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GENE TARGETING AND TRANSGENIC TECHNOLOGIES

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Recent progress in the development of molecular genetic methods enables the manipulation of genes in intact mammalian organisms. The power of such techniques to elucidate complex biological systems was initially recognized and exploited by developmental biologists and immunologists. More recently, the utility of these approaches for examining neural gene function in the context of the intact organism has led to their use in neuropsychopharmacology. Since the publication of the previous edition of this book, there has been an explosion in the application of molecular genetic technologies to study the regulation of complex behavior and its modulation by psychoactive drugs.

For several decades, the ability to manipulate genes in organisms such as yeast, fruit flies, and nematodes has produced important insights into the regulation of a wide variety of complex biological processes. Limitations in the use of such organisms for research in neuropsychopharmacology arise from the marked organizational differences between the mammalian brain and the systems that govern behavior in these organisms. By contrast, a substantial degree of homology exists in the organization of the central nervous system (CNS) and in the complement of genes expressed across mammalian species. Currently, the mouse genome is by far the most accessible mammalian genome to manipulation. Procedures exist in the mouse for introducing new genes, expressing elevated levels of endogenous genes, and eliminating or altering the function of identified target genes.

Mutant mouse models may be used for a number of purposes relevant to neuropsychopharmacologic research. For example, the impact of genetic mutations on the behavior of mutant mice may be examined, providing insights into the functional significance of particular gene products. In some cases, the manifestations (phenotypes) of these mu-

tations may resemble features of human neuropsychiatric diseases, providing animal models for studying neural processes relevant to such disorders. Furthermore, as genes that confer susceptibility to human diseases are identified, it will be possible to introduce corresponding mutations into the mouse genome, generating useful models for studying disease pathophysiology and treatment. Finally, genetic models will be useful for investigating mechanisms through which nonselective drugs influence neural function and behavior. For example, the contribution of a particular receptor subtype to the actions of a nonselective drug may be examined by studying its actions in animals with targeted loss-of-function mutations of that receptor gene.

This chapter provides an overview of the transgenic and gene targeting approaches used to manipulate the mammalian genome. We have divided these techniques into three categories: (a) transgenic technologies, in which exogenous gene sequences are inserted into the mouse genome; (b) gene targeting technologies, in which mutations are targeted to inactivate or otherwise modify an endogenous gene of interest; and (c) conditional genetic manipulations, in which mutations are restricted to particular stages of development or to particular regions of the CNS. In addition to a brief description of these technologies, examples of their application to neuropsychopharmacology are provided, as well as discussions of the benefits and limitations of each approach.

TRANSGENIC PROCEDURES

The ability to insert an exogenous (or foreign) gene into the mouse genome by direct injection into the pronuclei of zygotes was achieved just two decades ago (1). The term *transgenic* was applied to mice expressing exogenous DNA that had been produced using this technique (2). With this method, the gene of interest is inserted into a random locus in the mouse genome, and is expressed "in trans," i.e., not in its usual genetic locus. The techniques required for intro-

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ducing transgenes into the mouse genome have been highly refined, permitting their widespread use. Since the development of this technique, many thousands of lines of transgenic mice have been generated, and it has been the most widely utilized technique of genetic manipulation in mice.

Methods of Production of Transgenic Mice

Techniques for producing transgenic mice involve the microinjection of DNA constructs into fertilized mouse eggs (Fig. 19.1). DNA constructs used for the generation of transgenic mice typically consist of a gene of interest located 3′ to promoter sequences selected to produce a desired distribution of gene expression. The maximum length of the DNA sequence that may be successfully incorporated into the mouse genome is not known, and up to 70 kilobase (kb) DNA fragments have been successfully integrated. The transgene is linearized and purified from prokaryotic vector sequences. For optimal integration efficiency, about 1 to 2 picoliter (pL) of DNA at a concentration of 1 to 2 ng/μL (corresponding to a few hundred molecules of a 5-kb DNA fragment) is microinjected into the male pronucleus of a fertilized mouse egg. Although labor intensive, direct injection of DNA into the pronucleus results in much higher

rates of integration of transgenes than other known methods of transformation. After microinjection, the embryos are surgically transferred into the oviduct of pseudopregnant mice. Pseudopregnant females are generated by matings with vasectomized males. The act of copulation initiates the endocrine changes of pregnancy, providing a suitable uterine environment for the survival and implantation of the transferred embryos. The foster mothers give birth 19 to 21 days after oviduct transfer. For genotyping, DNA is typically isolated from mouse tail biopsies and screened for the presence of the transgene by Southern blotting or polymerase chain reaction (PCR). Typically, about 20% to 40% of the mice that develop to term possess the transgene. In the majority of cases, integration of the transgene occurs during the one-cell stage, so that the transgene is present in every cell of the transgenic mouse. Integration usually occurs at a single random chromosomal location, and, for reasons that are not fully understood, there are usually multiple copies of the transgene inserted as head-to-tail concatamers. Mice identified to possess the integrated transgene are referred to as *founders*. The founders are typically used in a breeding strategy to produce animals that are homozygous for the transgene insertion.

Uses of Transgenic Mice

Because transgenic mice often possess multiple copies of the transgene, this method can be used to produce animals with increased levels of expression of particular genes, i.e., mice that "overexpress" genes of interest. In addition, it can be used to express altered forms of a gene product in the distribution of the endogenous gene. One example is a transgenic line bearing a transgene composed of the Ca^{2+}/calmodulin-dependent protein kinase α subunit (CaMKIIα) promoter driving expression of a mutant form of CaMKIIα that conferred Ca^{2+}-independent activation. These mice exhibited an increased stimulation threshold for the induction of synaptic plasticity in the hippocampus, as well as deficits in spatial memory (3,4). Studies of these animals led to an enhanced understanding of the role of CaMKIIα in synaptic plasticity and spatial memory acquisition.

