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Inducible, Cell-Targeted Mutations In Mice: New Tools For Genetically Dissecting Behavior

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The first generation of genetic mutations in mice, conventional transgenics and knockouts, have provided important new insights into many aspects of brain function, including complex behavior. Moreover, as genetic variants that contribute to the development of mental disorders are found, mice bearing these genes represent novel and powerful animal models of the disorders. However, these mutations are complicated by the fact that they generally occur at early stages of development and in many and perhaps most tissues of the body. This complicates the use of these mice for studies of gene function in adult organisms. The last five years has seen the development of second generation mutations in mice, in which the mutation (either loss or addition of a gene) can be controlled temporally and spatially, that is, the mutation can be induced in adult animals and targeted to a particular brain region and neuronal cell type. Although not yet routine, such inducible and cell-type specific mutations represent powerful new tools to understand the role of a particular gene in complex behavior.

The draft of the human genome sequence that was published in 2001 indicates that the genome comprises roughly 35,000 genes. Based on alternative splicing of RNA transcripts and posttranslational processing of proteins, this means that the body contains on the order of several hundred thousand gene products, at least half of which are thought to be expressed in the brain. A critical step in understanding brain function is to determine the function that each gene (and each of its products) plays in the nerve cells where it is expressed. This process will gradually unravel the genetic and molecular determinants of neural cell functioning and ultimately of the complex behaviors subserved by networks of neurons in the brain.

The first creation of genetic mutant mice several decades ago led to revolutionary advances in our ability to study the contribution of individual genes and their products to the brain. These first-generation mutants can be divided into two broad categories. One variety can be referred to as *transgenic* mice, in which a gene of interest is added to the animal. Such a transgenic mouse contains extra copies of a normal gene, or perhaps copies of an altered gene; in either case the mouse would overexpress the protein encoded by the transgene. Transgenic mice are made by injecting a piece of DNA, containing the coding gene of interest (e.g., a neurotransmitter receptor) plus a promoter or regulatory region, into a mouse oocyte immediately after fertilization. The transgene then incorporates randomly

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into the genome in single or multiple copies. In the simplest case, the coding gene is controlled by a constitutively active promoter, which would ensure high levels of transgene expression from the earliest stages of development and in all tissues in the body. In more complicated situations, the transgene can be controlled by a promoter of interest, in which case the transgene would be expressed at the time of development and in the particular tissues where that promoter is normally active. For example, placing a transgene under the control of a neurofilament gene promoter would, in theory, target the transgene to all neurons in the body (but avoid expression in all nonneural tissues), with such expression occurring whenever the neurofilament gene is normally turned on during development.

In practice, however, such control over transgene expression has been inadequate: expression is seen in some regions where it does not normally occur and is not seen everywhere it should occur. Such unpredictable patterns of expression are due to several factors, including the small fragments (<10 kb) of promoters often used. The use of small promoter fragments can lead to erroneous patterns of gene expression, because such a fragment generally lacks all of the regulatory information contained within a gene, which might be distributed over more than 100 kb of DNA from distant 5' regions to intronic regions to distant 3' regions. Moreover, transgenes driven by small promoter fragments are more likely to be influenced by regulatory information contained in neighboring genes. Since the experimenter has no control over the location of the transgene's insertion site into the genome, constructing a transgenic mouse can be "hit or miss," with interesting patterns of expression occurring serendipitously. One way to overcome this limitation is to make a transgenic mouse with a much larger piece of DNA, for example, by placing the gene of interest within a 100-200 kb fragment of DNA (such as a bacterial artificial chromosome) containing a gene that exhibits the desired pattern of expression. However, we have still only characterized a relatively small number of genes that show expression in specific brain regions. As a result, the tools to precisely target transgenes to a specific brain region are not yet available.

