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ORIGINAL ARTICLE

Gene-Targeting Technologies for the Study of Neurological Disorders

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Abstract

Studies using genetic manipulations have proven invaluable in the research of neurological disorders. In the forefront of these approaches is the knockout technology that engineers a targeted gene mutation in mice resulting in inactivation of gene expression. In many cases, important roles of a particular gene in embryonic development have precluded the in vivo study of its function in the adult brain, which is usually the most relevant experimental context for the study of neurological disorders. The conditional knockout technology has provided a tool to overcome this restriction and has been used successfully to generate viable mouse models with gene inactivation patterns in certain regions or cell types of the postnatal brain. This review first describes the methodology of gene targeting in mice, detailing the aspects of designing a targeting vector, introducing it into embryonic stem cells in culture and screening for correct recombination events, and generating chimeric and null mutant mice from the positive clones. It then discusses the special issues and considerations for the generation of conditional knockout mice, including a section about approaches for inducible gene inactivation in the brain and some of their applications. An overview of gene-targeted mouse models that have been used in the study of several neurological disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, seizure disorders, and schizophrenia, is also presented. The importance of the results obtained by these models for the understanding of the pathogenic mechanism underlying the disorders is discussed.

Index Entries: Mouse; knockout; conditional; inducible; genetics; disease model; Alzheimer's disease; Parkinson's disease; Huntington's disease; seizure; schizophrenia.

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Introduction

Gene-targeting technology has revolutionized our ability to study gene function. It allows the generation of any type of mutation in mouse genes. The potential to manipulate virtually any gene of interest has been greatly facilitated by the publication of a draft sequence of the mouse genome (Mouse Genome Sequencing Consortium, 2002), which enabled researchers to readily obtain DNA clones containing genomic sequences of a given gene. The types of mutations that can be introduced include null mutations; deletion of specific functional domains; insertions; point mutations; and gene replacements in which an exogenous cDNA is targeted to an endogenous promoter, resulting in ectopic expression of the encoded protein. Generation of deletion mutations can be achieved by "knocking out" a genomic region, whereas insertion or exchange mutations can be introduced by "knocking in" the modified DNA. Since the differences among varying gene-targeting approaches are relatively minor, the most common of them, the production of null mutations, will be discussed here, with special reference to the extension of this application in the generation of null mutants under specified conditions. Since the first reports of null mutations in mice in 1987 (Hooper et al., 1987; Kuehn et al., 1987), hundreds of null mutant mice have been generated, and their analysis has provided important information regarding the functions of the disrupted genes. However, in many cases the targeted genes have proven to be required for normal embryonic development and survival, making it impossible to study gene function at postnatal stages, especially in the adult brain. To address this important limitation, methodologies have been developed to restrict gene disruption to specific embryonic or postnatal stages or to specific cell types or tissues. This has been made possible by the use of the Cre/loxP and Flp/FRT site-specific recombination systems to generate conditional knockout mice.

This review focuses on the description of the technical aspects of gene-targeting technologies and provides an overview of their applications for the study of neurological disorders. First, the technical principles that govern production of null mouse mutants are described, since this technology also constitutes the basis of more sophisticated gene-targeting approaches. The special issues of generating conditional mutations are then discussed, including an outline of systems for inducible gene inactivation in the brain. A section about genetic background considerations in gene targeted mutant mice follows. Finally, the importance and potential of gene-targeting technology in the research of neurological disorders is illustrated by an overview of mouse models that have provided valuable information about the pathogenesis of Alzheimer's disease, Parkinson's disease, Huntington's disease, seizure disorders, or schizophrenia.

Generation of a Null Mutation by Gene Targeting

Targeting a null mutation to a specific gene in the murine germline involves three principal steps. First, genomic sequences are obtained for the gene of interest and the desired mutation is introduced into the cloned gene, such that the resulting mutant allele is incapable of generating a functional protein product, following homologous recombination. Second, the targeting vector containing the desired mutation along with a drug-resistance marker is introduced into embryonic stem (ES) cells; resistant ES clones are then screened for the correct homologous recombination events. Owing to the low efficiency of homologous recombination, only one of the two genomic alleles of the targeted gene usually undergoes recombination, and thus, the positive ES clones are heterozygous for the desired mutation. Third, the positive ES clones are injected into mouse embryos at the blastocyst stage, and the blastocysts are implanted into host mothers. By virtue of their pluripotency, the injected ES cells have the potential to contribute to most or all of the cell lineages of the resulting chimeric mouse, including the germline. The chimeric mice are then mated with mice of appropriate genetic background to obtain F1 heterozygotes for the desired mutation, which are then bred to obtain F2 homozygotes. The strategy and procedure of these three steps are discussed in detail in the following sections.

Designing a Targeting Vector

When one chooses to study the function of a particular gene, the partial or complete cDNA sequence of this gene is often available, but sometimes not

the genomic sequence. In these cases, the first step is to isolate genomic clones containing the region that will be disrupted, which is usually one or more of the amino-terminal coding exons. The traditional way to achieve that is to use a cDNA probe (polymerase chain reaction [PCR]- or restriction endonuclease-generated) containing the exon(s) to be disrupted or part of it to screen a genomic library derived from the mouse strain to be used for gene targeting. Overlapping genomic clones should be obtained, subcloned and carefully mapped with restriction enzymes, and the intron–exon boundaries should be sequenced to locate the exon precisely within the genomic clone. After publication of a draft sequence of the mouse genome (Mouse Genome Sequencing Consortium, 2002), the task of cloning the genomic region of interest has become easier and is usually narrowed down to purchasing the sequenced genomic clone to which the region belongs and subcloning the sequence that will be targeted.

To make an effective targeting construct, it is important to select an appropriate region of the gene to be disrupted. Disruption is usually accomplished by replacement of an exon(s) of choice with an open reading frame, in the form of a selection marker. If the gene contains only one coding exon, then the choice is simple, but mammalian genes often contain many small exons separated by rather large introns. In order to maximize the chance of producing a null mutation, the first coding exon is often chosen, because a disruption there leads to the loss of the endogenous start codon. Although putative inframe start codons sometimes exist in the downstream coding sequence, aberrant translation initiating from these sites is likely to generate a nonfunctional truncated protein. An alternative choice is to disrupt a nonintegral coding exon(s) located close to the amino-terminal end, loss of which will cause a frameshift mutation.

In the construction of the targeting vector, a common strategy involves the replacement of the genomic sequence containing the critical exon(s) with a positive selection marker. The bacterial neomycin-resistance (*neo*) gene, driven by a mammalian promoter that is expressed in ES cells and linked to a downstream polyadenylation signal, is often used. The promoter of choice for expression of the *neo* gene is derived from the phosphoglycerate kinase gene (*PGK*), because it drives high levels of constitutive expression that is relatively independent of neighboring DNA sequences. Geneticin (G418) can then be used to select for neomycin-resistant ES clones in which the targeting sequence is stably integrated into the genome.