In many cases, it is desirable to express a gene with an anatomic distribution that does not mirror its native expression pattern in the mouse. Such ectopic expression of a gene may be achieved using a transgenic construct in which the gene of interest is preceded by promoter elements that direct expression in an anatomic distribution characteristic of another gene. An example of this approach is a transgenic line in which the D1 dopamine receptor promoter was used to drive expression of a cholera toxin subunit (which constitutively activates G_s) in cells that express D1 dopamine receptors (5). Studies of these animals revealed that chronic overstimulation (by constitutively activated G_s) of forebrain neurons expressing D1 receptors results in an abnormal behavioral phenotype that was likened to human compulsive
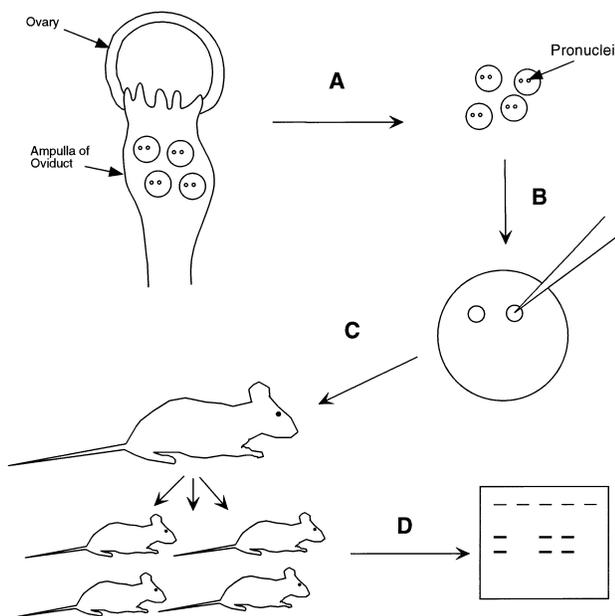
FIGURE 19.1. Procedure for production of transgenic mice. **A:** One-celled fertilized zygotes located in the oviduct ampullae of pregnant donor mice are surgically harvested. **B:** DNA encoding the gene of interest is microinjected directly into the pronucleus of the zygotes. **C:** Injected zygotes are surgically transferred into the oviducts of pseudopregnant female mice. **D:** DNA from the progeny can be analyzed by Southern blot or polymerase chain reaction (PCR) for the presence of the transgene.

behaviors. For most genes, the promoter elements necessary to reproduce the native patterns of expression are not well defined. A useful approach for identifying important promoter elements for genes of interest involves the generation of transgenic mice in which putative promoter sequences are used to direct expression of *reporter genes*, whose expression is readily determined in brain tissue. Comparisons may then be made between the pattern of reporter gene expression and that of the gene of interest (6–8).

It is also possible use transgenic approaches to reduce the expression of a particular gene product. This may be achieved using ''dominant-negative'' mutations, mutations that induce loss of function of a gene product when expressed in the heterozygous state. For example, transgenic constructs may be designed to express antisense RNAs that hybridize to native messenger RNA (mRNA) sequences, thus decreasing production of the gene product of interest (9–11). Alternatively, the function of gene products that aggregate into multimeric complexes may be disrupted by dominant-negative mutations that produce dysfunctional subunits of the complex. The most prevalent approach used to generate loss of function mutations, gene targeting, is described in the next section.

Considerations in the Interpretation of Transgenic Mouse Phenotypes

An important factor that frequently complicates the interpretation of studies with transgenic mice is the difficulty that may be encountered in achieving a desired anatomic distribution of transgene expression. Promoter elements are often quite large, and additional regulatory elements may at times be located great distances from the gene of interest. In addition, the site of integration often affects the pattern and level of transgene expression, so that founder mice generated with a common targeting construct may display different expression patterns. It may therefore be difficult to accurately duplicate a promoter's endogenous pattern of gene expression in the setting of a transgenic mouse. Commonly, expression patterns are assessed in multiple founders, and those with the most appropriate transgene expression would then be selected for a particular experiment.

Several additional considerations in the interpretation of phenotypes in transgenic mice warrant mention. For example, the number of copies of the transgene incorporated into the genome varies between founder mice. In some cases, concatamers can be unstable and susceptible to deletion of one or more copies of the transgene. In addition, the integration of the transgene may occasionally disrupt an endogenous gene. This could lead to the development of a phenotypic abnormality unrelated to the function of the transgene—this is estimated to occur in 5% to 10% of transgenic mice (12). This possibility may be assessed by determining whether similar phenotypes are present in animals derived independently from different founders, be-

cause the likelihood of two founders possessing the same transgene integration site is minimal.

GENE TARGETING PROCEDURES

A mutational approach has proved to be invaluable to investigators examining the roles of gene products in complex biological processes within prokaryotic and cultured eukaryotic cells. Recently it has become possible to apply this approach to a mammalian system. Gene targeting procedures enable the precise (site-specific) introduction of a mutation to one of the estimated 100,000 murine genes. Typically, mutations have been designed to eliminate gene function, resulting in the generation of ''knockout'' or ''null mutant'' mice. The introduction of mutations that produce more subtle alterations in gene function has also been achieved. Two major developments have made gene targeting experiments feasible: (a) the generation of totipotent embryonic stem (ES) cells, and (b) the elucidation of techniques to achieve homologous recombination in mammalian cells.