The second variety of mutant mouse is generated by a process called *homologous recombination*, where a chosen portion of a gene is replaced by an exogenous sequence. This recombination is carried out in embryonic stem (ES) cells, which are then injected into blastula stage mouse embryos that are in turn implanted into the uterus of a foster mouse. Resulting offspring are chimeric: only a fraction of the cells in the body possess the mutation. In some chimeric mice, the mutation will be present in germ cells and, consequently, subsequent generations of mice will no longer be chimeric for the mutation (i.e., the mutation will be expressed ubiquitously). Homologous recombination can be used to create a *knockout mouse*, in which a gene is disrupted or inactivated, or a *knockin mouse*, in which a gene of interest is added to an endogenous gene and therefore expressed when and where the endogenous gene is normally activated. The process can also be used to create more subtle mutations in endogenous proteins, for example, to mutate a single amino acid in a protein of interest or to replace a normal gene with a disease-causing variant. Generation of a mutant mouse by homologous recombination is considerably more time-consuming than traditional transgenic

approaches, yet offers much greater power in the types of mutations that can be created.

Hundreds of mutant mice have now been generated by these various techniques and have contributed in many important ways to our understanding of brain function. Perhaps most significantly, as the genetic differences that make certain individuals particularly vulnerable or resistant for a psychiatric disorder are identified, it has been possible to generate mice expressing that particular human genetic risk factor. Such mutants represent highly novel and extremely valuable animal models of the disorders, which can be used to study molecular mechanisms of disease pathogenesis and pathophysiology, and to develop new interventions to treat or prevent the disorder. Prominent examples of this approach, and its great promise, have come from recent work on Alzheimer's disease, Huntington's disease, Parkinson's disease, and Rett's syndrome, to name a few (e.g., see Chen et al., 2001; Dawson, 2000; Hock et al., 2001; Menalled & Chesselet, 2002).

Nevertheless, first generation transgenic and knockout mutants do not offer the experimenter control over the timing or spatial location of the mutations. This is not a problem for animal models of human disease, where the mutations are not controlled in such a manner as well. However, the lack of temporal and spatial control over mutations has seriously limited the ability to study the role of a given gene in the adult. For example, if a mutant mouse shows some behavioral abnormality, it is often difficult to ascribe that abnormality to the mutant gene's function in a particular brain region, since the mutation is typically expressed ubiquitously. Likewise, it is often difficult to ascribe an abnormality to a gene's role in the adult brain, since the mutation is typically expressed during critical stages of development. Another limitation with conventional mutants is that many knockouts are not viable as adult animals. This makes it impossible to use the mice to study the function of the gene of interest in the adult nervous system.

To overcome these obstacles, several approaches have been used in recent years to manipulate genes of interest in discrete brain regions of adult animals. These approaches include antisense oligonucleotides (Lebedeva & Stein, 2001; Robinson et al., 1997), viral-mediated gene transfer (Carlezon et al., 2001), and inducible, cell-targeted mutations in mice (Mayford et al., 1997). This review focuses on the latter approach. While creation of such inducible, cell-targeted mutant mice is still far from routine, and will always take more time and effort than the other approaches, the resulting mice represent uniquely powerful tools to understand brain function, including the generation and regulation of complex behavior. This review is not meant to be comprehensive; rather, it highlights important examples of the major methods currently under development.

Tetracycline Gene Regulation System

The tetracycline gene regulation system was one of the first systems developed for temporal and spatial control over transgene expression (Mansuy & Bujard, 2000). It utilizes a traditional transgenic approach, and is therefore relatively straightforward from a technical point of view. It involves two transgenes, as illustrated in Figure 1. The first transgene encodes an artificial fusion protein called the tetracycline transactivator (tTA) under the control of a promoter. The tTA protein consists of a fragment of the *tet* repressor (which binds

tetracycline) and the VP16 arm of a herpes simplex virus transcription factor. The result is a tetracycline inhibitable transcription factor. The second transgene encodes the gene of interest (e.g., a receptor) under the control of the Tetop promoter, which is activated by tTA. In a bitransgenic mouse, which contains both of these transgenes, expression of the gene of interest is inducible because the mouse can be fed tetracycline (or its derivative, doxycycline, which better penetrates the brain and binds more avidly to tTA) from conception through development to maintain expression in the off state; the mouse can then be withdrawn from doxycycline to induce transgene expression. This is also a cell-targeted system, since the transgene of interest will only be expressed in cells containing tTA, and that can be controlled by the choice of promoter to drive tTA in the first transgene.