The disrupted region is flanked by upstream and downstream genomic sequences—the left and right arms, respectively—which provide the substrate for homologous recombination events. Several factors that can be manipulated experimentally influence the frequency of homologous recombination. First, the rate of recombination is proportional to the length of the homologous sequences surrounding the disrupted region. The optimal length of homologous regions is often between 2 and 5 kb for each arm (although if PCR is to be used for screening, one arm should be shortened to 1–2 kb). Although longer flanking sequences may result in higher frequencies of recombination, it can be difficult to determine whether the G418-resistant ES clones contain the correct recombination events if the arms are too long. Second, any sequence differences between the cloned genomic DNA in the targeting construct and the genomic DNA of the ES cells reduce the rate of recombination drastically. Since as many as 1/500 nucleotides can differ between two mouse strains, the genomic library and ES cells should be derived from the same mouse strain. Third, the rate of recombination is heavily dependent on the specific locus, i.e., the sequence of the homologous region, but this variable is difficult to control since the sequence-dependence of homologous recombination is poorly understood. Finally, the observed proportion of integration events resulting from homologous recombination rather than random, nonhomologous integration can be increased by the addition of a negative selection to the positive selection outlined above (so-called "positive–negative selection"). For this purpose, cDNA encoding thymidine kinase (tk) or diphtheria toxin A (dtA), under the control of a strong promoter such as the *PGK* promoter, is inserted at the external end of either homologous region. Homologous recombination events will then result in excision of the tk or dtA cDNA, whereas it will be retained when the entire construct is randomly integrated in a nonhomologous fashion. In the presence of the cytotoxic nucleoside analogs, gancyclovir or FIAU, only those G418-resistant clones in which the tk cDNA has been removed by homologous recombination will survive. When dtA cDNA is used, clones that contain the dtA sequence

will die without the need for addition of any drug to the culture medium.

Before transfecting the targeting construct into ES cells, it is important to identify two external probes that will be used to screen for correct recombination events occurring at both the upstream and downstream homologous regions (left and right arms). If the initial screening is done by PCR, the structure of the targeted region must be confirmed by genomic Southern analysis with the external probes. Even if a correct homologous recombination event is identified within one arm, the recombination event at the other arm may be aberrant in some way about 10–30% of the time. The 5′ external probe should be a segment of genomic DNA located upstream of the left arm in the targeting construct, whereas the 3′ external probe is located downstream of the right arm. Since exons in mammalian genes are often surrounded by huge introns, the upstream and downstream flanking regions of the disrupted exon may be located within the neighboring introns. Since introns often contain repetitive sequences that are unsuitable for use as a probe in genomic Southern analysis, the identification of efficient external probes can be quite troublesome. Potential external probes may be tested before ES screening on wild-type genomic DNA to verify their competency. In addition to the external probes, a probe for the *neo* gene should also be used to confirm that the positive clones do not contain a randomly integrated targeting vector, in addition to the homologously recombined targeting sequence.

Screening for ES Clones Carrying Correct Recombination Events

Once a targeting vector is constructed, the linearized vector can be introduced into ES cells by electroporation. After application of a high voltage electrical pulse, the DNA passes through pores in the cell membrane. As mentioned earlier, the DNA may be incorporated into the genome either by random insertion or by homologous recombination at frequencies of around $1/10^4$ or $1/10^{5–7}$, respectively. The transfected cells are then selected in G418-containing medium. Five to 7 d later, the resistant colonies can be picked under a microscope and expanded in 96-well microtiter plates. Genomic DNA is then isolated from each clone, and analyzed for correct homologous recombination events by Southern analysis using one of the two external probes. The positive clones from this initial analysis should be further tested with the other external probe. At least two confirmed clones should be expanded for injection into blastocysts.

Great care should be taken at this stage to prevent differentiation of the pluripotent ES cells. ES cells are derived from the inner cell mass (ICM) of mouse blastocysts, and are capable of contributing to most or all tissues in chimeras. Under stringent culture conditions, these cells can maintain their embryonic developmental potential even after many passages, DNA transfections, and drug selections. Genetic alterations introduced into ES cells can be transmitted to the germline by the production of chimeras in which the germ cells are derived from the injected ES cells.

Although this step requires little previous experience to master, the following issues should be taken into consideration before starting the ES work. First, the culture conditions are important in the maintenance of ES cell pluripotency. ES cells grow on a feeder layer of embryonic fibroblasts (EF cells) that provide essential growth factors, such as leukemia inhibitory factor (LIF). LIF is required for the maintenance of ES cell pluripotency, and it is therefore also added to the culture medium. The water used for making medium should be purchased from a commercial source or purified by a Millipore Q filtration system. The quality of fetal calf serum used in the medium is also critical for the maintenance of ES cells. Different batches should be tested for their ability to support growth of pluripotent ES cells. Suitable batches should be ordered in large quantities and can be stored at –20°C for several years.

Second, ES cells should be propagated and maintained under strict sterile conditions. It is important to have a dedicated tissue culture facility including a laminar flow hood and a humidified CO_2 incubator. This practice reduces the risk of mycoplasma contamination from other tissue culture cells. While handling the cells, researchers should take extra precautions not to contaminate the culture, since mycoplasma infection does not have an obvious effect on cell growth or morphology, but can cause chromosome damage and reduce the efficiency of obtaining ES cell chimeras.

Third, it is crucial to obtain ES cells that have been tested for germline transmission. If this has not been done, it is important for investigators to determine

the pluripotency of the ES cells by testing their ability to generate high quality chimeras, before carrying out transfection experiments. The EF cells can be easily obtained from 13 to 16 d mouse embryos carrying a functional neomycin resistance gene (either transgenic or knockout animals), and are then treated with mitomycin C or γ-irradiation to be prevented from dividing and thus interfering with ES cell growth.

Generation of Chimeras and Homozygous Null Mutant Mice

Positive ES clones carrying the targeted mutation are injected into 3.5 d blastocysts, and then the injected blastocysts are returned to the uterus of pseudopregnant females. Resulting high-percentage male chimeras, as determined by the contribution of injected ES cells to the coat or eye color, are mated to an inbred line. Offspring with parental DNA derived from injected ES cells, as recognized by their coat or eye color, are genotyped for the presence of the mutant gene, since the recombinant ES cells are usually heterozygous. Mice bearing the mutant gene are heterozygous, and crosses among them generate wild-type, heterozygous, and homozygous progeny according to Mendelian ratios. The genotypes of the offspring should first be confirmed by Southern analysis using the same 5′ and 3′ probes as for ES cells. In the subsequent generations, the genotypes can be determined by PCR using a common primer for one strand and two different primers specific for either the wild-type or the mutant allele for the other strand, such that they give rise to PCR products of distinct sizes that represent wild-type or targeted allele.

Proficiency with blastocyst injections requires training by an experienced investigator, as well as the availability of a mouse colony and injection apparatus. Since ES cell lines are commonly derived from mouse strain 129, which has an agouti coat color, C57BL/6J mice are often selected as donors of blastocysts because of their black coat color, permitting a ready assessment of the resulting chimeras. Female mice from a hybrid strain are preferable for use as pseudopregnant recipients for the injected blastocysts, because they are better mothers, and are more likely to carry pregnancies to term. The injection apparatus includes an antivibration table, an inverted microscope with a cooling stage, and two micromanipulators.

