Establishing the contributions of inherited genes relative to experience in brain function forms a central theme of neuroscience. There is little doubt that both activity-dependent neuronal plasticity and genetically determined programs generate important influences on the developing and the adult nervous system. The ability to change behaviour in response to new environmental hazards and genetically determined programs generate important influences on the developing and the adult nervous system.

**Abstract**

Brain structure and function are determined in part through experience and in part through our inherited genes. A powerful approach for unravelling the balance between activity-dependent neuronal plasticity and genetic programs is to directly manipulate the genome. Such molecular genetic studies have been greatly aided by the remarkable progress of large-scale genome sequencing efforts. Sophisticated mouse genetic manipulations allow targeted point-mutations, deletions and additions to the mouse genome. These can be regulated through inducible promoters expressing in genetically specified neuronal cell types. However, despite significant progress it remains difficult to target specific brain regions through transgenesis alone. Recent work suggests that transduction vectors, like lentiviruses and adeno-associated viruses, may provide suitable additional tools for localized and controlled genetic manipulation. Furthermore, studies with such vectors may aid the development of human genetic therapies for brain diseases.

**Keywords:** transgenic mice • gene targeting • gene regulation • transduction vectors • adeno-associated virus • lentivirus

**Introduction**

Establishing the contributions of inherited genes relative to experience in brain function forms a central theme of neuroscience. There is little doubt that both activity-dependent neuronal plasticity and genetically determined programs generate important influences on the developing and the adult nervous system. The ability to change behaviour in response to new environmental hazards and
rewards is one of the major roles of the central nervous system. Adaptability, learning and memory have therefore become key avenues of neuroscience research. However, it is equally clear that many animal behaviours are stereotyped and encoded by hard-wired neural circuits specified genetically. Indeed, genetic programs are also likely to specify the rules for how and to what extent experience can change the brain to allow learning and the formation of memories. Thus, there are complex interactions between genetics, gene expression, activity-dependent neuronal plasticity and experience. In order to explore these interactions, neuroscientists are in great need of tools to make specific genetic manipulations. In this review we describe the enormous progress made over the last decades, particularly through use of genetically engineered mice, and we will point to new directions that offer greater spatiotemporal genetic control based on local gene delivery, in particular by viral transduction vectors. In addition to utility in basic neurobiological research for assessing the role of individual molecules in brain function, such vectors are also likely to be useful in gene therapy.

Genetic manipulation

Ideally, it would be possible to control gene expression in well-defined populations of neuronal cells at any given time during the development or adult life of an animal. One would like to be able to delete genes, change genes and add new genes to the genome in a temporally and spatially controlled manner. To this end remarkable advances have been made over the past decades, allowing genetic manipulation in different species, which has been greatly facilitated by the rapid progress of the large-scale genome sequencing projects.

Exogenous gene expression or ‘transgenesis’

For many studies it is useful to be able to express a gene of interest, be it the wild-type gene, a mutated gene or a foreign gene. Plasmids containing regulatory elements and a gene of interest can be added with relative ease to create transgenic animals of many species. In the worm, *Caenorhabditis elegans*, plasmids can be injected directly into the gonad, where the DNA is taken up in the developing eggs to form extrachromosomal arrays, which can become stably heritable. Co-expressed selective markers, like a mutant collagen gene, *rol-6*, which causes the worms to ‘swim’ in circles, rather than their usual sinusoidal pattern, can then be used to screen for the transgenic worms [1]. In the fruit fly, *Drosophila melanogaster*, transgenesis is also relatively simple. Fly embryos are injected with DNA plasmids, usually in the context of transposon sequences, like P-elements, to help germline integration, and eye color genes have been commonly used as selective markers [2]. Similar approaches can also be used for generating transgenic mice [3], even though obtaining eggs and bringing them to term is quite a bit more complex for mammalian species. As depicted schematically in Fig. 1, linearized plasmid DNA corresponding to a complete transcription unit is directly injected into the male pronucleus of fertilized mouse eggs (reviewed in Ref. [4]). The DNA generally inserts into the mouse genome.