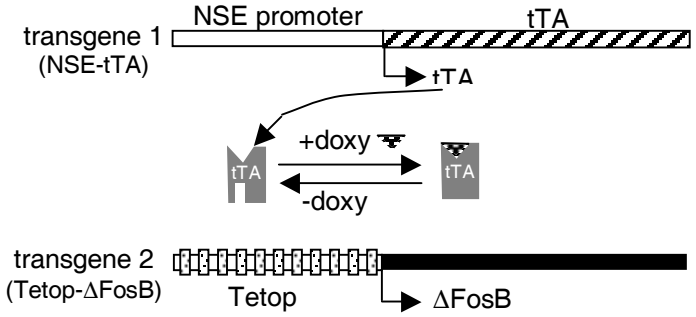

Figure 1. Illustration of the tetracycline gene regulation system. One transgene expresses tTA under the control of a promoter of choice. The other transgene expresses the protein of interest under the control of the Tetop promoter, which is activated by tTA. Doxycycline (doxy; a tetracycline derivative) binds to tTA and prevents this action. In the example shown (Kelz et al., 1999), a fragment of the neuron-specific enolase (NSE) promoter is used to drive tTA and the Tetop-driven gene is ΔFosB. In bitransgenic mice, removal of doxy causes the induction of ΔFosB selectively in cells in which the NSE promoter is active.

An advantage of this system is that genetically identical mice can be used to study the consequences of the mutation. Thus, bitransgenic littermate mice, all conceived and raised on doxycycline, can be divided into two groups at a chosen stage of development: one maintained on doxycycline, the other removed from doxycycline. Any effect of doxycycline per se can easily be controlled by studying wildtype animals. In this manner, one can investigate the effects of inducing the mutation at different stages of development, or even reversing any resulting phenotype by turning transgene expression on and then off again.

Indeed, the tetracycline gene regulation system has been used with considerable success to study the function of several genes in the adult nervous system. The first use of this technology for the study of brain function involved the expression of a constitutively active variant of CaM-kinase II (Ca^{2+}/calmodulin-dependent protein kinase II) selectively within the hippocampus of adult mice (Mayford et al., 1996). The resulting mice showed a decrement in long-term potentiation in the CA1 region of hippocampus as well as impaired functioning of place cells within hippocampus. These cellular phenotypes were associated with decrements in spatial learning, known to be a hippocampal-dependent task. More

recently, Frankland et al. (2001) used mice heterozygous for a null mutation in CaM-kinase II to study the function of the enzyme in cerebral cortex. These mutants show abnormalities in cortical, but not hippocampal, plasticity. Together, the findings provide support for the view that Ca^{2+} activation of this kinase is an important step in these cellular and behavioral forms of plasticity. They also underscore the utility of these experimental approaches to differentiate the roles played by a given molecule of interest in two different regions of adult brain.

Another use of the tetracycline system involved studies of the transcription factor ΔFosB, which is induced selectively in striatal regions of brain in response to chronic exposure to cocaine or many other drugs of abuse (Nestler et al., 2001). Striatal regions are known to be important neural substrates for the addicting properties of these drugs. ΔFosB is unique in that once it is induced in the brain it persists for a long time (weeks to months) due to its extraordinarily long half-life (~2 weeks *in vivo*). Therefore, ΔFosB represents a mechanism by which drug exposure could cause long-lasting behavioral abnormalities. Bitransgenic mice were generated where ΔFosB could be overexpressed selectively within the same subpopulation of striatal neurons in which drugs of abuse normally induce the protein (Chen et al., 1998; Kelz et al., 1999). Such ΔFosB overexpressing mice exhibit an interesting phenotype: they are more sensitive to the rewarding effects of cocaine and also show greater incentive drive for the drug (Kelz et al., 1999; Nestler et al., 2001). In a related series of experiments, mice generated, using the tetracycline system, to express a dominant negative inhibitor of ΔFosB selectively in striatum, showed the opposite phenotype (Peakman et al., 2000). (A dominant negative inhibitor describes a mutant protein which has no biological activity by itself but can antagonize the function of the normal, endogenous protein.) Together, these findings suggest that ΔFosB may be a molecular mechanism for long-lived drug sensitization and may also function as part of a sustained molecular switch that first initiates and then maintains a state of addiction.