Several days prior to injection, ES cells should be thawed and placed in several wells of a 24-well plate without the EF feeder layer, since the larger EF cells are prone to lysis and clog the injection pipets. On the injection day, ES cells should be treated with fresh medium 1 h before trypsinization, and then the dissociated cell suspension can be used for about 2 h if kept on ice to retard the lysis process. During the injection process, ES cells and blastocysts are stored in small drops of medium, and a cooling stage is used to reduce adherence of cells and increase the rigidity of the blastocysts. After injection blastocysts often collapse, and should be allowed to recover in injection medium in a 5% CO_2 incubator at 37°C. The blastocysts are then returned to the uterus of pseudopregnant females. Once the offspring are about 10 d old, it becomes rather easy to judge the quality of the chimeras from the coat color. If the coat color is mostly agouti, the chimera is likely to be largely derived from the ES cells, including the germ cells.

Time Frame for the Production of a Null Mutant Mouse

Generation of a null mutant requires at least 8 mo (if all goes smoothly). Construction of the targeting vector takes about 2 mo if isolation of genomic clones from a library is required or about 1 mo if a genomic clone is purchased. Isolation and identification of mutant ES clones can be accomplished in 2 mo. Blastocyst injection and subsequent matings to obtain homozygotes requires another 5 mo.

Generation of Conditional Mutations by the Cre/loxP, Flp/FRT or Inducible Systems

A major limitation of the use of knockout technology to study gene function in the nervous system is the embryonic lethality often caused by null mutations, precluding the characterization of gene function in the adult brain. In addition, the elimination of gene function in all tissues of an animal limits the ability to attribute a phenotype to the loss of gene function in a particular tissue or cell type. In these cases spatial and/or temporal restrictions on gene disruption can be achieved, so that gene function is

turned off at a specific time and/or in specific tissues or cell types.

This technology, of generating conditional knockout mice, relies on the use of site-specific recombinases. Cre is a bacteriophage P1-encoded recombinase that catalyzes homologous recombination between two 34 bp loxP recognition sites, resulting in the excision of the intervening DNA sequences (Sternberg and Hamilton, 1981). LoxP sites may be introduced into a targeting vector so that they flank the genomic region to be deleted (a "floxed" gene, for flanked by loxP sites). It is crucial that these insertions do not interfere with the normal expression (e.g., transcription, splicing, translation) of the gene, and therefore, the loxP sites are often introduced in intronic region without affecting the splice donor or acceptor sequences. The targeting vector is then introduced into the genome of ES cells by homologous recombination and mice homologous for the floxed locus are generated from the ES cells carrying the floxed gene. These mice are then bred with a mouse expressing the Cre recombinase as a transgene under the control of a tissue-specific promoter, or with a knockin mouse in which the Cre recombinase is expressed under the control of an endogenous promoter following gene targeting. Progeny bearing both alleles of the floxed gene and the Cre transgene will undergo deletion of floxed sequences in those cells or tissues in which Cre is expressed, thus generating a pattern of gene disruption dependent on the spatial and temporal pattern of Cre expression. This technique allows inactivation of any gene in any tissue at any stage of embryonic or postnatal life, depending on the availability of transgenic mice that express Cre in spatially and temporally restricted manner. An extension of this system is the inducible control of Cre expression on application of an exogenous agent, thereby conferring further temporal regulation of the disruption of the floxed gene.

Designing a Targeting Vector

Once the exon(s) to be disrupted is selected, a loxP site and a loxP–*PGK*–*neo*/*tk*-loxP cassette should be inserted in the flanking introns. Any other promoter capable of driving high levels of expression in ES cells and other positive and negative selection markers may also be used, instead of the *PGK* promoter and the *neo* and *tk* markers. The targeting vector thus comprises the upstream homologous region, the upstream loxP site, the region to be disrupted, the downstream loxP–*PGK*–*neo*/*tk*-loxP cassette, and the downstream homologous region (Fig. 1). An additional negative selection marker, such as dtA driven by the *PGK* promoter, can be inserted external to either homologous region in order to decrease the number of ES clones carrying the targeting vector by random integration. The *neo*-resistant ES clones are screened for correct homologous recombination events in both homologous regions by Southern analysis using both external probes.

At this stage, the positive ES clones contain not only the loxP sites, but also positive- and negative-selection genes (*neo* and *tk*, respectively) under the control of the strong *PGK* promoter. The presence of the exogenous *PGK* promoter may affect the expression of the targeted gene and its neighboring genes (Olson et al., 1996). In addition, expression of the *tk* gene may prevent germline transmission of the targeted locus (Gondo et al., 1994). Therefore, the *neo*–*tk* cassette should be removed by transient transfection of Cre in the ES cells, as discussed in the next section.

This technique can be used in a similar way to generate subtle mutations, such as deletion of a functional domain, or "knocking in" a point mutation or an exogenous cDNA to an endogenous promoter. The difference with these applications is that only two loxP sites are required. The targeting constructs in such cases include the upstream homologous region, the targeting region containing a deletion, point mutation or an exogenous cDNA, the selection cassette flanked by loxP sites, the downstream homologous region, and the additional negative selection marker.

Removal of the Selection Cassette in ES Cells

To remove the positive- and negative-selection cassette flanked by loxP sites, a small amount of supercoiled Cre-encoding plasmid is introduced into the targeted ES cells by electroporation. After selection in gancyclovir-containing medium for 5 d, the surviving clones are picked and expanded. DNA prepared from the ES clones is tested for the loss of the cassette by Southern analysis. Since three loxP sites are present in the targeted clones, three different patterns of deletions can occur (Fig. 1). One of the three loxP sites is located upstream of the targeting region, and the other two flanking the selection

