GENE DELIVERY INTO THE BRAIN USING VIRAL VECTORS

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The delivery of recombinant genes into the brain is becoming an increasingly important strategy for answering questions about the molecular mechanisms of brain function. Answers to these questions may be applied to many of the disorders that affect the brain. For example, an understanding of the mechanisms by which repeated exposure to drugs of abuse increases their stimulant and rewarding properties in animal models will almost certainly lead to new ways of treating addiction in humans. If we are able to decipher the molecular events underlying long-term changes in neurotransmitter release, we will find new approaches to diseases such as the epilepsies, in which neurotransmitter release is altered. Knowledge of the molecular means by which neurotransmitters shape neuronal development and plasticity, or how trophic factors regulate neuronal health, will lead to insights into how defects in these pathways cause specific psychiatric and neurodegenerative diseases.

Unfortunately, the brain does not yield easily to genetic intervention. The terminally differentiated state of most neurons in the brain precludes the use of vectors, such as conventional retroviruses, that are dependent on cell replication for stable maintenance in the cell. In addition, the molecular mechanisms of specific brain disorders may be restricted to subsets of neurons at specific times during development and maturity. Therefore, strategies for manipulating gene expression in the brain must utilize vectors that persist stably in postmitotic cells and that can be targeted both spatially and temporally in the nervous system. A number of such gene delivery systems have been developed over the last decade. Rather than giving a superficial overview of the field, this chapter highlights the use of herpes simplex virus (HSV) as a vector for gene transfer into neurons, with emphasis not only on its potential but also on its flaws; compares and contrasts HSV-mediated genetic intervention in neurons with that mediated by other viral vectors; and gives detailed examples of the practical uses of this technology by describing the use of HSV vectors to study the molecular basis for drug addiction.

HERPES SIMPLEX VIRUS: THE PROTOTYPIC VECTOR

HSV possesses multiple features that make it an ideal vector for delivery of genes into the nervous system. In particular, it accepts large molecules of exogenous DNA; it infects non-dividing cells from a wide range of hosts with high efficiency; it enables strong expression of foreign genes; it is episomal, and thereby does not cause integration effects; its infection of postmitotic cells is persistent; and HSV-1 particles can be concentrated to relatively high titers. Because of these characteristics of HSV-1, and because it is neurotropic, it is currently one of the best viral vectors available for functional analysis of genes in the nervous system.

Amplicon vs. Genomic HSV-1 Vectors

There are two types of replication-deficient HSV vectors: those in which the foreign DNA of interest is cloned into the viral genome itself (genomic vectors), and those that are composed of a plasmid carrying minimal HSV sequences that allow it to be packaged into virus particles with the aid of a helper virus (amplicon vectors). A number of genes within the wild-type HSV genome are dispensable for its growth in cells *in vitro*. This knowledge was used to create "crippled" recombinant HSV-1 viruses that could be used as vectors for gene transfer into cells (1). This type of genetically engineered genomic vector has been used by a number of investigators and is described in detail by Fink et al. (2).

The idea of the amplicon vector originated with the discovery of defective HSV-1 particles (3,4) that appeared in and interfered with HSV-1 stocks that were passaged at high multiplicities of infection (MOIs). Examination of the

genomes of these defective HSV-1 particles revealed that they carried only a minimal subset of DNA sequences from the wild type genome (3–5). These sequences included an origin of DNA replication and a cleavage/packaging site (the "a" site). It was found that incorporation of these two sequences into a plasmid (the "amplicon") gave the plasmid the ability to be replicated and packaged into virus particles when it was introduced into a cell that was superinfected with wild-type virus (which supplied HSV replication and virion assembly functions in trans). The plasmid sequences that were packaged into virus particles consisted primarily of 150-kilobase (kb) concatamers of the original plasmid (3–6).

The chief advantage of this amplicon type of vector, which is now packaged with replication-defective helper viruses, is that cloning manipulations are relatively easy due to the small size (5 to 10 kb) of the plasmid. One disadvantage is that production of amplicon vectors requires a copropagated HSV helper virus, resulting in viral stocks that are a mixture of helper and amplicon viruses. In the past, cytotoxic effects of these stocks limited the amount of vector that could be used to infect cells. Even though the replication-incompetent helper viruses could not cause lytic infections in normal cells, cytopathic effects resulted both from proteins present in the HSV-1 particles and from expression of HSV-1 immediate-early (IE) genes (7). Occasionally, wild-type HSV-1 revertants appeared during the amplicon packaging process, exacerbating the cytotoxicity of the virus preparations (7,8). However, recent improvements in the amplicon packaging procedure, which are discussed in the following section, have largely overcome these problems. The development of a helper virus-free packaging system for the HSV vector (9) has virtually eliminated any lingering cytotoxicity in the preparations, although the helper-free system yields relatively low titers of virus and still needs improvement in that area.