![Fig. 1](image-url) Generation of transgenic mice. A. The example of the GFP-M mouse [5], made by classic transgenesis. Depiction of: 1. the transgene vector, with a Thy1 promoter driving GFP expression; 2. direct injection into the mouse egg pronucleus; and 3. offspring for screening. The micrographs are confocal images of a cell within a 100 μm PFA-fixed coronal section from medial cortex of the brain of a GFP-M mouse. The scale bar on the left is 100 μm, while that for right hand image, zooming in on dendrites (boxed on the left), is 10 μm. B. The example of the αCaMKII T286A mouse [20], made by targeted knockin of embryonic stem cells. Depiction of: 1. the mutagenesis of the targeting vector (the C in blue is a ‘silent’ mutation which generates a new restriction enzyme cleavage site); 2. transfection of embryonic stem cells; 3. homologous recombination of the targeting vector with the genomic locus; 4. selection of correct clones; 5. blastocyst injection; and 6. screening of chimeric mice. On the right is a depiction of the wild-type and point mutant complexes of αCaMKII. During a transient calcium rise wild type αCaMKII can autophosphorylate and acquire long-lasting calcium independent activity. The autophosphorylation occurs at T286, which is mutated in the gene-targeted mouse. In the lower right is shown the physiological result that LTP is nearly absent in the mutant mice (electrophysiology figure reprinted with kind permission from Ref. [20]).
A  ‘transgenic’ through random insertion - e.g. GFP-M mouse
1. transgene vector
   Thy1 GFP
2. pronuclear microinjection
3. implant surrogate mother and screen offspring

B  ‘gene targeting’ by homologous recombination - e.g. CaMKII T286A
1. targeting vector
2. ES cell transfection and selection
3. homologous recombination
genomic locus
4. select correctly targeted clones
5. blastocyst injection
6. screen chimeric offspring

Wild type CaMKII

Point Mutation CaMKII T286A

\[ \text{Ca}^{2+}/\text{CaM independent activity} \]

\[ \text{Ca}^{2+}/\text{CaM independent activity} \]

\[ \text{inactive without Ca}^{2+}/\text{CaM} \]

\[ f\text{EPSP (% of baseline)} \]

\[ \text{Time (min)} \]
chromosomal DNA at a random location in concatemeric arrays. The expression of the transgene is then regulated both by the promoter and by the surrounding elements where the transgene inserted into the genome. The injected eggs are implanted into a surrogate ‘mother’ mouse. Screening of the viable progeny by southern blotting or PCR of genomic DNA is usually necessary to identify founders for breeding.

A nice example of this transgenic approach was applied by Feng and co-workers [5] to the neuronal expression of green fluorescent protein (GFP) and its variants under a Thy1 promoter, which had been engineered to be neural specific [6, 7] (Fig. 1A). In these experiments, red, yellow and green variants of GFP were cloned into plasmids, all driven by the identical Thy1 promoter elements. The DNA was injected into mouse eggs and 25 independent stable transgenic mouse lines were obtained. Analysis of these lines showed that the expression pattern of each line was different! For instance in some mouse lines, all retinal ganglion cells fluoresced brightly, while in others many cortical cells were bright. Two of the most useful lines, GFP-M and YFP-H, had very brightly fluorescing neurons, which were sparsely distributed. This ‘golgi-like’ pattern of fluorescence has allowed elegant in vivo imaging studies of spine formation and elimination, using two photon laser scanning microscopy [8, 9].

Although the transgenic approach has produced many valuable mouse lines, it suffers from the lack of control of the integration site into the genome. This generates uncertainty in the expression profile of the gene as illustrated above by the varied expression patterns in the different lines of the Thy1-GFP mice. To gain greater control of transgene expression, the gene of interest can be flanked by very large regions of DNA in the form of Bacterial Artificial Chromosomes (BACs). In some studies, this BAC approach has allowed the native expression pattern of genes to be recapitulated [10, 11]. However, this has not consistently been successful, because even such large constructs can apparently be affected by their genomic context. Potential epigenetic modifications (i.e. chromosomal DNA methylation or histone acetylation) are further confounding factors in analysis of expression in transgenic mice. To gain greater precision in the genetic manipulation it is therefore necessary to specifically target endogenous genes.

**Gene targeting**

In the worm *Caenorhabditis elegans* and in the fly *Drosophila melanogaster*, gene disruption or knockout (KO) has primarily been carried out through forward genetic screens. In the worm, chemical mutagenesis (for instance with ethylmethylsulfonate or psoralen/uv) inducing random mutations and deletions has been used extensively followed by selection and screening. P-element hopping has been used to disrupt genes in the fly, again in a random fashion followed by selection and screening. Such random mutagenesis approaches have proven extremely powerful in providing ‘unbiased’ screens to determine the genes involved in many biological processes, including learned behaviours [12, 13].