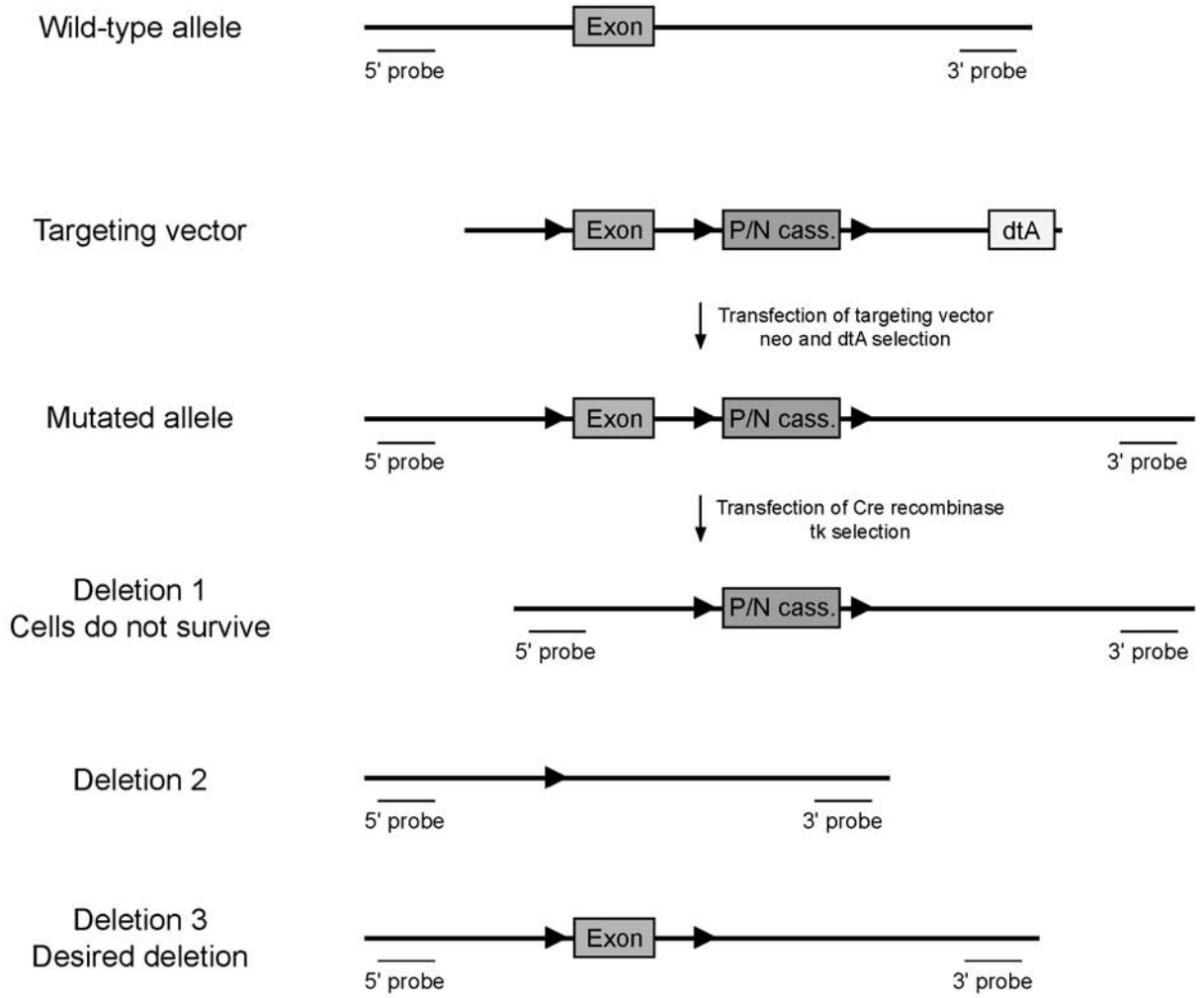

Fig. 1. Gene targeting with three loxP sites. The steps involved in the generation of a floxed allele using a strategy with three loxP sites are depicted. The targeting vector contains the upstream homologous region (left arm), the upstream loxP site, the exon to be disrupted, the positive/negative selection cassette (P/N cass.)—such as the *PGK–neo/tk* cassette—flanked by loxP sites, the downstream homologous region (right arm), and an additional negative selection marker, such as the *dtA* gene driven by the *PGK* promoter. After transfection into ES cells and positive and negative selection for the *neo* and the dtA genes, respectively, ES clones are screened for correct homologous recombination events using the 5′ and 3′ external probes. Positive clones are then transfected with a Cre-expressing plasmid which will delete sequences between two loxP sites, and undergo negative selection for the *tk* gene. Three possible deletion events can occur: (1) the targeted exon is deleted and the selection cassette remains; these cells will not survive the selection process because of the presence of the *tk* gene, (2) both the targeted exon and the selection cassette are deleted, and (3) only the selection cassette is deleted and the targeted exon remains, which is the desired pattern. Dark triangles: loxP sites.

cassette are located downstream of the targeting region. Therefore, ES cells carrying a deletion that leaves the selection cassette intact will not survive in gancyclovir-containing medium, since the *tk* gene is present in their genome (*see* null mutation section). The remaining two types of deletion—removal of the selection cassette vs removal of both the targeting region and the cassette—can be distinguished