Present-Day Amplicon Vectors: Advantages and Disadvantages

Genomic and plasmid defective HSV-1 vectors have been used to manipulate neuronal physiology both *in vitro* and *in vivo*. These studies have been promising, but they have also revealed limitations of the current HSV vector systems. Because HSV is too fragile to purify on cesium chloride gradients, it could not be concentrated in the same way that encapsulated viruses such as adenovirus were concentrated. Moreover, as noted above, nonspecific cytopathic effects of the defective vectors restricted the number of viral particles that could be used to infect neurons. Finally, lack of persistence of high expression levels from the viral recombinants has hampered long-term *in vivo* studies and has limited the usefulness of the vectors for both experimentation and gene therapy.

Recent improvements in the amplicon packaging proce-

TABLE 20.1. CHRONOLOGY OF IMPROVEMENTS IN AMPLICON VECTOR PACKAGING SYSTEM

- 1985—Use of wild-type HSV-1 as helper virus and first amplicon plasmid (5)
- 1988—Use of HSV-1 tsK IE3 mutant as helper virus (64)
- 1990—Use of D30EBA IE3 deletion mutant and M64A complementing cell line (8)
- 1996—Use of 5d/1.2 IE2 deletion mutant and 2-2 complementing cell line (10)
- 1996—Improvements in transfection procedure and development of methods to purify and concentrate the virus result in amplicon/helper ratios of up to 100:1 (previously 1:100) and titers of up to 2×10^8 infectious units/mL
- 1997—Helper-free packaging by transient transfection of HSV cosmids (19)

HSV, herpes simplex virus.

dure (Table 20.1) have corrected some of the limitations listed above. The most widely used second-generation helper virus was HSV-1 tsK, with a temperature-sensitive single-base mutation in the *ICP4* (*IE3*) gene. Because revertants of this mutant arose at a finite frequency during the packaging procedure, lytic virus was present in some preparations (7,8). The frequency of revertants was decreased with the development of an efficient packaging system using a deletion mutant of *IE3* (8) as helper virus. However, occasional lytic virus particles continued to appear, albeit at a greatly reduced frequency, presumably as a result of recombination between the helper virus and the sequences flanking both sides of the *IE3*-containing fragment present in the permissive host.

A key breakthrough was made by Lim et al. (10) when they compared three replication-defective HSV-1 mutants [KOS strain 5dl1.2, deleted in the ICP27 (IE2) gene; and strain 17 D30EBA and KOS strain dl120, both deleted in the ICP4 (IE3) gene] for their usefulness as helper virus for packaging an amplicon vector. Historically, *IE3* mutants have been preferred because they express fewer HSV-1 genes under nonpermissive conditions than do ICP27 mutants. However, Lim et al. found that use of the *IE2* mutant 5dl1.2 yielded higher vector titers than did use of the IE3 mutants, with no increase in cytotoxicity. In addition, wild-type lytic virus was virtually absent in stocks made using 5dl1.2 in conjunction with the permissive host 2-2 cells, likely due to the fact that 5dl1.2 is a more complete deletion than D30EBA, sharing little sequence with the IE2-containing fragment present in the permissive host.

To achieve a favorable ratio of recombinant vector to helper virus, the stocks derived from transfection of the packaging cells followed by superinfection with helper virus are passaged three times on the permissive host. The recombinant vector is packaged as long concatamers, which contain multiple origins of replication, conferring a selective replicative advantage on the vector-containing virus relative to the helper virus. Therefore, the efficiency of the initial transfection of vector DNA into the packaging line is critical to the success of the packaging. Lim et al. (10) showed that the transfection of the vector DNA at the start of the packaging procedure was significantly more efficient using Lipofectamine (Life Technologies) than using calcium phosphate, and thereby achieved a favorable ratio (≥1) of vector to helper. Since then, we have achieved vector/helper ratios greater than 100.