Discussion of ΔFosB illustrates the importance of using inducible systems to study the role of genes in the adult nervous system. Thus, conventional FosB gene knockout mice exhibit a phenotype that is not completely compatible with the more recent findings from the inducible system. FosB knockouts show no sensitization to cocaine (consistent with results from the inducible system), but show heightened sensitivity to initial cocaine exposures (inconsistent with the inducible system; Hiroi et al., 1997). Yet, we have much greater confidence in the inducible system for several reasons. First, the FosB knockouts lack not only ΔFosB but also full-length FosB, which is induced by cocaine and other drugs of abuse in striatum, but only after acute drug exposures. (The influence of full-length FosB on cocaine action is not known.) Abnormalities seen in the FosB knockouts could, then, reflect loss of FosB. Second, the loss of ΔFosB and full-length FosB occurs not only in striatum but throughout the brain and peripheral tissues, and occurs at the earliest stages of development. As a result, abnormalities seen in the knockout could reside in any of several brain areas and could even represent a developmental consequence of the knockout. Third, ΔFosB is not expressed at appreciable levels under basal conditions; hence it is difficult to understand how loss of ΔFosB per se could directly mediate the enhanced initial drug sensitivity in the FosB knockouts.

The importance of an inducible system can also be demonstrated by analysis of bitransgenic mice that overexpress another transcription factor, CREB (cAMP response element binding protein; Chen et al., 1998). Overexpression of CREB selectively in the striatum of adult mice leads to the induction of dynorphin, a neuropeptide whose expression is known to be regulated by CREB *in vitro* and *in vivo*. In contrast, when CREB overexpression occurs throughout the life of the animal (by keeping them off doxycycline), basal levels of dynorphin expression are depressed (Sakai et al., 2002). This result emphasizes the misleading findings and interpretations, concerning the function of a gene in the adult, that can come from analysis of conventional mutant mice.

Despite the value of the tetracycline gene regulation system, there are two glaring limitations. First, the timing of gene induction is relatively slow due to the duration of time that is required to wash doxycycline out of the animal. It typically takes several weeks to obtain maximal levels of transgene expression, even when the lowest effective doses of doxycycline are used (Chen et al., 1998; Kelz et al., 1999). Even this period can allow compensations that might cloud experimental interpretations. One way to overcome this limitation is to use a mutant form of tTA, termed rtTA or reverse tTA, which is activated rather than inhibited by doxycycline. In this case, gene expression can be induced in a matter of days in a bitransgenic animal by administration of doxycycline (Mansuy et al., 1998). However, use of rtTA has been limited by substantial levels of "leak" expression, that is, expression seen even in the absence of doxycycline.

The other major limitation of the tetracycline system, as stated earlier for conventional transgenics, is the lack of availability of promoters with which to obtain spatial control of gene expression. Thus, the hippocampal-enriched expression of CaM-kinase II mentioned above was obtained by use of a CaM-kinase II promoter driving tTA expression (Mayford et al., 1996), and the striatal-enriched expression of ΔFosB was obtained by use of a neuron-specific enolase promoter driving tTA expression (Kelz et al., 1999). In both cases, tTA expression should have been much more widespread than was observed and indeed was more widespread in many other lines of these mice. The restricted expression patterns obtained in the selective lines were presumably due to the insertion sites of the transgenes, and this is entirely serendipitous. To gain more control over resulting expression patterns, investigators are now making mice in which tTA is included within a larger segment of DNA or even knocked into a particular gene locus via homologous recombination. Whether such efforts will result in greater spatial control of gene expression remains to be seen.