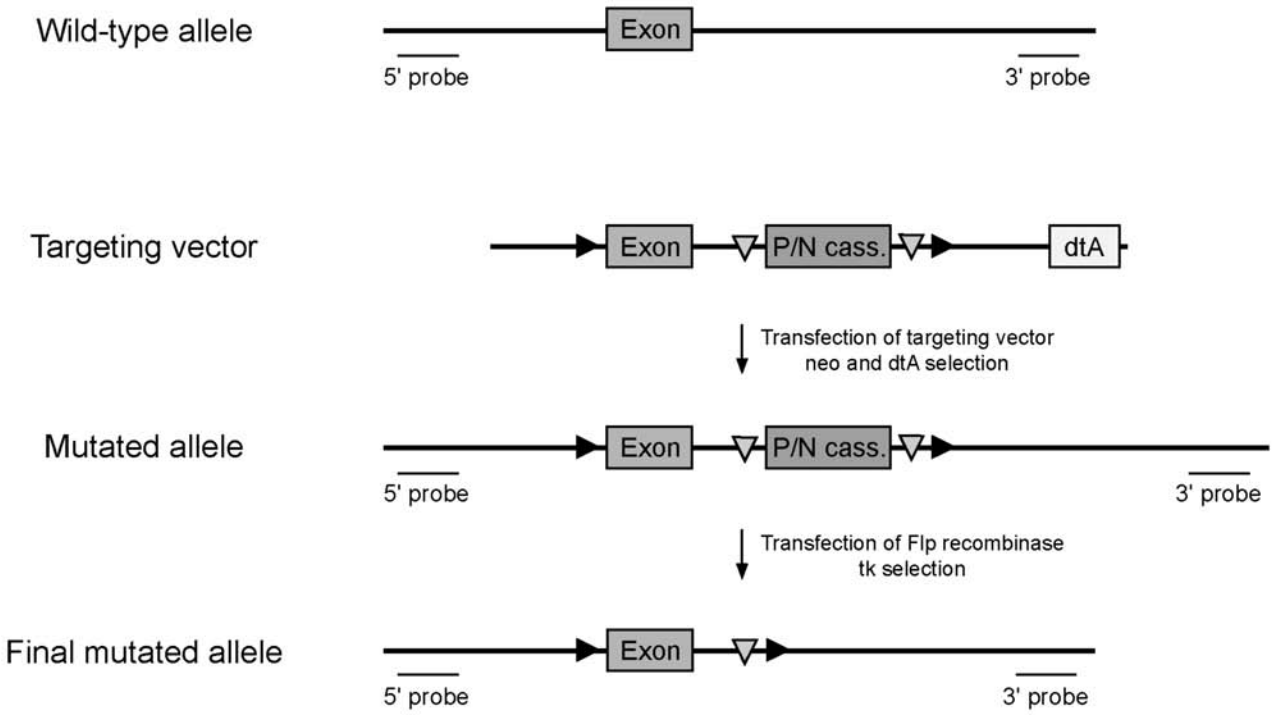

Fig. 2. Gene targeting with loxP and FRT sites. A strategy for the generation of a floxed allele by a combined Cre/loxP and Flp/FRT system is shown. The targeting vector comprises the upstream homologous region (left arm), the upstream loxP site, the exon to be disrupted, the positive/negative selection cassette (P/N cass.)—such as the *PGK–neo/tk* cassette—flanked by FRT sites (light triangles), the downstream loxP site, the downstream homologous region (right arm), and an additional negative selection marker, such as the *dtA* gene driven by the *PGK* promoter. As in the strategy with three loxP sites, the first step includes transfection into ES cells, positive and negative selection for the *neo* and the *dtA* genes, and screening of ES clones for correct homologous recombination events using the 5′and 3′ external probes. Subsequent removal of the selection cassette from positive ES clones is accomplished by transfection with a Flp-encoding plasmid, which will excise the sequence between the two FRT sites, with only one possible deletion pattern. Dark triangles: loxP sites.

by different banding patterns on Southern analysis. The ES clones carrying the desired deletion—removal of the selection cassette alone—will be expanded for injection into blastocysts. It is important that the strategies applied for ES cell screening be appropriate so as to ensure the selection of the correct site-recombination event, since positive ES clones may bear heterogeneous deletion patterns due to persistent Cre activity following ES cell division. If initial screening is carried out by PCR, positive clones should be further tested by Southern analysis, which provides a better estimation of the precentage of correctly recombined cells in the clone, based on band intensity. Thorough analysis should be performed also for genotyping of the F1 mice, since the heterogeneity in the Cre-mediated deletion patterns in ES cells will most likely be reflected in the mouse genotypes.

The technical difficulty of the removal of the selection cassette from a locus that contains three loxP sites is because of the very low frequency of the recombination event removing only the selection cassette, as opposed to the higher frequency of complete excision of all sequences between the outermost loxP sites. To overcome this hurdle, a combined Cre/loxP and Flp/FRT system has been developed (Fig. 2). Much like the Cre recombinase, the Flp recombinase in yeast catalyzes a site-specific

recombination reaction between two 47 bp recognition sequences (FRT sites) (Kimball et al., 1995), resulting in the excision of intervening sequences. This system, the applicability of which has been initially tested in ES cells and transgenic mice (Dymecki, 1996), has been successfully used for the removal of the selection cassette from a floxed allele in ES cells, e.g., in the generation of forebrain-specific calcineurin knockout mice (Zeng et al., 2001) that exhibit schizophrenia-like behavioral abnormalities (Miyakawa et al., 2003) as discussed later. With the addition of this system, the targeting vector still includes the upstream homologous region, upstream loxP site, the targeting region, the downstream selection cassette, the downstream homologous region, and the additional negative selection marker. However, the positive- and negative-selection cassette contains two FRT sites flanking the selection markers, and a loxP site located downstream of the FRT flanked markers (FRT–*PGK*– *neo/tk*–FRT–loxP). To remove the selection cassette in ES cells, Flp is transiently transfected into the ES cells to catalyze recombination between the two FRT sites. Since only one type of reaction can occur, it appears to be a more efficient method for removal of the selection cassette. The resulting ES clones, in which the two remaining loxP sites flank the genomic region to be deleted, are then used to generate homozygous mice.

Inducible Gene Inactivation

Another valuable extension of gene targeting technology is the ability to knock out genes inducibly. Most current approaches combine inducibility with conditional knockout technology by subjecting Cre expression to inducible control. The application of such systems to the central nervous system raises the important issue of the accessibility of the inducing agent to the target tissue, since the accessibility of many agents to the CNS is limited by the blood–brain barrier. Several inducible systems that have been successfully used for gene inactivation in the brain are discussed below.

The first inducible Cre-expressing transgenic mice were generated based on the ability of the inducible promoter of the mouse *Mx1* gene to be transiently activated in many tissues on application of interferon-α or -β (Kuhn et al., 1995). In this study, the inducible Cre mice, in which Cre was expressed under the control of the *Mx1* promoter, were crossed to mice carrying a floxed gene and the efficiency of

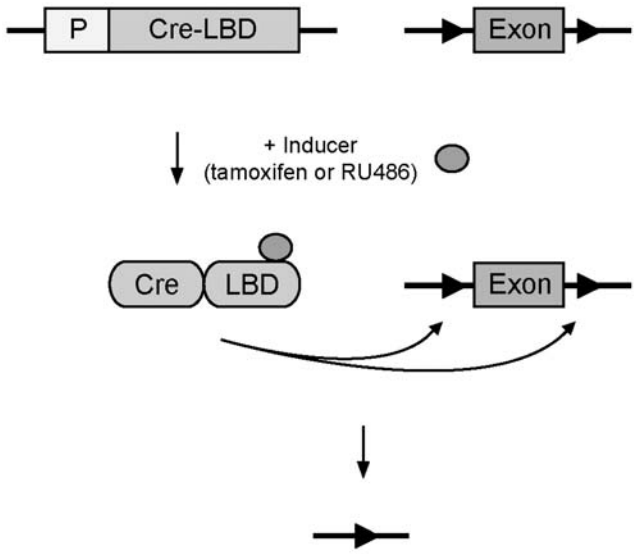

Fig. 3. Estrogen/progesterone inducible systems. The diagram illustrates the principles of inducible control of Cre/loxP-mediated gene inactivation by the use of estrogen or progesterone analogs. Cre recombinase is expressed under the control of a promoter of choice (P) as a fusion protein with the ligand-binding domain (LBD) of the estrogen or the progesterone receptor, engineered to bind only to the synthetic analogs tamoxifen or RU486, respectively, and not to the respective endogenous steroids. Mediated by the LBD, Cre activity is induced upon application of tamoxifen or RU486, thereby generating a gene inactivation pattern dependent on the temporal course of inducer administration, in addition to the control conferred by the expression properties of the promoter used.

Cre-mediated deletion in different tissues was assessed by Southern analysis. Deletion was quite efficient in hepatocytes and lymphocytes, but rather inefficient in the brain, rendering this system of little utility to neuroscientists.

In another approach for the inducible activation of Cre, induction is mediated by a ligand-binding domain that is fused to Cre (Fig. 3). Cre activity can be specifically induced by the synthetic estrogen analog tamoxifen but not by endogenous estradiol, when expressed as a fusion protein with a mutated ligand-binding domain of the estrogen receptor (Feil et al., 1996). Although in this study, expression of the fusion Cre protein, which was driven by the cytomegalovirus (CMV) promoter, removed less than 15% of target sequences in the

brain, use of the prion protein promoter with the same system resulted in more efficient recombination in various regions of the brain (Weber et al., 2001). The Cre transgenic mice from the latter study were successfully used for the inducible disruption of frataxin, resulting in morphological and behavioral phenotypes similar to those in Friedreich ataxia (Simon et al., 2004). In a similar system, Cre recombinase, fused to a truncated ligand-binding domain of the progesterone receptor, was induced in the brain of transgenic mice by the synthetic steroid RU486 but not by progesterone (Kellendonk et al., 1999). Expression of the fusion protein in these mice was under the control of regulatory elements of either the *CaMKIIα* or the *Thy-1* gene and strong induction of recombination was observed in the cortex and hippocampus (Kellendonk et al., 1999).