Random mutagenesis, however, relies on the availability of large numbers of animals. In general, one would like to screen several ‘genomes’ worth of animals. If, as estimated, in mice there are ~30,000 genes, then one would like to screen ~100,000 animals. Whereas this is possible for worms and flies, this is very difficult for larger more complex animals such as the laboratory mouse, although forward genetic screens are nonetheless utilized [14]. A secondary drawback of this strategy is the inherent imprecision of random mutagenesis. The exact nature of the genetic lesion can be subtle (such as small deletions or point mutations) and therefore often hard to pinpoint. The use of highly transposable elements and other selective tricks has facilitated forward genetic screening of mice [15–18].

The development of mouse embryonic stem cells has led to important progress in the generation of gene-targeted genetically engineered mice. The major advantage is that the stem cells can be manipulated like other cultured cells. They can be grown, the population expanded, and, notably, specific stem cell clones can be selected. Plasmid constructs to manipulate the genome of embryonic stem cells can be introduced by ordinary transfection techniques as used for other tissue culture cells. Furthermore, by including DNA homologous to the genome, the exogenous DNA can insert into the genome in the endogenous locus [19]. This process of homologous recombination is not very efficient, and therefore markers and resistance genes are typically inserted to aid the selection process.
For example, Giese and co-workers [20] generated gene-targeted mice containing a point mutation of an autophosphorylation site of the alpha Ca\(^{2+}\)/calmodulin dependent kinase II (\(\alpha_{\text{CaMKII}}\)) gene (Fig. 1B). The kinase \(\alpha_{\text{CaMKII}}\) is a major component of the postsynaptic density, where it is able to phosphorylate several important targets involved in regulating the strength of a synapse [21]. Introduction of activated \(\alpha_{\text{CaMKII}}\) into neurons increases the strength of synapses [22, 23]. Additionally, active \(\alpha_{\text{CaMKII}}\) can bind NMDA receptors in the synapse, directly affecting plasticity, depending upon the subunit composition of the NMDA receptor [24]. Kinase activity is regulated by calcium and calmodulin as its name suggests, such that increased calcium concentrations push the enzyme into an activated state in which autophosphorylation at a threonine at position 286 (Thr286) of the protein occurs. Once autophosphorylated, the activity of the enzyme becomes independent of calcium. Therefore, a transient calcium elevation can produce sustained kinase activity, suggesting that \(\alpha_{\text{CaMKII}}\) is a switch-like molecule [25, 26] which could underlie step-like all-or-none synaptic potentiation [27–29].

To directly test the role of Thr286 autophosphorylation of \(\alpha_{\text{CaMKII}}\), Giese et al. (1998) made a point mutation in the mouse genome, changing the amino acid residue to one (Ala) which cannot be phosphorylated. To make gene-targeted mice carrying this change (T286A), they generated the point mutation by PCR and substituted it into a cloned genomic region of the \(\alpha_{\text{CaMKII}}\) gene. This targeting construct carried a selectable marker, \textit{neo}, which allows selection of eukaryotic cells for resistance to G418, an aminoglycoside targeting the ribosome. The mutant \(\alpha_{\text{CaMKII}}\) DNA was transfected into mouse embryonic stem (ES) cells and in some stem cells the exogenous DNA carrying the point mutant \(\alpha_{\text{CaMKII}}\) gene was inserted in its correct position in the mouse genome through homologous recombination. Individual clones of the transfected stem cells carrying the mutant \(\alpha_{\text{CaMKII}}\) gene were selected for in culture, and healthy cells from stem cell clones containing the properly targeted homologous integration were then introduced into early stage blastocysts (Fig. 1B). These blastocysts were implanted into surrogate mother mice. First generation offspring were chimeric mice, meaning that part of the mouse is derived from the cloned ES cells and other parts of the mouse are derived from the wild-type cells of the blastocyst. Coat color markers from the parental strain of the ES cell relative to the strain from which the blastocyst was obtained aid identification of ‘high-chimeric’ offspring, which were then bred and screened for germline transmission. Subsequent analysis of the mice carrying the point mutation revealed deficits in both synaptic plasticity and learning [20], suggesting that autophosphorylation of \(\alpha_{\text{CaMKII}}\) indeed plays an important role in learning and memory.