Gene Targeting Methods

ES cells are derived from 3.5-day-old mouse embryos, at the blastocyst stage of development (Fig. 19.2). Blastocysts are cultured individually under conditions that permit the proliferation of the inner-cell mass cells, which are those cells that would normally become the fetus. These cells are then disaggregated, and individual ES cells clones are grown. Under optimal conditions, ES cells retain the ability to contribute to all of the tissues of the developing fetus. The derivation of ES cells was pioneered using embryos derived from the 129/Sv strain of mice, a strain that has been most commonly used in studies of early embryonic development. Although this mouse strain is not ideal for the study of behavior (see below), most ES cell lines in current use are 129/Sv-derived.

Homologous recombination is the process by which a mutation is targeted to a precise location in the genome. A targeting construct is generated that typically consists of a target gene sequence into which a loss-of-function mutation has been engineered (Fig. 19.2). Most targeting constructs are designed to achieve homologous recombination events in which recombination at the target locus results in replacement of native target sequences with construct sequences. In mammalian cells, fragments of DNA preferentially integrate into the genome at random locations, at rates that greatly exceed homologous recombination. Therefore, targeting constructs are designed for use in selection strategies that enrich for ES clones in which homologous recombination has occurred. In the commonly used positive-negative selection strategy (13), a portion of a protein-coding exon is replaced by sequences that confer resistance to the drug neomycin. This mutation serves two purposes: (a) to inacti-
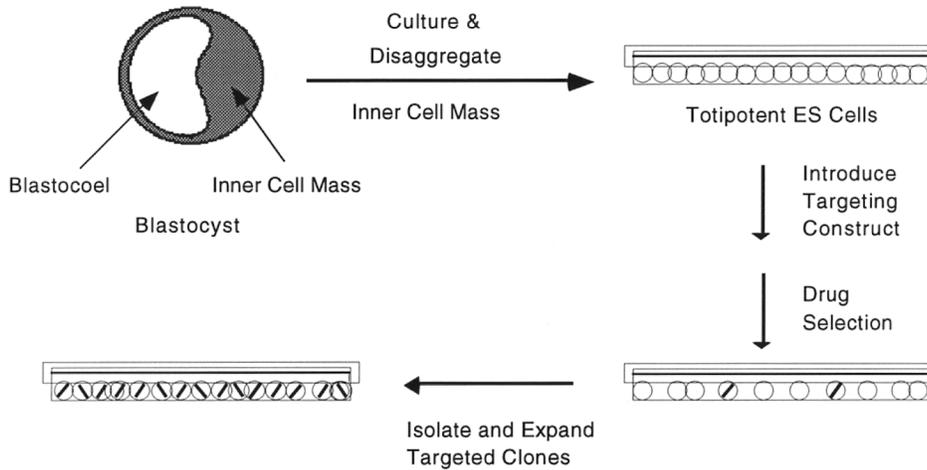
FIGURE 19.2. Gene targeting in embryonic stem (ES) cells. **A:** ES cells are derived from the blastocyst inner cell mass. A targeting construct is introduced into ES cells by electroporation. Cells are subjected to drug selection to enrich for homologous recombinant clones (*striped cells*). Homologous recombinant clones are isolated for blastocyst injection. **B:** A targeting construct is produced in which the second exon of the gene of interest is replaced by a neomycin resistance cassette (Neo). A thymidine kinase (TK) cassette is included for negative selection. Homologous recombination results in the incorporation of engineered mutation into endogenous gene locus. *Arrows* indicate the junction of construct sequences and native locus.
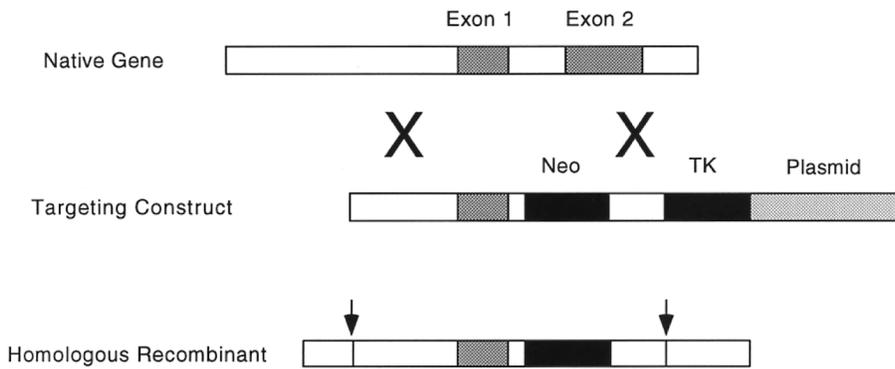
vate the gene product, and (b) to provide a marker that enables the selection of cells that have integrated the construct. This exogenous DNA fragment is flanked by regions of DNA that are homologous to the native gene. Adjacent to one of these homologous regions is a gene encoding thymidine kinase. Treatment with the drug ganciclovir will kill cells that express this gene.

The targeting construct is typically introduced into ES cells by electroporation. In this step, cells are subjected to an electric current that facilitates the internalization of the DNA construct. Those cells that failed to incorporate the targeting construct are killed by the addition of neomycin to the culture medium (positive selection). The majority of the remaining cells have incorporated the entire DNA construct (including the thymidine kinase gene) at random sites throughout the genome. By contrast, during homologous recombination, nonhomologous regions of the construct that are not flanked by homologous sequences are excluded from the integration event. Therefore, homolo-

gous recombinant clones will not contain the thymidine kinase gene. Thus, the addition of a second drug, ganciclovir, will selectively kill cells that have randomly incorporated the construct (negative selection), thereby enriching for targeted clones. ES cell clones that survive this double drug selection are then screened for homologous recombination by PCR or Southern blot analysis. The homologous recombinant clones, which are heterozygous for the introduced mutation, are then used to generate chimeric mice.