Cre-loxP System of Gene Recombination

A major breakthrough in the generation of genetic mutations in mice involved the application of a bacterial recombinase, termed Cre, to mammalian systems (Laska et al., 1992; Nagy, 2000; Sauer & Henderson, 1988). Cre excises a span of DNA from a gene that is flanked by so-called loxP sites (a 34 nucleotide sequence of DNA). As illustrated in Figure 2, the Cre-loxP system can be used to generate knockout mice. Like the tetracycline system, this involves two mutants. One mouse contains a "floxed" gene; that is, a critical part of a gene is flanked by loxP sites. The other mouse is a transgenic that expresses Cre under the control of

some promoter of choice. Partial spatial and temporal control over recombination can be achieved by placing Cre under the control of a promoter with the desired pattern of expression. For example, synapsin-Cre mice would theoretically induce recombination solely within neurons (Ma et al., 1999), since the synapsin promoter directs gene expression to neurons. Moreover, the knockout would occur at relatively late embryonic times when the promoter first becomes active. Such mice are often called *conditional knockouts*, since the knockout occurs only in some tissues and at later stages of development compared to the conventional knockouts described above.

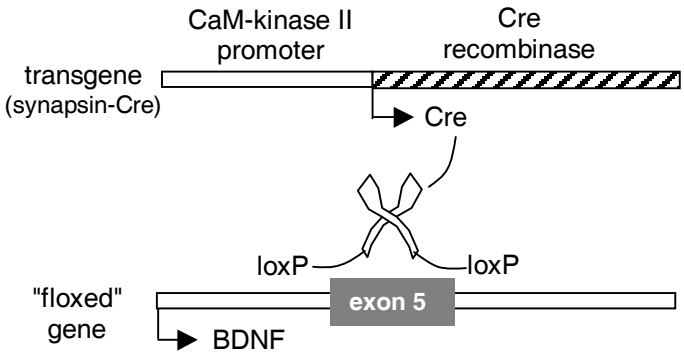

Figure 2. Illustration of a conditional knockout using the Cre-loxP system. One transgene expresses Cre under the control of a promoter of choice. The second mutated gene contains loxP sites flanking a critical region of the gene to be knocked out. In the example shown (Rios et al., 2001), Cre is driven by the CaM-kinase II promoter, and removes exon 5 (flanked by loxP sites) from the BDNF gene. The resulting bi-mutant mice show a loss of BDNF, which occurs at the time of development and selectively in those cells in which the CaM-kinase II promoter is active.

Conditional knockouts are being used increasingly to study brain function. They have already enabled the development of adult knockout mice, where the conventional knockout of the gene is fatal. For example, conventional knockout mice lacking NT-3 (neurotrophin-3) or BDNF (brain-derived neurotrophic factor) die shortly after birth due to a variety of peripheral abnormalities. In contrast, utilizing the Cre-loxP system, it has been possible to knockout NT-3 or BDNF at later embryonic stages and selectively within the nervous system (Akbarian et al., 2001; Ma et al., 1999; Rios et al., 2001). In one study, this was accomplished by crossing mice with a floxed NT-3 locus with nestin-Cre mice; the nestin promoter targets neurons and glia and becomes active around embryonic day 10. This has resulted in the generation of adult animals that completely lack NT-3 in the brain but have no apparent peripheral defects.

Another example of the successful use of the Cre-loxP system comes from research on the subtypes of glutamate receptors important for long-term potentiation in hippocampus. A transgenic mouse line expressing Cre under the control of the CaM-kinase II promoter tends to direct Cre expression to the hippocampus (this is serendipitous and due to the insertion site of the transgene, as mentioned earlier for CaM-kinase II-tTA mice). Relatively selective knockout of the NMDA glutamate receptor subunit NMDAR1, which is an obligatory subunit of NMDA receptors, causes impairments in hippocampal long-term potentiation

and place cell function, and in spatial memory (McHugh et al., 1996; Shimizu et al., 2000; Tsien et al., 1996a, b).