For a viral vector to have utility for gene therapy, cytopathic effects of the virus must not outweigh the beneficial effects of the transgene. Unfortunately, in the past, investigators have had difficulty generating nontoxic HSV-based replication-defective vectors. They often were toxic to neurons in vitro (7). Significant necrosis, often accompanied by inflammation and gliosis, was identified at injection sites with some genetically engineered HSV-1 vectors used for in vivo studies (see, e.g., ref. 11). However, the achievement of a more favorable ratio of vector to helper, and the virtual elimination of wild-type virus in the vector preparations (10) has greatly reduced the cytotoxicity of present-day defective HSV-1 amplicon vectors (see, e.g., refs. 12–14). An additional improvement to the packaging procedure, the banding of the virus on a sucrose step gradient, followed by a high-speed centrifugation to pellet the virus, has reduced further the cytotoxicity of the virus preparations. It simultaneously removes toxic factors present in the crude cell lysates, and enables concentration of the vector to titers exceeding 10⁸/mL.

A troubling problem that has not yet been resolved for any viral vector used in the brain, except perhaps for the lentiviral vector (see below), is that of persistence of expression. Numerous investigators have had the experiences reported by During et al. (15) and Lim et al. (10), in which an initial peak in expression of an HSV transgene *in vivo* or *in vitro*, respectively, has been followed by loss of the bulk of the expression by 1 to 2 weeks postinfection. Interestingly, superinfection with helper virus 5dl1.2 1 week postinfection rescued expression of a transgene expressed under the control of the IE 4/5 promoter (10). Apparently, transactivating factors provided by the helper virus reactivated transcription of the transgene.

Two recent developments suggest that the problem of persistence of expression is not insoluble. Use of a 9-kb fragment of the tyrosine hydroxylase promoter to drive reporter gene expression in an HSV-1 amplicon vector resulted in prolonged gene expression *in vivo* (16), suggesting that neuronal, unlike viral, promoters in HSV-1 vectors have the potential to produce stable gene expression. Additionally, the development of hybrid amplicons that incorporate elements that allow autonomous replication of the episome (17) or that incorporate adeno-associated virus (AAV) elements for genomic integration of the amplicon (18–20) have resulted in vectors that support long-term gene expression both *in vitro* and *in vivo*.

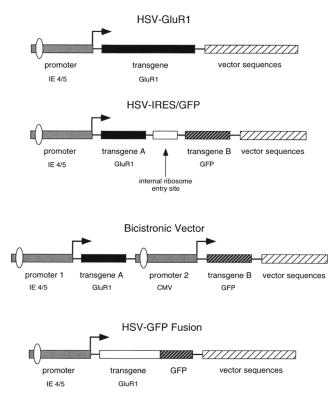


FIGURE 20.1. Different vector constructions for coexpressing two genes from a viral vector. Glutamate receptor GluR1 and green fluorescent protein (GFP) are used as examples.

An improvement in gene transfer methods in general has been the incorporation of the gene for the green fluorescent protein (GFP) into many vectors. Coexpression of GFP allows the investigator to detect cells infected by the vector with a fluorescence microscope, whether they are fixed or alive. There are at least three ways to "mark" vector-infected cells with GFP (Fig. 20.1). In the example shown, the objective is to coexpress GFP with the AMPA receptor GluR1. They can be expressed on a single transcript (HSV-IRES/ GFP) by putting an internal ribosome entry site (IRES) between the two genes, which are then transcribed from the same promoter. The IRES enables independent translation of the two coding sequences even though they are present on the same messenger RNA (mRNA). In theory, the two coding sequences should be expressed at similar levels, but in practice the translation of one of the two coding sequences on the mRNA often occurs at the expense of the other. Alternatively, they can be expressed from two independent transcriptional units, in a bicistronic vector. The addition of an extra transcriptional cassette to the vector makes it larger and more unwieldy to use; and the level of transcription from one promoter is independent of the level of transcription from the other, so that the transgenes may be expressed at very different levels. Third, the GFP can be fused to the transgene product, so that they are expressed

as a single protein. This is a trickier construction, since the two coding sequences must be placed in frame with each other. However, the added benefit of being able to track the subcellular location of the transgene product makes this the option of choice in many instances.