Following the isolation of homologous recombinant ES cell clones, these cells are microinjected into the fluid-filled blastocoele cavity of 3.5-day-old embryos at the blastocyst stage (Fig. 19.3). The injected embryos are then surgically transferred into the uterus of pseudopregnant females. These animals will then give birth to chimeric mice, which are derived partly from the injected ES cells and partly from the host embryo. For example, ES cells derived from a brown strain of mice are often injected into embryos derived from black C57BL/6 mice, resulting in chimeras with coats
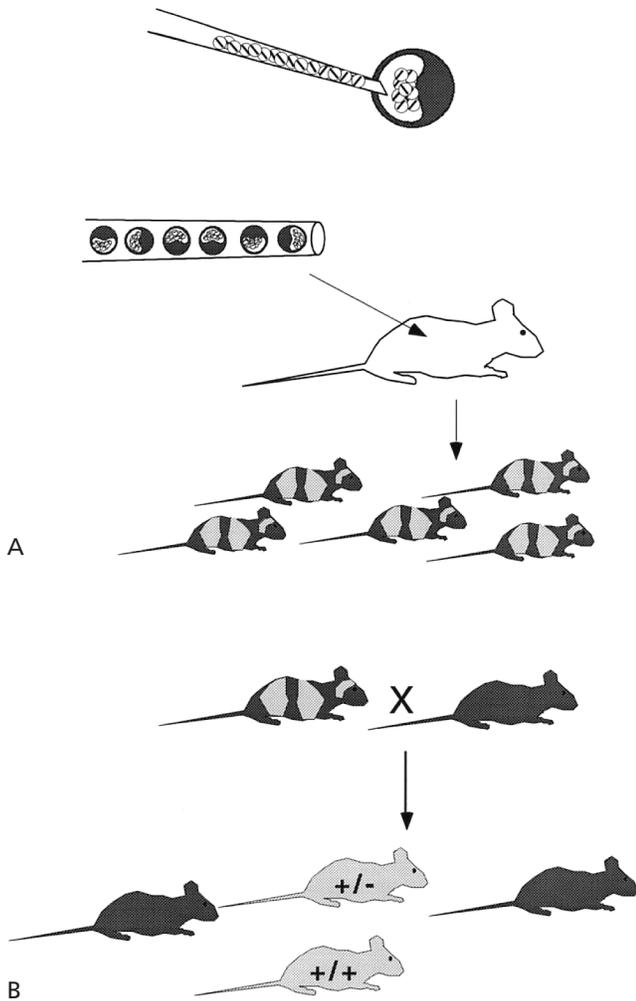
FIGURE 19.3. Generation of gene targeted mice. **A:** Homologous recombinant ES cells are injected into the blastocoele cavity. **B:** Injected blastocysts are surgically transferred into the uterus of pseudopregnant female mice for the production of chimeric mice. **C:** In this example, chimeric mice are bred with C57BL/6 animals. Germ-line transmission is indicated by coat color. One-half of animals with the coat color indicative of the ES cell line will be heterozygous for the targeted mutation and the other half wild type. Heterozygous animals may be bred for the production of homozygous mutant mice.

Uses of Gene Targeted Mice

Studies of null mutant mice provide novel insights into the functional roles of neural genes and, in some cases, animal models relevant to human neuropsychiatric disorders. An illustrative example is a recent study of mice lacking the hypothalamic neuropeptide orexin (14). Observations of homozygous mutant mice revealed an unanticipated phenotypic abnormality. The mutants displayed frequent episodes of inactivity characterized by the sudden collapse of the head and buckling of the extremities. Subsequent electroencephalogram (EEG) analysis revealed these episodes to be similar to narcoleptic attacks observed in humans and in a strain of narcoleptic dogs. Moreover, a mutation of an orexin receptor gene was found to underlie the canine syndrome (15). Thus, the null mutant phenotype revealed a novel role for orexin in sleep regulation. In addition, this line of mice represents an important animal model for examining the pathophysiology and treatment of narcolepsy.

Another example illustrates the potential utility of null mutant mice to uncover mechanisms underlying the behavioral effects of psychoactive drugs. The nonselective serotonin (5-hydroxytryptamine, 5-HT) receptor agonist m-chlorophenylpiperazine (mCPP) interacts with several subtypes of 5-HT receptors. Although this compound typically reduces locomotor activity in rodents, it produced a paradoxical hyperlocomotor response in a line of 5-HT_{2C} receptor null mutant mice (16). This response to mCPP was blocked by pretreatment with a 5-HT_{1B} receptor antagonist, indicating that the absence of 5-HT_{2C} receptors unmasked a hyperlocomotor effect of mCPP on 5-HT_{1B} receptors in mutant mice. These results provide a model whereby genetic endowment may contribute to the development of a paradoxical drug response. When a compound alters the function of multiple gene products with opposing influences on behavior, then mutations or allelic variation of these genes may lead to paradoxical effects.

Although gene targeting techniques are most commonly used to generate animals with null mutations, subtle mutations may also be introduced that alter, but do not eliminate, function. The benefits of such an approach are highlighted by a mutation of a gene encoding the α_1 subunit of the γ-aminobutyric acid A ($GABA_A$) receptor (17). The mutation produced a single amino acid change, rendering the α_1 subunit–containing subpopulation of $GABA_A$ receptors insensitive to benzodiazepines, without affecting their responsiveness to GABA. The resulting animals exhibited reduced sensitivity to the sedative and amnestic effects of diazepam, but no change in sensitivity to the anxiolytic-like effects of this drug. These results indicate that benzodiazepine site ligands devoid of activity at α_1 subunit–containing $GABA_A$ receptors may act as anxiolytics devoid of some of the side effects typically associated with benzodiazepines, a prediction borne out by a recent report of the behavioral effects

containing black and brown patches. The extent to which the ES cells have colonized the animal may be roughly approximated by the extent of the brown contribution to the coat. It is most important that ES cell derivatives colonize the germ cells of the chimera, so that the targeted mutation can be propagated to subsequent generations. If chimeras are mated with C57BL/6 mice, then the germ line transmission of ES cell–derived genetic material is indicated by the generation of brown offspring. Half of these brown mice will be heterozygous for the targeted mutation. These heterozygous mice are then bred to produce homozygous mutant mice that completely lack the normal gene product.

of such a compound (18). These insights would not have been obtained using a conventional gene targeting approach, because a null mutation of the α_1 subunit gene would profoundly perturb brain GABA signaling.