Despite the great potential of these types of conditional mutants, and there is still a great deal more work to be done with them, there are important limitations that must be considered. The first is the lack of availability of promoters needed to generate the Cre-expressing mice—a limitation emphasized earlier for any transgenic project. Particular care must be taken in analyzing the conditional mice, since Cre expression and hence the knockout may not occur everywhere it is expected to occur and may occur in anomalous locations. The synapsin-Cre mouse mentioned earlier is illustrative, since many conditional knockouts generated with the mouse do not mediate a knockout throughout the nervous system (e.g., see Ma et al., 1999). Investigators are attempting to overcome this obstacle by knocking Cre into a known genetic locus by homologous recombination (e.g., see Hebert & McConnell, 2000). However, this might result in disruption of the endogenous gene, which would complicate interpretation of resulting mice.

Another limitation with the conditional mutants is that the knockout is not truly inducible: the experimenter cannot induce the knockout (or other mutation) selectively in adult animals. One way to overcome this limitation is by use of viral vectors: to inject a virus encoding Cre into a discrete brain region of a mouse with a floxed gene (DiLeone et al., 2001). Recombination will be brain-region specific (localized to the site of the virus injection) and inducible (it will occur shortly after the surgery). Of course a problem with this approach is that it requires intracranial surgery and viral infection, which might confound the experimental findings. Another problem is that viral expression usually does not affect all neurons in an injected area. An alternative method to obtain inducible recombination is to use modified versions of Cre, the subject of the next section.

Inducible Cre Systems

Inducible Cre refers to Cre enzymes whose catalytic activity is under some built-in molecular control. The most widely used inducible Cre consists of Cre fused to a steroid hormone receptor (e.g., estrogen or progesterone receptor; Feil et al., 1996, 1997). The fusion protein displays no Cre activity in the absence of the steroid hormone, but is activated upon binding the hormone. When a mouse expressing an inducible Cre is crossed with a mouse containing a floxed gene, recombination should only occur when the animal is exposed to the steroid hormone that activates the Cre (Figure 3). To date, such inducible Cre systems have been used successfully in cell culture systems and for peripheral tissues. The system is relatively new, however, and has only been applied to the nervous system in a few cases to date (Kellendonk et al., 1999; Kitayama et al., 2001). One concern is whether activity of the Cre is truly off in the absence of the steroid hormone; a low level of Cre activity seen in the absence of exogenous hormone might be sufficient to mediate recombination. It is even conceivable that naturally occurring surges in endogenous hormone might induce the knockout. Another concern is the effect of administering a steroid hormone to an animal, which would be expected to exert its own effects on the brain. On the other hand, this can be readily controlled by administering the steroid to wildtype animals.

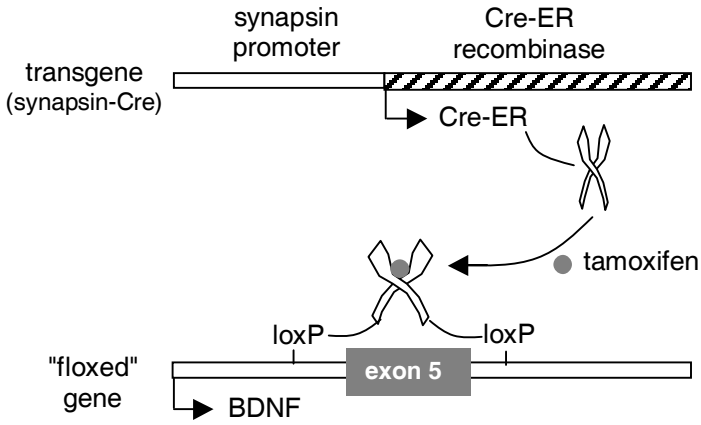

Figure 3. Illustration of an inducible Cre system. One transgene expresses a modified Cre-ER (estrogen receptor) fusion protein under the control of a promoter of choice. The second mutated gene contains loxP sites flanking a critical region of the gene to be knocked out. In the hypothetical example shown, Cre-ER is driven by the synapsin promoter, and removes exon 5 (flanked by loxP sites) from the BDNF gene. The resulting bi-mutant mice would show a loss of BDNF selectively in those cells in which the synapsin promoter is active, but only upon administration of the estrogen receptor agonist, tamoxifen, which activates Cre-ER.