COMPARISON OF HSV-1 WITH ALTERNATIVE VECTORS FOR GENE TRANSFER INTO NEURONS

Despite the problems that remain with the HSV-1 vector, it is a gene delivery system that has come of age. In addition, numerous alternative and increasingly user-friendly means of gene transfer into the brain are now available (Table 20.2). Adenovirus vectors, like HSV-1 vectors, infect postmitotic cells and can enter a broad range of mammalian cell types. They have the additional advantages that they

TABLE 20.2. COMPARISON OF VIRUSES USED TO MANIPULATE GENE EXPRESSION IN THE BRAIN

Advantages	Disadvantages
Herpesvirus vectors	
Broad host and cell type range	Occasional cytotoxicity
Episomal (no possibility of insertional activation of host genes)	Lack of persistence of expression
Can accommodate up to 15 kb of foreign DNA	
High level of expression of foreign genes within hours	
Can be concentrated to high titers	
Helper virus–free stocks possible	
Adenovirus ve	ectors
Broad mammalian host and	Elicits host immune
cell type range	response
Episomal (no possibility of insertional activation of host genes)	
Growth to high titers (~10 ¹⁰ /mL)	Accommodates <10 kb of foreign DNA
High level of expression of	5
foreign genes	
Expression is relatively persistent	Genetic manipulation is unwieldy
Adeno-associated vectors	
Broad mammalian host and cell type range	Can accommodate only 4.7 kb of foreign DNA
Nonpathogenic	
Helper virus-free stocks possible	
Expression is relatively persistent	
Lentivirus vectors	
Integrates into host chromosome	Can accommodate only 6–8 kb of foreign DNA
Expression is persistent	Low titers
	Potent human pathogen

kb, kilobase.

can be concentrated to very high titers ($\geq 10^{10}$ /mL). However, the use of adenovirus vectors continues to be restricted by the robust host immune response that they elicit (21, 22).

At present, HSV amplicon vectors can accommodate larger pieces of foreign DNA, on the order of 15 kb, than can the adenovirus vectors, which can only contain a maximum of 6 to 8 kb (although the new "gutless" adenovirus vectors can take up to 37 kb of foreign DNA). Foreign genes are cloned into easy-to-manipulate amplicon vectors that can be packaged directly into viral particles as head-to-tail repeats in the presence of the helper virus, with no intermediate recombination step required. This enables rapid construction of a large number of recombinant vectors simultaneously, and is particularly useful for those who are doing mutation analysis, and who wish to work with multiple genes. Such ease of cloning is not possible with the genomic HSV and adenovirus vectors.

Direct in vivo transfer of genes into the brain has been achieved using not only herpes virus and adenovirus vectors, but also adeno-associated virus vectors (see refs. 23,24 for review) and lentivirus vectors (see ref. 25 for review). In contrast to other viral vectors, adeno-associated virus vectors do not cause an immune response or toxicity. Interestingly, the ability of adeno-associated virus vectors to transduce and express transgenes is not equivalent in all regions of the brain (26,27). Only in some regions will neurons bind and internalize the virus, with resultant long-term expression. Stability of expression of lentivirus vectors in the brain is their greatest advantage. Long-term expression of β-galactosidase and GFP was observed in rat neurons for at least 9 months following intracerebral injection of the vectors, with no sign of tissue pathology or immune response (28, 29). Progress has been made in achieving biosafety with these vectors, by eliminating viral sequences nonessential for transduction.

GENE DELIVERY INTO THE BRAIN AS A TOOL FOR NEUROPSYCHIATRIC RESEARCH: DRUG ADDICTION

Overview

The bulk of the published work on gene transfer technologies has focused on their use for gene therapy (see ref. 30 for an excellent review). To date, the delivery of recombinant genes into the brain as a strategy for answering questions about the molecular mechanisms of brain function has utilized primarily HSV vectors. One of the most successful uses of this strategy has been in the field of addiction research. Exposure to drugs of abuse causes many changes in gene expression within the brain. A major challenge in addiction research is to determine which of these changes have a direct influence on behavior. Viral vectors offer the ability to study individual changes in gene expression in

discrete brain regions. In the case of addiction, it is thus possible to mimic certain aspects of the drug-exposed state without ever administering the drugs themselves. The ultimate goal of such studies is to understand the "biobehavioral" mechanisms of addiction, that is, to establish direct, causal relationships between drug-induced changes in biology and drug-induced changes in behavior. Examples of behavioral changes in addiction that may result from drug-induced alterations in biology (gene expression) are compulsive drug use (drug-taking) and craving (drug-seeking).