Considerations in the Interpretation of Targeted Mutant Phenotypes

In interpreting behavioral phenotypes, attention must be paid to the effects of genetic background. The phenotypic consequences of many targeted mutations may be influenced by modifying genes that differ among various inbred strains (19). In some cases, phenotypic abnormalities have been lost when mutations were bred to a new genetic background (20). It may therefore be useful to examine the persistence of mutant phenotypes in the context of several genetic backgrounds. In one example, three groups independently generated lines of mice with null mutations of the 5-HT$_{1A}$ receptor subtype (21–23). Interestingly, although each group placed this mutation on a different genetic background, all observed enhanced anxiogenic-like behaviors in the mutant lines. Thus, particularly strong evidence is provided for a contribution of the 5-HT$_{1A}$ receptor to the regulation of anxiety.

Another potential problem arises from the common use of ES cells derived from 129/Sv mice. Mice of this strain are susceptible to structural abnormalities of the CNS, such as agenesis of the corpus callosum, and are impaired in several behavioral assays (19,24). This potential problem may be addressed through breeding programs to place targeted mutations on different inbred backgrounds, and by the generation of ES cell lines derived from other inbred strains. It has been recommended that the C57BL/6 and DBA strains be used as standards, due to the extensive body of data relating to the behavioral characterization of these strains (19).

In addition to the above strain considerations, the standard application of gene targeting technology has several inherent limitations. The null mutations engineered into knockout mice are typically constitutive, i.e., they are present throughout embryonic and postnatal development. Therefore, the potential for developmental perturbations is a major caveat to the interpretation of mutant phenotypes in adult animals. It may be difficult to determine whether a mutant phenotype reflects a normal adult role for the targeted gene or an indirect effect of the mutation attributable to perturbed development. Such an effect may lead to an overestimation of the functional significance of the gene product in the adult animal. Conversely, if significant compensation for the loss of a gene product occurs during development, then the severity of the mutant phenotype may underestimate the functional significance of the gene product. The nature of such compensatory mechanisms and the extent to which they exist may be difficult to determine.

The above considerations also pertain to the analysis of transgenic mice carrying constitutive mutations.

Another limitation of the standard gene targeting technology is that the mutations are ubiquitous, present in all of the cells of the animal. Thus, if a neural gene of interest is also expressed in peripheral tissues, then the absence of the gene product peripherally could lead to a lethal or altered phenotype, independent of its neural role. Moreover, for genes that are widely expressed in the CNS, it may also be difficult to anatomically localize the brain region(s) that underlie the mutant phenotypes. New techniques to overcome these problems by achieving region-specificity and inducibility of targeted mutations are under development, and are described in the next section.

PROCEDURES FOR ENGINEERING CONDITIONAL MUTATIONS

New technologies are under development for circumventing the limitations of standard gene targeting approaches by creating mutations that may be induced in adult animals and/or restricted to particular brain regions. Although these strategies are not yet in widespread use, it is likely that rapid advances in this area will lead to an exponential increase in the generation of such "conditional mutations" over the next several years.

Cell Type–Specific Mutation Strategies

When a null mutation of a gene results in a mutant phenotype, limitations in the interpretation of that phenotype can arise because the gene is inactivated in every cell in which it was expressed in the mouse in the wild-type (WT) state. Therefore, the observed abnormal phenotype may arise from the absence of the functioning gene product in a peripheral organ system, the peripheral nervous system, or the CNS—i.e., in any of those regions in which the gene is normally expressed. It is possible that the absence of a gene product in the periphery may lead to embryonic lethality, precluding use of the mutant for the studies of neural function. For genes that are widely expressed within the CNS, it may be difficult to identify neural circuits through which mutations produce behavioral perturbations. The ability to inactivate genes in restricted subpopulations of the cells that normally express them will be a valuable asset in studies to uncover the neural mechanisms underlying neural phenotypes.

Recent efforts have focused on a mutational strategy developed to exert spatial control over the pattern of expression of genetic changes introduced into mice. This approach utilizes somatic cell recombination rather than germ cell (or embryonic stem cell) recombination to inactivate a gene in restricted populations of cells or tissues. In this approach, a tissue-specific promoter is used to direct expression of one

of the site-specific recombinases (25) to limit gene inactivation to only those cells expressing the recombinase. The two recombinase systems that have been utilized for genetic manipulation in mice have been the Flp-frt system from yeast (26), and the Cre-lox system from bacteriophage P1 (27–29), with the large majority of reports using this technique utilizing the Cre-lox system.