Tetracycline (Tc) control of transcriptional activation has received a great deal of attention for use with inducible knockout systems. The Tc-controlled transactivator (tTA) is a fusion protein of the *Escherichia coli* tetracycline repressor (tetR) and the transcriptional transactivation domain of viral protein 16 (VP16) of herpes simplex virus (Gossen and Bujard, 1992). In this system (Fig. 4), the expression of the tTA fusion protein is driven by a tissue- or cell type-specific promoter. The transgene of interest is placed under the control of a minimal promoter with multiple upstream tet operator (tet-O) sites. In the absence of Tc and its analogs, activation of the transgene results from the binding of tTA to the tet-O sites. In the presence of Tc or its analogs, the interaction of tTA with the operator sites is inhibited, resulting in repression of transgene transcription and thus providing an on/off switch for gene expression. This system was proven very promising to neuroscientists when it was successfully used in the mouse brain to turn off transgene expression, suggesting that the blood–brain barrier is sufficiently permeable to Tc analogs (Mayford et al., 1996). It has also been used in combination with the Cre/loxP system in the generation of inducible and reversible NR1 knockout mice, in which a tTA-controlled NR1 transgene reversibly rescued the knocked-out NR1 in the CA1 region (Shimizu et al., 2000), as well as in the generation of a transgenic line expressing Cre under *CaMKIIα*-driven tTA control (Lindeberg et al., 2002). Another application of the system has been the expression in transgenic mice of a tTA-regulated dominant-negative inhibitor of the CREB family transcription factors under the control of the *CaMKIIα* promoter (Pittenger et al., 2002).

A modification of the tTA system is the reverse Tc-controlled transactivator (rtTA) system (Gossen et al., 1995). The rtTA contains four amino acid changes in the tet repressor portion of tTA that render DNA binding dependent on the presence of Tc or its analogs, so that gene expression is induced rather than inhibited by their administration (Fig. 4). This system has been combined with the Cre/loxP system to provide rtTA-regulated Cre expression, with the expression of rtTA driven by the *retinoblastoma* gene promoter or the whey acidic protein promoter (Utomo et al., 1999). It has also been applied for the reversible expression of a calcineurin inhibitor under the control of the *CaMKIIα* promoter, transiently decreasing calcineurin activity in various regions of the brain, which improved LTP and memory specifically during the period when the inhibitor was expressed (Malleret et al., 2001).

For more information about inducible mouse models including examples of inducible overexpression and applications in other tissues, the reader is referred to a series of comprehensive reviews (Metzger and Feil, 1999; Lewandoski, 2001; Mills, 2001; Tronche et al., 2002; Morozov et al., 2003).

Genetic Background Considerations in the Generation of Gene-Targeted Mutant Mice

One of the most controversial issues in the use of gene targeting technology is the possible contribution of genetic background to the mutant phenotype, which is especially important in the study of gene function in the brain where a major part of the phenotypic characterization is often the analysis of performance in behavioral tests, which can be strongly influenced by the genetic background. ES cells are in most cases derived from mouse strain 129, whereas strain C57BL/6J is commonly used for crossing with chimeric mice leading to the F1 generation that includes the first heterozygotes for the targeted mutation. By some researchers it is considered optimal to analyze the mutant mice in a pure genetic background, which can be essentially achieved by backcrossing hybrid mice to an inbred strain for a high number of generations, thereby

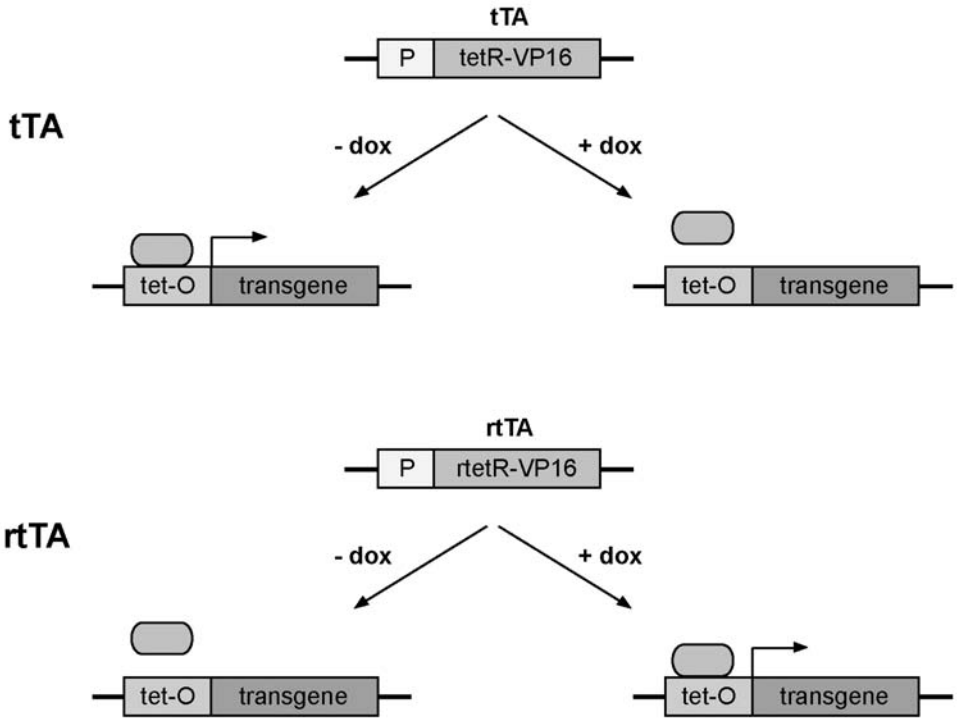

Fig. 4. Tetracycline inducible systems. Regulation of transcriptional activation by the tTA and the rtTA systems is depicted. The first system makes use of tTA, which is a fusion protein of the *E. coli* tetR and the transcriptional transactivation domain of viral protein 16 (VP16) of herpes simplex virus. Expression of tTA is driven by a promoter of choice (P). Expression of the transgene of interest is under the control of a minimal promoter with multiple upstream tet-O sites. In the absence of Tc analogs, such as doxycycline (dox), transgene expression is facilitated by the binding of tTA to the tet-O sites. In the presence of doxycycline, this binding is inhibited, resulting in repression of transgene expression. In the second system, rtTA is used, which contains four amino acid changes in the tetR part that render binding to the tet-O sites dependent on the presence of doxycycline, so that gene expression is induced rather than inhibited by doxycycline administration. rtetR: reverse tetracycline repressor.

eliminating genetic segregation of genes that can contribute to different phenotypes when derived from different strains.