Biobehavioral Studies of Addiction

Addiction Circuitry

Much research on the neuronal circuitry involved in drug addiction has focused on the mesolimbic dopamine (DA) system. The dopaminergic projection from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAc) of the forebrain has been implicated in the habit-forming (rewarding) effects of many types of abused drugs, including stimulants (cocaine, amphetamine) and opiates (heroin, morphine) (31,32). However, the neural events that mediate the acute rewarding effects of abused drugs are not understood, nor are the neuroadaptations that presumably underlie the transition from occasional drug use to compulsive drug use. In rats, repeated exposure to drugs of abuse appears to cause increases in sensitivity ("sensitization") to the rewarding effects of drugs (33–35), a phenomenon that may contribute to the addiction process (36). This altered sensitivity is presumably a consequence of altered gene expression, and the VTA-NAc circuitry is a logical starting point for biobehavioral studies. Because several robust and reliable drug-induced neuroadaptations have been discovered within this circuitry (37), it has been the focus of gene transfer studies in which the behavioral significance of altered gene expression has been assessed. To date, the biobehavioral significance of three specific, drug-induced changes in gene expression have been studied using viral-mediated gene transfer—the ability of drugs (cocaine, morphine) (a) to increase expression of the AMPA (glutamate) receptor subunit GluR1 in VTA, (b) to alter expression of GluRs in the NAc, and (c) to increase the activity of the transcription factor CREB (cAMP response element binding protein) in the NAc.

GluRs in the VTA

AMPA receptors are made up of various combinations of the subunits GluR1, GluR2, GluR3, and GluR4 (collectively called GluRs) (38,39). Repeated intermittent exposure to morphine selectively elevates expression of GluR1 in the VTA (40). Relative levels of GluR1 and GluR2 expression in the dopamine neuron-rich VTA are important because the subunit composition of AMPA receptors con-

trols their function. High expression of GluR1 favors the formation of Ca²⁺-permeable (GluR1-homomeric) AMPA receptors (13,38,39), which presumably increases sensitivity to the excitatory (depolarizing) effects of glutamate (12). Conversely, high expression of GluR2 favors the formation of Ca²⁺-impermeable (heteromeric) AMPA receptors, since this subunit contains a motif that blocks Ca²⁺ conductance (38). Because repeated drug exposure is known to selectively increase the electrophysiologic responsiveness of VTA dopamine neurons to AMPA receptor agonists (41), it is possible that drug-induced alterations in GluR1 expression in this region contribute to drug-induced behavioral changes, such as sensitization. However, this question is difficult to address using traditional methods. For example, AMPA agonists and antagonists cannot be used to study the relationship between GluR1 expression and sensitized drug responses because they affect AMPA receptor function generally and thus do not mimic morphine's selective effects. This biochemistry-behavior relationship could be studied by performing intra-VTA microinjections of HSV-GluR1, which caused ~75% increases in GluR1 expression within this region (13).

It was first necessary to determine if viral-mediated elevations in GluR1 expression within the VTA would increase sensitivity to the locomotor-stimulating effects of morphine, a hallmark of behavioral sensitization (36,42). Low doses of morphine induced significantly more activity in rats with viral-mediated elevations in GluR1 expression within the VTA. Furthermore, increased activity in rats given HSV-GluR1 was seen only in response to morphine, and was not evident after saline. The transient nature of the HSVmediated elevations in transgene expression allowed the behavioral adaptations to be correlated with the time course of GluR1 expression: when morphine was given only on days 7 and 8 after microinjection—when elevations in GluR1 expression had dissipated—the rats that were given HSV-GluR1 were no longer more sensitive to the stimulant effects of morphine. When some rats given HSV-GluR1 were tested with morphine on days 3 to 4 and on days 7 to 8, significant increases in sensitivity to the locomotorstimulating effects of the persisted in rats given HSV-GluR1, despite the fact that GluR1 labeling in the VTA had dissipated. Thus when morphine is given while GluR1 expression in the VTA is elevated, increased sensitivity to morphine outlasts viral-induced increases in GluR1 expression. These data suggest that altered expression of GluR1 in the VTA underlies, at least in part, the development and expression of sensitized behavioral responses to morphine.