To address some of these concerns, mutant forms of steroid hormone receptors have been generated; these are less affected by endogenous hormones and more sensitive to low doses of synthetic ligands that show lower affinity for the wildtype receptor (Feil et al., 1996, 1997). In addition, the inducible Cre systems are now being combined with tissue-specific promoters to obtain spatial, in addition to temporal, control over gene expression. There remains a great deal of excitement with the inducible Cre systems, although more time is needed to evaluate their ultimate utility for investigation of the CNS.

Combining the Tetracycline and Cre-loxP Systems

An alternative to the use of inducible Cre systems is to combine the Cre-loxP system with the tetracycline system. This would involve three mutant genes, as illustrated in Figure 4. The first gene encodes tTA under the control of some tissue-specific promoter. The second gene encodes Cre under the control of Tetop. Mice containing both of these transgenes would then be crossed with a mouse with some floxed gene locus. In theory, the tri-mutant mouse, when maintained on doxycycline, should be normal. Upon removal of doxycycline, recombination should be induced selectively in those neuronal cell types that express Cre. Early experience with this approach is encouraging (Monteggia et al., 2001), although the system is limited by the same factors that limit the tetracycline system in general, in particular, the lack of availability of suitable tissue-specific promoters, and the long time it takes to wash tetracycline or doxycycline out of the animal. Another limitation of the combined Cre-loxP-tetracycline system is that it requires more extensive breeding. The system involves three mutant mice, which makes the breeding of such animals considerably onerous.

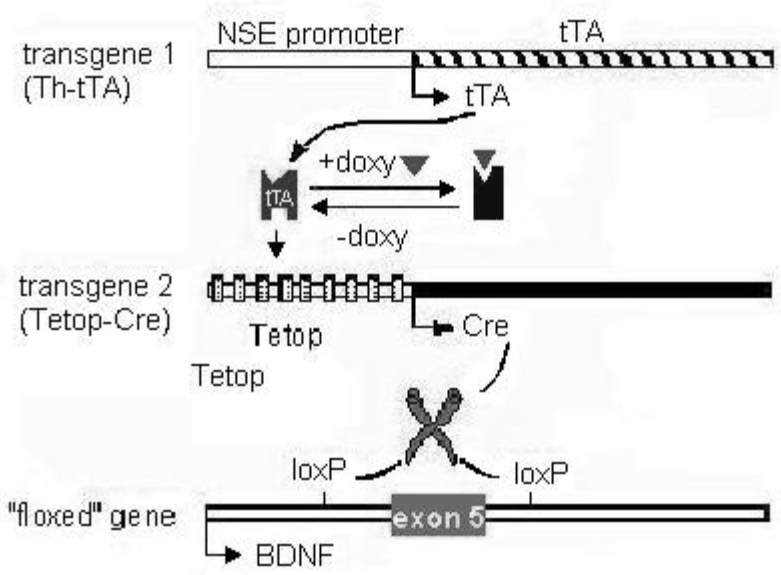

Figure 4. Illustration of a combined tetracycline-Cre-loxP system. One transgene expresses tTA under the control of a promoter of choice. A second transgene expresses Cre under the control of the Tetop promoter. The third mutated gene contains loxP sites flanking a critical region of the gene to be knocked out. In the example shown (Monteggia et al., 2001), a fragment of the neuron-specific enolase (NSE) promoter is used to drive tTA. In tri-mutant mice, removal of doxycycline (doxy) causes the induction of Cre selectively in cells in which the NSE promoter is active; the Cre then inactivates the BDNF gene in those cells.

The Future

The goal of current research is to generate a mouse in which any given gene of interest can be turned on or off, or even its sequence altered, in a selected population of neurons at any given stage in the life of the animal. This review highlights the impressive progress that has been made in less than a decade in generating mice where the mutation is under some temporal and spatial control. It is also clear from this discussion that much works remains to achieve the exquisite degree of control that is needed, let alone make the technology readily applicable to the scientific community at large. This simply reflects the difficulty of the tasks ahead. The ultimate endpoint remains essential: to define the function that every gene and gene product serves, across many different types of nerve cells and at different stages of life, in determining all aspects of brain function, including complex behavior.

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