Cre (cyclization of recombination) recombinase is a 38-kd protein from bacteriophage P1, which recognizes and catalyzes reciprocal DNA recombination between two loxP (locus of crossing over of P1) sites. The loxP site is the 34–base pair (bp) recognition sequence for Cre composed of a palindromic 13-bp sequence separated by a unique 8-bp core sequence (Fig. 19.4A). A gene or gene segment with flanking loxP ("floxed") sites will be excised by homologous recombination in the presence of Cre, leaving a single loxP site marking the point of excision and re-ligation of upstream and downstream DNA (Fig. 19.4B). This approach, then, involves generating two independent lines of mice—a line bearing loxP sites, and a transgenic line in which Cre expression is driven by a tissue-specific promoter. Animals with a gene or gene region of interest flanked by loxP sites (floxed) are generated by gene targeting. Because the loxP sites are relatively small and placed in intronic regions, they do not typically interfere with normal gene transcription. Of course, WT patterns and levels of expression need to be documented in these floxed mouse lines, because inadvertent placement of lox sites into promoter elements or RNA splice sites could disrupt gene expression. The Cre mice are most commonly generated by creating a transgenic line of mice in which Cre expression is driven by a tissue-specific promoter. As discussed above in the section on transgenic mice, variability in transgenic expression patterns requires several lines of Cre mice that need to be generated and assayed for patterns of Cre expression. An alternative strategy is to use gene targeting procedures to place Cre under the control of an endogenous promoter (30). The advantage of this approach is that Cre expression should closely approximate the WT expression pattern of the gene it is replacing because the original gene's promoter remains in its endogenous location. A potential disadvantage is that Cre may disrupt expression of the gene into which it has integrated. Once a line exhibiting the desired pattern of Cre expression is identified, it is crossed with an appropriate floxed line to commence a breeding strategy resulting in the generation of animals with a restricted pattern of gene inactivation (Fig. 19.4C).

Several lines of Cre mice have been reported in which expression is restricted to subpopulations of cells within the CNS (31–35). The first example of this approach was the inactivation of the glutamate receptor subunit NMDAR1 in CA1 pyramidal neurons of the hippocampus, with expression in other brain areas mostly intact (31). NMDAR1 is the predominant *N*-methyl-D-aspartate (NMDA) receptor subunit and is widely expressed in most CNS neurons. It had been previously demonstrated that widespread gene inactivation in NMDAR1 null mutants resulted in perinatal lethality (36,37). When the mutation was restricted to hippocampal CA1 neurons, animals were viable and exhibited impaired spatial learning and impaired plasticity at CA1 synapses (31). Thus, spatial restriction of neural mutations can be used to uncover particular brain regions or cell type through which gene inactivation alters behavior.

The utility of this approach for producing cell-type–specific inactivation of genes is enhanced by the fact that the components of the system produced in one laboratory can be "mixed and matched" with components from another laboratory. That is, Cre lines generated for use with a particular floxed gene may also be used with other floxed genes when a similar pattern of gene inactivation is desired. Collaborative efforts to generate databases of Cre and floxed lines will speed and simplify the production of animals with restricted patterns of gene inactivation.

Inducible Mutation Strategies

As described above, the absence of a gene product throughout development complicates the interpretation of mutant phenotypes. Efforts are currently under way to overcome this limitation through the use of gene expression systems that may be induced in the adult animal. Strategies are under exploration for achieving this goal using a variety of compounds, such as tetracycline, steroid receptor antagonists, and ecdysone to induce gene expression. Although these approaches have yet to be optimized for general use, this development is likely to be close at hand. The tetracycline system has been the most utilized and best developed approach to inducible gene regulation.