The effect of elevated GluR1 expression in the VTA on the rewarding effects of morphine was examined using place conditioning, a classic conditioning procedure in which rats learn to associate the rewarding effects of a drug with a distinctive environment. Rats given HSV-GluR1 into the rostral (anterior) portion VTA spent more time in morphine-associated environments than did control rats, indi-

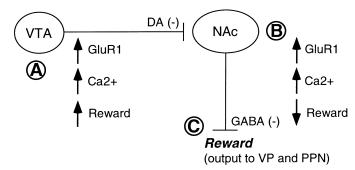


FIGURE 20.2. Simplified schematic of putative reward circuitry; see ref. 39 for detailed discussion. Treatments that increase excitation in the ventral tegmental area (VTA) (A) are rewarding, presumably because they promote the inhibitory actions of dopamine (a D2-like receptors) in the nucleus accumbens (NAc) (B). Inhibition of NAc GABAergic output neurons, in turn, decrease inhibitory influences on reward processes in other areas of brain reward circuitry (C), including the ventral pallidum (VP) and peduncular pontine nucleus (PPN) (63). Elevations in GluR1 expression in the VTA (A) increase drug reward, presumably because the accompanying changes in Ca²⁺ flux increase the excitability and/or neuronal function of VTA dopaminergic neurons (as in ref. 49). Conversely, elevations in GluR1 in the NAc decrease drug reward (B), presumably because the accompanying changes in Ca²⁺ flux increase the excitability of NAc GABAergic neurons that normally inhibit reward processes in distal regions (C). (Based on refs. 13, 43, and 55.)

cating an increase in morphine reward (13,43). Since prior treatment with morphine intensifies its rewarding actions in the place-conditioning paradigm (33), these data suggest that the behavioral consequences of morphine preexposure are mimicked by HSV-mediated expression of GluR1 in the VTA.

Together, these data demonstrate that specific changes in motivational states can result from altered expression of a single, localized gene product. Drug-related increases in GluR1 expression in the VTA, a region known to be involved in the induction of sensitization (42,44), may themselves be sufficient to explain sensitization (13,41), or they may lead to Ca²⁺-dependent adaptations (45) that also contribute to changes in drug sensitivity (Fig. 20.2). Thus these studies have added strength to the hypothesized association between the VTA and sensitization, and identified biobehavioral relevance for the drug-induced regulation of the GluR1 protein in the VTA.

GluRs in the NAc

There are two important reasons for interest in how altered expression of GluRs in the NAc affects the actions of drugs of abuse. First, GluR2 is a target gene of Δ FosB, a stable and long-lasting variant of the fos family of transcription factors that is regulated in the Nac by drugs of abuse (46). Second, GluR1 expression is elevated the NAc in cocaine-sensitized rats during long-term drug withdrawal (47).

Exposure to cocaine and other drugs of abuse causes the rapid and transient expression of the immediate-early gene c-fos in the striatum (including the NAc) (48,49). Repeated drug exposure decreases expression of the transient forms of c-fos, but increases expression of a more stable and longlasting form of the transcription factor, Δ FosB (50,51). The accumulation and sustained transcriptional activity of Δ FosB in the NAc could mediate long-lasting neural and behavioral adaptations that accompany repeated drug exposure (37). Consistent with this notion, inducible transgenic mice that express Δ FosB spontaneously (i.e., without prior drug treatment) in the NAc during adulthood have increased sensitivity to the locomotor-stimulating and rewarding effects of cocaine (46). The proximal cause of the increase in drug sensitivity is presumably not increased expression of Δ FosB per se, but rather increased expression of a target gene (or genes) whose transcription is regulated by this factor. The Δ FosB-overexpressing mice also had large increases in GluR2 expression in the NAc, implicating this AMPA receptor subunit in the increased drug sensitivity. To examine whether elevated GluR2 expression in the NAc was sufficient to cause increases in sensitivity to the rewarding effects of cocaine, rats were tested in the placeconditioning paradigm after microinjections of HSV-GluR2 into this region (46). This treatment dramatically increased sensitivity to the rewarding effects of cocaine, mimicking the effects of increased expression of Δ FosB. Together, these findings provide strong evidence that the increase in cocaine sensitivity seen in Δ FosB transgenic mice is attributable, at least in part, to elevated expression of GluR2 in the NAc.

For comparison, rats were given microinjections of vectors expressing other GluRs into the NAc, and tested with cocaine in the place-conditioning paradigm. Rats given microinjections of HSV-GluR1 into the NAc spent dramatically less time than control rats in the cocaine-associated environments, suggesting that elevated expression of this AMPA receptor subunit in this region increases sensitivity to the aversive effects of the drug. Additionally, some rats were tested after intra-NAc microinjections of HSV-GluR2Q, which expresses unedited GluR2. This form of GluR2 lacks the final transcriptional edit $(Q \rightarrow R)$ that produces the motif that blocks Ca²⁺ flux (38,39). Use of this construct showed that the ability of GluR2 to increase cocaine reward appears to be directly related to diminished Ca²⁺ permeability, because overexpression of GluR2Q does not increase cocaine reward, but rather causes effects that more closely resemble those of increased GluR1 expression (as would be expected).

