapse, following what may often be protracted abstinence from drug taking, is a logical consequence of the conditioning process. It happens when the drug-seeking habit is reactivated by drug-related cues. Initially, the addict may retrieve from memory devastatingly compelling drug-related experiences (often described subjectively as 'craving'). This leads to further drug-seeking and drug-taking behaviour, sometimes even without the pleasurable anticipation associated with earlier drug experiences.

Relapse seems to be triggered by three main events[27]. The first is taking the drug itself (although the effects of certain drugs,

Box 2 Common terms in psychopharmacology

Co-morbidity The statistically significant co-occurrence of distinct diseases or disorders within the same individual or group.

Conditioning Forms of learning by association. They may be either instrumental (in which voluntary actions are controlled by their outcomes; see reinforcers below) or Pavlovian (in which a temporal correlation between events is detected and learned by an animal, even in the absence of voluntary control).

Drug (substance) dependence A term used synonymously with 'drug addiction'. It refers to the compulsive nature of drug seeking and taking, which precludes other forms of adaptive behaviour, impairing social and other forms of functioning. 'Physical dependence' refers to the need to take drugs to prevent aversive (usually involuntary 'autonomic') bodily symptoms caused by drug withdrawal.

Habit Technically, a form of instrumental learning in which a stimulus elicits a response without reference to the goal (or reinforcer) that originally motivated the learning. Aberrant 'habit' learning may accurately describe the later stages of drug addiction, being a product of certain brain memory systems.

Homeostasis Classical physiological mechanisms of body regulation, which keep systems under equilibrium.

Reinforcement A reinforcer increases the likelihood of the act that produced it being repeated. The resultant strengthening of behaviour can be seen as a form of memory, by which voluntary actions with certain outcomes are consolidated into long-term memory.

such as morphine and cocaine, may substitute for one another). Second is a conditioned stimulus — for a rat or mouse, this could be a noise associated with the drug, which predicts its availability for self-administration. And the third is induction of a state of stress (although the pharmacological precipitation of withdrawal following opiate dependence does not seem to be effective). These three triggers are all thought to lead to similar neural events associated with drug taking (including the release of dopamine in the nucleus accumbens), which are enough to activate drug-seeking behaviour. Conditioning and memory retrieval may be good targets for treatment of addicts. For example, a drug that selectively stimulates another dopamine receptor (D3) greatly reduces cue-induced cocaine-seeking behaviour[28]. And damage to the part of the amygdala that connects with the nucleus accumbens prevents cue-induced relapse for the same drug[29].

Inhibitory processes in the brain normally hold potentially maladaptive behaviour in check. Presumably, similar processes also restrain many of us from over-indulging on drugs in the first place. Such self-control is usually attributed to neural networks involving the prefrontal cortex and striatum (Fig. 1). In fact, some of the general behavioural and cognitive characteristics of drug abusers — including impulsivity (a tendency to act without foresight), risk taking and apparently poor decision-making abilities — resemble the effects of damage to the frontal lobes. For example, in decision-making tests, chronic amphetamine abusers perform similarly to patients with damage to the ventromedial (but not dorsolateral or medial) prefrontal cortex. A group of mainly opiate abusers, by contrast, shows only part of this pattern of deficit[30]. The similarity may be explained, in part, by findings that chronic amphetamine abusers have reduced serotonin function in the orbitofrontal cortex *post mortem*[31]. People who abuse 'ecstasy' show global reductions in binding of serotonin to the 5-HT transporter, based on measurements using positron-emission tomography[32].

Correlations and causality

The observed correlations between drug taking and neuropsychological changes raise several points. First, the cognitive consequences of drug abuse and addiction may lead to problems of rehabilitation that far outreach just reducing drug-seeking behaviour. Second, the additional induced behavioural changes may accelerate the progression to addiction, for example, by impairing self-control. Third, the causal relationships between drug taking and neural, as well as neuropsychological, impairments are not clear. There may be a co-morbidity of drug-taking behaviour with other impulsive or risk-taking behavioural traits, because of genetic or developmental factors. Alternatively, drug taking could produce neural or neurotoxic 'side effects' that facilitate the drive to addiction. At present we cannot distinguish between these possibilities in humans although, for amphetamine users, the degree of the behavioural deficit on the decision-making task correlated with the length of time they had been abusing the drug[30].