Since the introduction of the Tet system by the Bujard laboratory in 1992, many laboratories have validated the utility of this approach to inducible gene regulation, and many refinements/improvements in the system have been introduced (38). This system is based on the regulatory elements of a tetracycline resistance operon of *Escherichia coli*, in which the transcription of tetracycline resistance genes is negatively regulated by the tetracycline repressor (tetR) (Fig. 19.5). When tetracycline is present, tetR binds the tetracycline and loses its capacity to bind to the operator sequences (tetO) located within the promoter of the tetracycline resistance operon, and transcription is permitted. By creating a fusion protein composed of the tetR and a potent transcriptional activator, VP16, a tetracycline-dependent transactivating factor (tTA) was produced that retained the DNA binding and activation specificity of the tetR. The desired regulatable gene of interest is placed under tetO plus a minimal promoter (P_{min}), that contains the basic promoter elements required for transcription in all cell types. Activation of this system requires the binding of the tTA to the tetO operator sequence (39). The presence of tetracycline,
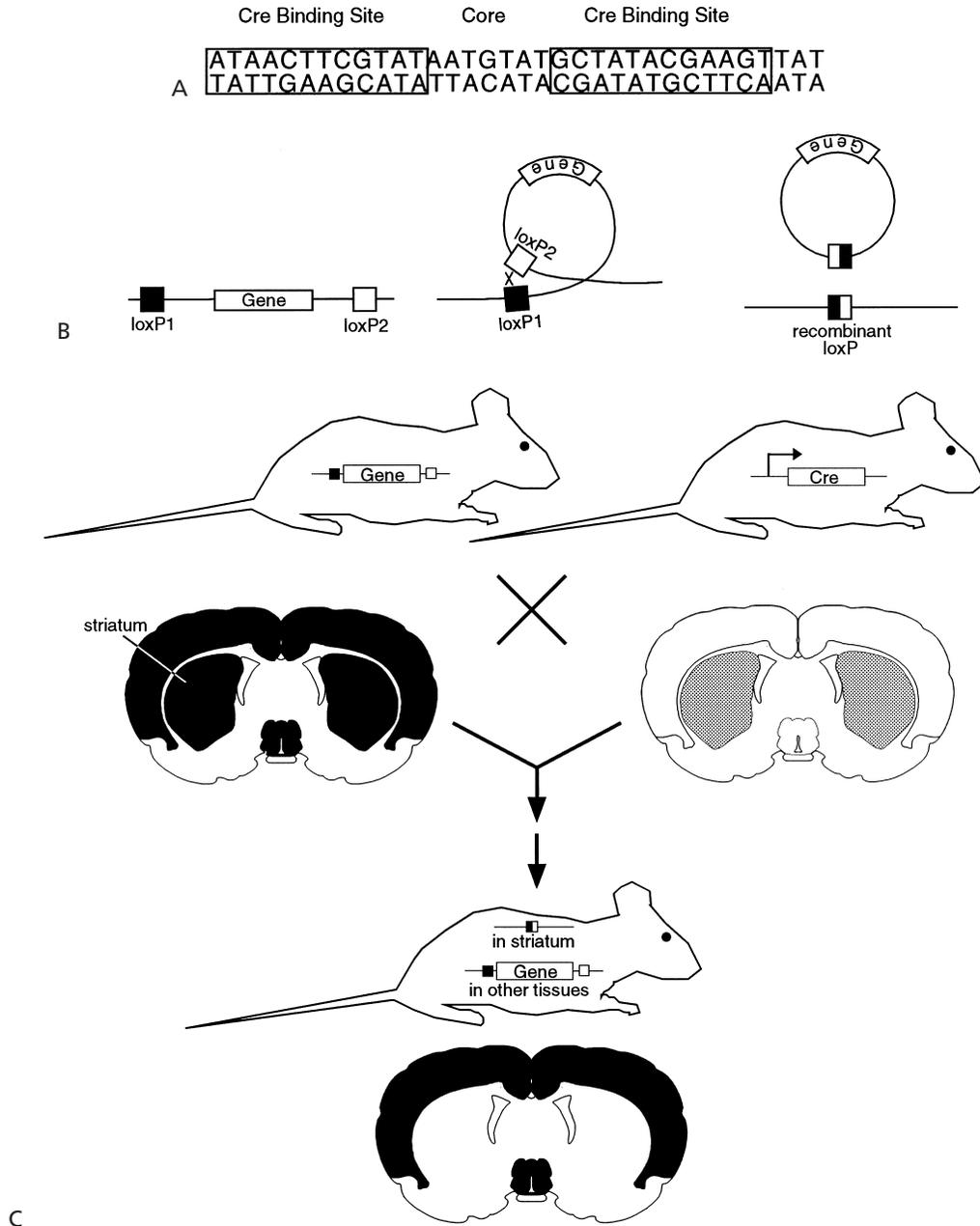
FIGURE 19.4. Strategy for cell-type–selective mutations using Cre-mediated recombination. **A:** The loxP DNA sequence indicating the core region and the inverted repeats that constitute Cre binding sites. **B:** In the presence of Cre, a gene flanked by tandomly oriented loxP sites (floxed gene) will be excised by homologous recombination. The recombination occurs in the core region of the loxP site, leaving a single recombinant loxP site in the genome after Cre excision. **C:** Use of the Cre-lox system to generate cell-type–specific gene inactivation in mice. A "floxed mouse" is generated by gene targeting to introduce loxP sites flanking a gene of interest. The wild-type expression pattern of this gene is shown (*black*) in the coronal section beneath the floxed mouse. In this example, there is expression of the gene in the cortex (ctx), striatum (str), and hypothalamus (hypothal). This floxed mouse can be crossed to a transgenic mouse expressing Cre in a distribution dictated by the promoter used in the transgene construct. In this example, Cre expression is shown (*gray*) and is limited to the striatum. A breeding program is pursued to produce animals in which expression of the floxed gene is normal except in the striatum, where the expression of Cre results in the excision of the floxed gene.
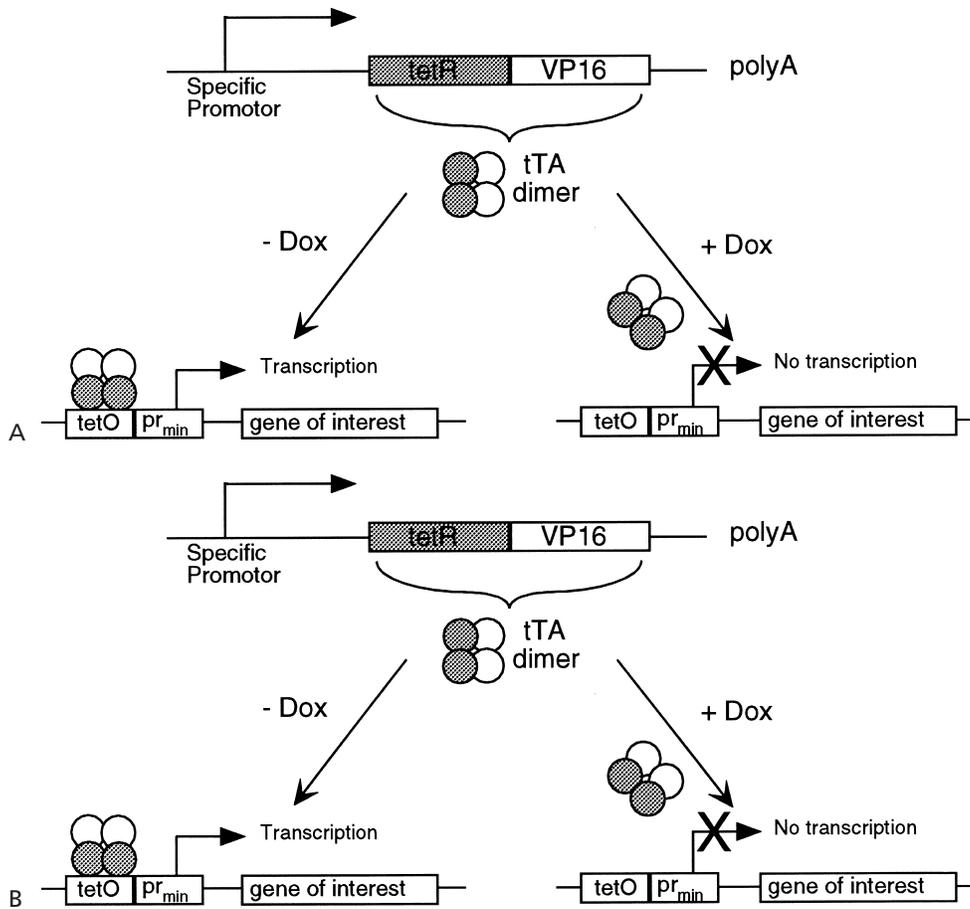
FIGURE 19.5. Tetracycline-regulated expression systems. **A:** Tet-off system. The tetracycline-controlled transactivator, tTA, is a fusion protein consisting of the tetracycline repressor (tetR) domain and a transcriptional activation domain (VP16). tTA homodimerizes, and in the absence of tetracycline (or the tetracycline analogue doxycycline) activates transcription of the gene of interest that has been placed downstream from tetO and a minimal promoter. Binding of doxycycline (Dox) to the tTA dimer prevents the binding of tTA to tetO, and transcription of the gene is prevented. Therefore, the tTA system has been called the Tet-off system, because in the presence of doxycycline, transcription is prevented. **B:** Tet-on system. In the Tet-on system, the tetracycline-controlled activator has been mutated to reverse the action of doxycycline on the transactivator. By contrast with tTA, doxycycline binding to rtTA enables the complex to bind to tetO and activate gene transcription. In the absence of doxycycline, rtTA is unable to bind to tetO and cannot activate transcription. Therefore, the rtTA system is also called the Tet-on system, because doxycycline activates transcription of the regulated gene.