There are many possible explanations for how altered Ca²⁺ flux in the NAc might influence drug reward, considering the role of Ca²⁺ in cellular functions including membrane depolarization, neurotransmitter release, signal transduction, and plasticity (52,53). Certainly, cocaine-induced changes in the excitability of NAc neurons have been re-

ported: repeated exposure to cocaine makes neurons in this region significantly less excitable than normal at short (3-day) withdrawal periods (54). Studies with $\Delta FosB$ (46) suggest that these electrophysiologic adaptations are associated with increases in the rewarding efficacy of cocaine, because elevations in GluR2 expression (which would be expected to minimize Ca^{2+} flux and/or neuronal excitability) increase cocaine reward, whereas elevations in GluR1 (which would be expected to increase Ca^{2+} flux and/or neuronal excitability) decrease (or oppose) cocaine reward.

Together, these data support the working hypothesis (55) that altered Ca²⁺ flux and/or neuronal excitability in the NAc has important consequences on motivated behaviors (Fig. 20.2). Moreover, they suggest that altered GluR1 expression in this region seen during long-term (3-week) cocaine withdrawal (47) might also be associated with important changes in the rewarding efficacy of the drug. Regardless, the use of HSV vectors has identified biobehavioral relevance for the drug-induced regulation of GluRs in the Nac.

CREB in the NAc

Chronic cocaine exposure increases 3',5'-cyclic adenosine monophosphate (cAMP) formation and protein kinase A (PKA) activity in the NAc (37). Direct stimulation of PKA in the NAc counteracts the rewarding properties of cocaine (56), suggesting that drug-induced up-regulation of the cAMP system is a neural mechanism of drug tolerance. Increased PKA activity leads to increased CREB phosphorylation, which activates CREB-mediated gene transcription and could be an important step in producing long-lasting neuroadaptations. To determine the functional role of CREB and its transcriptional consequences in the NAc, its expression in this region was increased directly by microinjecting HSV-CREB (57). In other rats, a dominant negative mutant CREB (mCREB) was overexpressed, which is transcriptionally inactive and competes with endogenous CREB for cAMP response element binding sites (CREs) (58).

The effects of elevated CREB expression in the NAc on cocaine reward was studied with place conditioning. Although the effects of a low (threshold) dose of cocaine were not altered by control treatments, this dose established dramatic conditioned place preferences in rats given bilateral microinjections of HSV-mCREB (which acts as a CREB antagonist) into this region. This rewarding effect was "inversed" to aversion in rats with elevated expression of CREB in the NAc; rats given HSV-CREB avoided drug-associated environments, suggesting that this dose of cocaine was made aversive by gene transfer. When cocaine was administered a week (rather than 3 days) after microinjections of the HSV vectors into the NAc, cocaine was devoid of rewarding or aversive effects. This finding confirms that the behavioral consequences of HSV viral vectors are transient and reversi-

ble, and have a time course of efficacy that parallels that of transgene expression.

Dose-response analyses revealed that microinjections of HSV-mCREB and HSV-CREB in the NAc were producing, respectively, approximately parallel leftward (more rewarding) and rightward (less rewarding) shifts in the effects of cocaine. At a high dose of cocaine, there were no differences in the preferences for the drug-associated environment between rats given HSV-mCREB and those given vehicle, consistent with observations that there is an upper limit to the magnitude of place preferences that can be established (59). Treatment with high doses of cocaine established place preferences in some rats given HSV-CREB, suggesting that the aversive consequences of increased levels of CREB in the NAc can be counteracted by more drug.

One explanation for these findings is that elevated CREB expression in the NAc increases local dynorphin function. Dynorphin is the endogenous ligand for κ opioid receptors (60), and κ opioid agonists have aversive actions in the nucleus accumbens shell (NASh) (61). To determine if dynorphin is involved in the cocaine aversion caused by HSV-CREB, brain receptors for dynorphin were blocked with the long-lasting κ receptor antagonist norBNI. Treatment with norBNI [intracerebroventricular (ICV)] before cocaine place conditioning blocked the aversive effects associated with cocaine in animals given HSV-CREB into the NAc, but not in rats given microinjections of vehicle or HSVmCREB. The fact that only the aversive properties of cocaine are altered significantly by nor-Binaltorphimine (nor-BNI) suggests that microinjections of HSV-CREB into the NAc enhance the aversive aspects of cocaine via increased stimulation of κ opioid receptors by dynorphin.