However, there are several reasons to argue for the use of a hybrid background. Many strain-specific alleles that are responsible for poor performance in certain behavioral tests are recessive and their effect can be counteracted by the respective allele of a different strain. Indeed, several behavioral traits are less variable in hybrid mice than in inbred strains (Wolfer et al., 2002). Also, it has been suggested that the phenotypes of different hybrid lines (e.g., hybrid of strain A/strain B vs hybrid of strain B/strain C) should be more similar than the phenotypes of different inbred lines, and thus, employing a hybrid background can enable better comparison between results from different laboratories (Banbury Conference on Genetic Background in Mice, 1997). The same report proposes the use of F1 hybrids between the strains 129/J and C57BL/6, whenever possible, and suggests a breeding strategy for F1 production that involves generation of a 129/J and a C57BL/6 congenic strains by backcrossing the chimeric mice to 129/J or C57BL/6 inbred strains, respectively. In another report, a strategy for addressing the significance of the targeted mutation to a behavioral phenotype vs a possible contribution of other genes linked to the targeted gene is suggested, when hybrid mice from the F2 generation are used (Zimmer, 1996). According to this strategy that requires the use of polymorphic markers located near the mutated gene, the offspring

of wild-type F1 inter-breedings are also subjected to the same behavioral test and their phenotypes are compared with those of the F2 mutant mice.

Gene-Targeted Mouse Models for the Study of Neurological Disorders

The genetics that govern neurological conditions are among the most complicated in living systems. This reflects the high complexity of the nervous system and the very precise specificity that defines the function and plasticity of neurons and neuronal networks. Brain functions and behaviors are complex traits that involve distinct patterns of neuronal activity, and this is in part because of specific profiles of gene activation, suppression, and altered expression. Therefore, many genes that are expressed in the brain have various different functions either in the same cell, in different cells or brain regions, or in different developmental stages. For this reason, several genes that are involved in neurological disorders by altered function in certain regions of the adult brain are also important in other tissues or brain regions and/or during neurodevelopment. In order to overcome these multiple functions of a given gene and to focus on its importance in the disorder that is involved, the conditional knockout technology has been successfully used to inactivate genes only under the spatial and temporal course that is relevant to the respective disorder. This section presents an overview of gene targeting mouse studies that have provided valuable information about the genetics of several neurological disorders. In some of the mouse models that are discussed, the targeted gene(s) is an established genetic factor for the disorder, such as the presenilin genes in Alzheimer's disease or the parkin gene in parkinsonian syndromes. In other models discussed here, the mutant mice exhibit phenotypes similar to characteristics of a disorder supporting a role of the targeted gene in its pathogenesis, as in the case of β-catenin dorsal telencephalon-specific knockout mice that display phenotypes that resemble features of epilepsy (Campos et al., 2004). Special emphasis is given to conditional knockout studies.

Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia in the elderly population and is manifested by memory decline, followed by a more generalized loss of cognitive abilities. Neuropathologically, AD is characterized by neuronal and synaptic degeneration, and the appearance of amyloid plaques and neurofibrillary tangles. Early onset familial AD is caused by mutations in either of three genes, amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2), of which mutations in PS1 result in the earliest disease onset (Hutton and Hardy, 1997). PS1 and PS2 are components of a catalytic complex with γ-secretase activity responsible for the proteolytic cleavage of various proteins, including APP and the Notch receptors (De Strooper et al., 1998, 1999).

As a model system for the in vivo study of loss of presenilin function, *PS1*-null mice have been generated and shown to be perinatally lethal, and bear severe skeletal and CNS deficits at the embryo stage (Shen et al., 1997). These results demostrated important roles of PS1 in neurodevelopment, but the early lethality precluded the study of PS1 function in the adult brain. To achieve that, the Cre/loxP system has been used to generate conditional knockout mice in which PS1 is selectively inactivated in excitatory neurons of the postnatal forebrain (*PS1* cKO mice) (Yu et al., 2000, 2001). These mice are viable with no obvious morphological abnormalities and exhibit only a minor impairment in spatial memory (Yu et al., 2001). *PS2*-null mice, in contrast to *PS1*-null mice, are also viable with no major abnormalities (Herreman et al., 1999; Steiner et al., 1999). However, presenilin conditional double knockout (*PS* cDKO) mice, generated by the crossing of *PS1* cKO and *PS2*-null mice, exhibit memory and synaptic plasticity deficits shortly after PS1 inactivation is initiated, which progress with age and are accompanied by neuronal degeneration in the cerebral cortex (Saura et al., 2004). These impairments are associated with several molecular alterations including reduction in the expression of CREB/CBP downstream genes (Saura et al., 2004) and up-regulation of imflammatory molecules (Beglopoulos et al., 2004). Interestingly, conditional knockout mice lacking the transcription factor CREB and its family member CREM in the postnatal forebrain show age-dependent neurodegeneration (Mantamadiotis et al., 2002), raising the possibility that the neuronal loss in *PS* cDKO mice may be mediated by the decrease in CREB/CBP target genes. These results suggested that loss of presenilin function in the adult brain leads to phenotypes

that resemble neuropathological features of AD (Saura et al., 2004), and illustrate the importance of the conditional knockout technology in AD research.

APP is a member of a protein family, which also includes amyloid precursor-like proteins APLP1 and APLP2. Null mouse mutants for either of the three family members are viable and fertile (Zheng et al., 1995; von Koch et al., 1997; Heber et al., 2000). However, although double knockout mice for APP/APLP1 are viable, double knockout mice for APP/APLP2 or for APLP1/APLP2 show early postnatal lethality, suggesting functional redudancy between APLP2 and both other members of the family (von Koch et al., 1997; Heber et al., 2000). Given the importance of the results obtained by conditionally inactivating presenilins in the postnatal forebrain, it would be very interesting to generate and analyze similar conditional knockout mice for the amyloid precursor family of proteins.

Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder, in which the major clinical features are tremor, rigidity, and akinesia, and age is an important risk factor (Lang and Lozano, 1998). The most typical neuropathological signs of PD are death of dopaminergic neurons in the substantia nigra and the appearance of cytoplasmic inclusions called Lewy bodies. Mutations in the parkin gene, that encodes a ubiquitin protein ligase, are a major cause of early-onset familial parkinsonism (Vaughan et al., 2001). *Parkin* mutations are mainly inherited in an autosomal recessive manner, suggesting a loss-of-function mechanism as causative to the disease. Several groups have generated *parkin* null mouse mutants. Morphological analyses showed no loss of dopaminergic neurons in the substantia nigra of these mice (Goldberg et al., 2003; Itier et al., 2003; von Coelln et al., 2004). However, these mouse models display several pathological and behavioral phenotypes, such as increased extracellular dopamine concentration in the striatum (Goldberg et al., 2003), inhibition of amphetamine-induced dopamine release (Itier et al., 2003), impairments in mitochondrial function (Palacino et al., 2004), and loss of catecholaminergic neurons in the locus coeruleus (von Coelln et al., 2004). Recently, mutations in the genes *DJ-1* and *PINK1* were identified to be associated with autosomal recessive early-onset parkinsonism (Bonifati et al., 2003; Valente et al., 2004). It is of high interest to examine the effect of inactivation of these genes in mice by analyzing knockout models and to compare the phenotypes with those of parkin knockout mice, as well to generate and analyze multiple null mutants for combinations of *parkin*, *DJ-1* and *PINK1*.