or other suitable ligand such as doxycycline, prevents tTA from binding to tetO and activating transcription of the gene of interest. This is referred to as the *tet-off* system—that is, when tetracycline is present, transcription is off. A *tet-on* system has also been developed, in which tetracycline induces transcription of the gene of interest. It utilizes a reverse tetracycline transcriptional activator (rtTA), designed so that it would bind to tetO and activate transcription only in the presence of tetracycline-related compounds (38). Doxycycline is most frequently used because it is a potent regulator in both the tet-off and tet-on systems (38), and can be easily supplied to mice through their water or food supply (40,41).

The tet-off and tet-on systems are binary systems—i.e., they require two genetic elements to be introduced into mice. First, a tissue-specific promoter can be used to express tTA or rtTA in a region or cell-type specific manner; then the gene of interest is inserted behind tetO and a minimal promoter. This can be achieved by creating two separate transgenic lines of mice and then cross-breeding to produce bigenic lines. In these lines, expression of the gene of interest may be induced by doxycycline (tet-on) or by the discontinuation of doxycycline treatment (tet-off). For example, the

tet-off system has been used to investigate the effects of the transcription factor ΔFosB on psychostimulant responses. A line of mice was generated in which expression of a ΔFosB transgene was suppressed by continuous doxycycline treatment throughout development. Discontinuation of treatment in adult animals led to overexpression of the transgene in the nucleus accumbens and to augmentation of the rewarding and locomotor stimulant properties of cocaine (42). The utility of the tet-on system has also been demonstrated. For example, a line of mice was developed to examine the role of the Ca^{2+}-activated protein phosphatase calcineurin in synaptic plasticity. Treatment of these animals with doxycycline induced calcineurin overexpression in restricted forebrain regions, associated with deficits of neuronal plasticity and spatial learning (41).

Rather than generating regulatable gain of function mutants with the Tet system, regulatable loss of function mutants can also be generated by combining the Tet system with the Cre-lox system (43,44). In this arrangement, a cell-type–specific promoter drives rtTA expression and Cre is linked to tetO and a minimal promoter. In the presence of doxycycline, Cre is expressed in the cell type specified by the promoter used to drive rtTA expression, and somatic

cell recombination excises floxed DNA fragments in those cells—achieving an inducible cell-type–specific knockout. This inducible knockout approach may be utilized to circumvent concerns discussed above in the interpretation of gene knockout phenotypes.

In these inducible knockout mice it must be remembered that although the excision of the floxed gene can be induced relatively quickly, the appearance of any phenotype resulting from the absence of the gene product will occur gradually, depending on the degradation rate of the relevant mRNA and the half-life of the protein. Another important limitation of strategies utilizing the Tet system relates to the inherent "leakiness" of the tetO operator; i.e., low levels of unwanted gene expression may occur during periods in which gene expression is expected to be turned off. This may be problematic when the inducible transgene is toxic or has significant effects even when expressed at very low levels. Recent findings with tetracycline controlled transcriptional silencers indicate that it may be possible to modify the tet system to substantially reduce unwanted gene expression (45). In addition, work has begun on alternative inducible gene expression systems with low levels of basal expression. One such system utilizes the insect hormone ecdysone as an induction signal.

SUMMARY

The development of transgenic and gene targeting technologies is significantly enhancing understanding of cellular and molecular functions of genes and their contributions to neural processes relevant to clinical disorders. In some instances, novel roles for receptor subtypes have been revealed by the observation of unexpected phenotypic abnormalities in mutant animals. Mutant strains are also providing models for studying the pathophysiology and treatment of particular neuropsychiatric diseases. In addition, some mutant mouse models are useful for investigating the mechanisms of action of psychoactive drugs. Although mutant mouse models represent powerful tools in neuropsychopharmacology, they do not replace older methods of investigation. These models may be best used as components of integrated multidisciplinary research efforts spanning multiple levels of analysis.

This chapter briefly surveyed the most common strategies used for manipulating the mouse genome, and cited the advantages and limitations of each approach. Progress in the development of conditional mutagenesis strategies will address many of the current limitations, and facilitate uncovering the neural mechanisms through which mutations alter neural systems to impact behavior. Although this field is still in its infancy, an exponential increase in the application of molecular genetic technologies is anticipated to contribute substantially to our understanding of brain function in health and disease.

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