These results suggest that drug-induced increases in CREB activity (62) is a homeostatic change that opposes drug reward. Mimicking increases in CREB activity by increasing levels with HSV-CREB or by stimulating PKAinduced phosphorylation (56) decreases the rewarding effects of cocaine. Moreover, these data implicate κ opioid receptors in the valence (reward versus aversion) of cocaine action, and suggest that CREB-mediated transcription in the NAc is a "drug reward rheostat" (Fig. 20.3), in part via effects on dynorphin expression. These data also suggest a sequence of D1 receptor-mediated intracellular events, culminating with altered gene transcription, through which exposure to cocaine influences subsequent responsiveness to the drug. Augmented release of dynorphin could inhibit local DA release through actions at κ opioid receptors on terminals of mesolimbic DA neurons that innervate the NAc (61). Diminished release of dopamine in the NAc may itself be aversive, or it may unmask other actions of cocaine that are aversive or that oppose drug reward. Regardless, these viral vector studies have identified biobehavioral relevance for alterations in CREB function in the NAc.

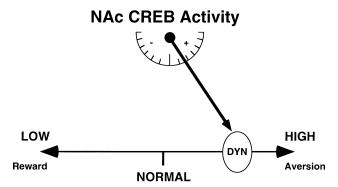


FIGURE 20.3. Schematic depiction of CREB activity in the NAc functioning as a cocaine "reward rheostat." Elevated expression of CREB increases CREB-mediated transcription of dynorphin (a measure of CREB activity). Elevated dynorphin, in turn, decreases cocaine reward at high doses of drug, and makes cocaine aversive at low doses of drug. Conversely, disruption of CREB activity by overexpression of dominant-negative CREB (mCREB) decreases dynorphin transcription, which increases cocaine reward. (Based on ref. 57.)

Conclusions

The use of viral-mediated gene transfer in addiction research is leading to an understanding of where certain changes in gene expression occur within the cascade of molecular events that lead to the addicted phenotype. This approach complements and extends the predominantly pharmacologic approaches that previously have been used in addiction research, and in fact can be thought of as "genetic pharmacology." Understanding the molecular basis of addiction will facilitate the development of effective pharmacologic treatments that specifically target proteins, enzymes, or transcription factors within the addiction cascade. These therapeutics may be the prototypes for a new generation of "smart" pharmacotherapies that are designed to negate or even reverse changes in the molecular structure of the brain that characterize specific brain disorders.

GENE DELIVERY INTO THE BRAIN AS A MEANS FOR GENE THERAPY

The recent rapid advancements in gene transfer technologies have raised hopes that central nervous system (CNS) gene therapy, the introduction of genes into the brain to ameliorate neuropsychiatric diseases, is closer to reality. However, a number of major methodologic advances must be made before it can become a reality. First, and most important, stability of transgene expression must be achieved. Second, not only stability but also inducibility and regulatibility of transgene expression are a priority, since the level of transgene product is often critical. Third, the transgene capacity of most vectors must be increased, so that not only the gene(s) of interest but also appropriate regulators or

inducible promoters can be delivered to the brain. Fourth, because of the small volume of material that can be delivered stereotactically, it will be necessary to increase both the viral titers and the transduction efficiencies for all the known vectors. Fifth, a high degree of cell specificity of gene transfer must be achieved, by the use of targeted vectors that selectively infect particular cell types, cell-specific promoters, and routing via normal neuronal projections in the brain. Finally, nontoxic vectors that do not induce an immune response must be developed.

The development of gene therapy for neuropsychiatric diseases suffers, in addition, from many of the same problems that drug therapeutics research on these disease does. Neurodegenerative disorders such as Alzheimer's disease pose particular problems for gene therapy because neurons in the CNS cannot undergo regeneration. Therefore, gene therapeutic approaches must target the remaining brain cells, provide suitable replacements for the dying cells, or enable regeneration of CNS neurons. Another set of hurdles arises from the complex etiology of most neuropsychiatric disease. It is not clear that a single gene product will cure any of these diseases. In addition, the molecular mechanisms of different neuropsychiatric diseases may be restricted to subsets of neurons at specific times during development and maturity. Consequently, as noted above, optimal strategies for gene therapy must utilize vectors that persist stably in postmitotic cells and that can be targeted both spatially and temporally in the nervous system. Present-day viral vectors have come of age for use in basic research, but vectors useful for gene therapy in the brain are still a work in progress.

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