Huntington's Disease

Huntington's disease (HD) is a type II trinucleotide repeat disorder characterized by movement disturbances and dementia, and by neuronal loss most prominently in the striatum. The cause of the disease is an expansion of a CAG trinucleotide repeat in the first exon of the *HD* gene, which encodes the cytoplasmic protein huntingtin, resulting in an abnormally long polyglutamine stretch (The Huntington's Disease Collaborative Research Group, 1993). Although the autosomal-dominant nature of HD and several studies have suggested a gain-of-function mechanism, loss of huntingtin function is also considered a mechanism that could contribute to the pathogenesis of the disease (Cattaneo et al., 2001; Menalled and Chesselet, 2002; Rubinsztein, 2002). Substantial support for the loss-of-function hypothesis came from analysis of huntingtin conditional knockout mice (Dragatsis et al., 2000). Germline inactivation of huntingtin results in embryonic lethality of homozygous mice (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995), indicating essential roles of hungtingtin in brain development, but rendering huntingtin null mice unsuitable as HD models. Mice with selective inactivation of huntingtin in the brain and testis, however, are viable, but exhibit progressive neurodegeneration, motor phenotypes, and early lethality (Dragatsis et al., 2000). The loss-of-function hypothesis is also suggested by findings that mutant huntingtin can recruit normal huntingtin into aggregates (Huang et al., 1998; Kazantsev et al., 1999; Narain et al., 1999; Wheeler et al., 2000), as well as by results showing that normal huntingtin upregulates the expression of brain-derived neurotrophic factor, a function that is lost when huntingtin is mutated (Zuccato et al., 2001).

Seizure Disorders

Epilepsy, a neurological disorder that is characterized by recurrent seizures and affects 1–2% of the population, can be caused by pathways resulted

from mutations in more than 70 genes that affect cortical synchronization (McNamara, 1999; Noebels, 2003). Genetic studies in mice have contributed significantly toward the identification of genes that could be associated with epilepsy, based on the seizure phenotype caused by the engineered mutation. β-Catenin is a signaling protein that has been associated with various neurological conditions, including AD with its function being altered by PS1 mutations (Zhang et al., 1998; Xia et al., 2001). Findings that β-catenin is increased by pilocarpine-induced status epilepticus (Fasen et al., 2002) or by electroconvulsive seizure treatment (Madsen et al., 2003) have suggested that β-catenin may also be implicated in the pathogenesis of epilepsy. Although β-catenin-null mice are lethal at the embryo stage (Haegel et al., 1995), mice conditionally lacking β-catenin only in the dorsal telencephalon are viable, but exhibit severe morphological abnormalities in the brain, such as reduced cortical size and absence of hippocampal structures (Campos et al., 2004). In addition, these mice display increased seizure susceptibility after PTZ injection, further suggesting a potential role of β-catenin in epileptogenesis (Campos et al., 2004).

Additional conditional knockout mouse models that present seizure phenotypes are mice, mutant for cannabinoid receptor type 1, *Tsc1*, or *Pten*. In mice with selective inactivation of cannabinoid receptor type 1 in principal forebrain neurons, seizures induced by kainic acid are more severe than in control mice (Marsicano et al., 2003). In addition, protective mechanisms that are activated in control principal hippocampal neurons by kainic acid administration are absent in the mutant mice, leading the authors to conclude that the endogenous cannabinoid system provides protection against acute excitotoxicity in the adult central nervous system (Marsicano et al., 2003). Another mouse model with a phenotype related to the pathology of epilepsy comes from the astrocyte-specific inactivation of *Tsc1*, a gene involved in tuberous sclerosis complex, which is a disorder characterized by several neurological abnormalities including seizures (Uhlmann et al., 2002). In these mice, astrocyte-specific loss of the protein hamartin, which is the product of the *Tsc1* gene, results in progressive epilepsy as well as in dysfunctional glutamate homeostasis, among other phenotypes (Uhlmann et al., 2002; Wong et al., 2003). Mice lacking the tumor suppresor PTEN in granule cells of the cerebellum and dentate gyrus exhibit seizures, ataxia, increases in brain size and in soma size of mutant neurons, and die prematurely, providing an animal model of Lhermitte–Duclos disease (Backman et al., 2001; Kwon et al., 2001). Treatment of these mice with an inhibitor of the central growth mediator mTor efficiently reduced seizure frequency and mortality, and prevented or reversed soma enlargement, suggesting a potential therapeutic application of mTor inhibitors in Lhermitte–Duclos disease or other neurological disorders related to PTEN deficiency (Kwon et al., 2003).

Schizophrenia

Schizophrenia is a genetically complex neurological disorder with the genes involved each contributing only a small increment in risk and their effect quite likely modified by other genes or environmental factors (Harrison and Weinberger, 2004). It has been proposed that gene predisposition converges to an alteration in synaptic plasticity, especially in NMDA receptor-mediated responses, affecting higher-order cortical function (Harrison and Weinberger, 2004). Several genetic mouse models with phenotypes related to schizophrenic behavior have been developed, such as Dvl1-null mice (Lijam et al., 1997) and mice with reduced expression of the NMDAR1 subunit (Mohn et al., 1999). One of these models comes from the conditional elimination of calcineurin activity in the adult forebrain (Zeng et al., 2001; Miyakawa et al., 2003). Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase involved in several cellular processes and Ca^{2+}-dependent signal transduction pathways (Rusnak and Mertz, 2000). Forebrain-specific calcineurin knockout mice display a variety of behavioral abnormalities that resemble characteristics of schizophrenia, such as increased locomotor activity, reduced social interaction, impaired prepulse inhibition and latent inhibition, and enhanced sensitivity to the locomotor stimulatory effects of the NMDA receptor blocker MK-801 (Miyakawa et al., 2003). In an accompanying paper, the *PPP3CC* gene, which encodes the calcineurin γ catalytic subunit was identified as a potential schizophrenia susceptibility gene, further supporting the hypothesis that altered calcineurin signaling contributes to the pathogenesis of schizophrenia (Gerber et al., 2003).

Outlook

The technology of targeted gene modification in mice has been one of the most rapidly progressed ones in biology over the last 17 yr and has contributed substantially to our understanding of the genetics of neurological conditions. Conditional knockout studies are at the cutting edge of this technology and have been proven invaluable for the in vivo functional study of genes, complete inactivation of which results in early lethality. In the study of neurological disorders, several gene targeted mouse models have been developed and have revealed important information about molecular, cellular or behavioral functions controlled by the targeted gene, its implication in the pathogenesis of the disorder, or potential treatments. The powerful capabilities of temporal and spatial restriction in gene inactivation that the conditional knockout technology confers hold great promise for its even more extensive and elaborate use in the future.

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