Many questions remain. First, with drugs such as cocaine or amphetamine, does the dopamine system become less important when people become addicted, as control of behaviour devolves to other neural systems that may mediate the habit-based learning? Second, how do we explain addiction to drugs that have distinct pharmacological actions? Most drugs of abuse have some common modes of action centred on the mesolimbic dopamine system, but they may also affect parts of the interactive memory systems that impinge on the striatum[24]. Third, how do (possibly genetic) differences between people affect addiction — from the initial response to the drug to, for example, habit-formation mechanisms? Fourth, to what extent might chronic exposure to drugs lead to neurochemical changes that affect the habit-learning process? Finally, can molecular correlates of various phases of the addiction process be identified; for example, the 'switch' from 'drug-misuse' to addiction[19]? And what are the implications for the treatment of drug abuse, whether psychological or pharmacological, before and after this switch?

The examples given in this article illustrate the potential complexity of the factors that may influence addiction and its treatment. Drug-seeking habits can be consolidated and, in some cases, provoked or disinhibited by other effects of drug misuse. The remaining questions can be addressed at both the molecular and neuropsychological levels — it is to be hoped that the answers will lead to new treatments and therapies. □

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1. Wise, R. A. & Bozarth, M. *Psychol. Rev.* **94,** 469–492 (1987).
2. Hoebel, B. G. *et al. Psychopharmacology* **81,** 158–163 (1983).
3. Bozarth, M. A. & Wise, R. A. *Life Sci.* **28,** 551–555 (1981).
4. Rossetti, Z. L. *et al. Eur. J. Pharmacol.* **221,** 227–234 (1992).
5. Koob, G. F. & Le Moal, M. *Science* **278,** 52–58 (1997).
6. Volkow, N. D. *et al. Nature* **386,** 830–833 (1997).
7. Uhl, G. *Neuropsychopharmaology* **20,** 3–9 (1999).
8. Phillips, T. J. *et al. Nature Neurosci.* **1,** 610–615 (1998).
9. Maldonado, R. *et al. Nature* **388,** 586–589 (1997).
10. Harris, G. C. & Aston-Jones, G. *Nature* **371,** 155–157 (1994).
11. Rocha, B. A. *et al. Nature* **393,** 175–178 (1998).
12. Volkow, N. D. *et al. Nature* **386,** 827–830 (1997).
13. Markou, A. *et al. Neuropsychopharmacology* **18,** 135–174 (1998).
14. Rocha, B. *et al. Nature Neurosci.* **1,** 132–137 (1998).
15. Parsons, L. *et al. J. Neurosci.* **18,** 10078–10089 (1998).
16. Self, D. W. & Nestler, E. J. *Drug Alcohol Depend.* **51,** 49–60 (1998).
17. Mendelson, J. H. *et al. Neuropsychopharmacology* **8,** 263–271 (1998).
18. Robinson, T. E. & Berridge, K. C. *Brain Res. Rev.* **18,** 247–291 (1993).
19. O'Brien, C. P. & McLennan, A. T. *Lancet* **347,** 237–240 (1996).
20. Whitelaw, R. *et al. Psychopharmacology* **127,** 213–224 (1996).
21. Grant, S. *et al. Proc. Natl Acad. Sci. USA* **93,** 12040–12045 (1996).
22. Breiter, H. C. *et al. Neuron* **19,** 591–611 (1997).
23. Tiffany, S. T. *Psychol. Rev.* **97,** 147–168 (1990).
24. White, N. M. *Addiction* **91,** 921–949 (1996).
25. Schultz, W. *et al. Science* **275,** 1593–1599 (1997).
26. Herman, B. H. *et al. Neuropsychopharmacology* **13,** 269–293 (1995).
27. Shaham, Y. *et al. J. Neurosci.* **16,** 1957–1963 (1996).
28. Pilla, M. *et al. J. Psychopharm.* **11S,** A5 (1997).
29. Meil, W. M. & See, R. E. *Behav. Brain Res.* **87,** 139–148 (1997).
30. Rogers, R. D. *et al. Neuropsychopharmacology* **20,** 322–339 (1999).
31. Wilson, J. M. *et al. Nature Med.* **2,** 699–703 (1996).
32. McCann, U. D. *et al. Lancet* **352,** 1433–1437 (1998).