Homologous recombination in ES cells can be used to delete genes or portions of a gene (knockout), to insert sequence elements like target sites for recombination (knockin), or even to substitute genomic sequences, for instance, with a minigene or those from another organism. Multiple controls are always required, however, to confirm changes in and potential expression from targeted alleles. For instance, alternate splicing might allow unexpected gene products to persist. It is not always straightforward to prove that a gene targeting event has not resulted in, for instance, a change in another element (like expression of a microRNA) or a dominant negative effect due to truncation of a protein product, rather than a simple loss of function. However, genetic strategies, such as analyzing heterozygotes carrying the targeted allele in combination with a known loss of function allele, can help. Even though homologous recombination allows the creation of precise genetic alterations in mice, these changes are present in every cell and throughout the development and lifetime of the mouse. If a gene contributes to development, then it is not possible to separate its impact during development from its function in the adult. Equally, if a gene functions in different brain areas, it is not possible to delineate its role in each area. Considerable effort has therefore been expended to develop genetic manipulations that can be temporally and spatially regulated.

**Temporal and spatial control of genetic manipulations**

Increased control of genetic manipulation has been achieved through utilization of genetically engineered mice harbouring regulatory functions in
addition to the transgene or targeted gene. Below we outline two important strategies, which have been successfully developed for controlling genes in the mouse brain.

**Conditional knockout by site-specific recombination (Cre-loxP)**

The first technique and the one that has been used most extensively derives from a particular type of recombination mediated by the enzyme Cre recombinase, which was cloned from bacteriophage P1 [30, 31]. The Cre recombinase acts at specific 34 base pair DNA sequences called loxP sites, inducing recombination between molecules of the bacteriophage DNA. Cre recombinase was also found to work in mammalian cells, inducing site-specific recombination between loxP sequences [32]. The first successful application of this genetic control system to neuroscience research in the mouse was carried out by Joe Tsien in Susuma Tonegawa’s laboratory, who used this system to generate mice lacking functional NMDA receptors specifically in the CA1 region of the hippocampus [33, 34]. This cell-specific KO required two different mouse lines, which were generated in the Tonegawa group (Fig. 2A).

In one mouse line, loxP elements were inserted into intronic and downstream sequences surrounding the second half of the NMDA receptor 1 gene (NMDAR1), utilizing ES cells for the knockin, as described above. The modified NMDAR1 gene in this mouse is termed a 'flox'-ed gene, since it is flanked by loxP elements. Importantly, the native expression and function of the NMDAR1 gene was not altered in these floxed NMDAR1 (fNR1) mice. In other words, phenotypically, the fNR1 mouse is wild-type. In order to delete most of the NMDAR1 gene in a region specific manner, a second mouse expressing the Cre recombinase under the control of the c-fos promoter was crossed with the floxed NMDAR1 mice. The Cre-mediated excision of the floxed gene leading to knockout (KO) is illustrated in Fig. 2A.

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**Fig. 2** Strategies for spatiotemporal genetic control. A. Conditional knockout. One transgenic mouse expresses Cre recombinase in a specific spatiotemporal pattern determined by the promoter and the genomic context. The other mouse carries a floxed gene (flanked by loxP elements). Crossing these two mouse lines allows Cre mediated excision of the floxed gene leading to knockout (KO). The micrograph shows the hippocampal CA1 specific activity of the Cre recombinase driven by the c-fos promoter in mouse line T29-1. βgal staining is evident as the dark blue curve in the sagittal brain slice (image reprinted with kind permission from Ref.[33]). B. Inducible and reversible expression. The rtTA2 is expressed in a transgenic mouse line, which is crossed with a mouse carrying a tetO-regulated transgene. Doxycycline (Dox) can be added to the mouse food or water supply, entering the brain and binding to rtTA2. The Dox-rtTA2 complex binds to the tetracycline operator (tetO) sequences and induces expression of linked genes.
of the αCaMKII promoter was generated by standard transgenic techniques, and so inserted randomly into the mouse genome. As described for the Thy1-GFP mice (Fig. 1) the location of the integration of the transgene in the genome plays a significant role in the expression pattern of the transgene. Tsien and co-workers screened 9 of 11 different transmitting lines of Cre-transgenic founder mice carrying 1–20 copies of the transgene for their mRNA expression patterns. Four lines expressed the correct 2.6kb Cre mRNA in a forebrain restricted pattern; four expressed no detectable Cre mRNA; and one expressed an abnormally large mRNA of 9kb, which included the Cre sequence. Recombination activity was analyzed by breeding the Cre-expressing mice with an indicator mouse carrying a ‘floxed stop’ lacZ gene. Remarkably, three of the five lines showed Cre/loxP recombination confined to the CA1 region of the hippocampus, even though the mRNA data suggested that neocortical mRNA expression was just as high in at least one line. The spatially localized Cre recombinase activity is restricted to the CA1 pyramidal cells of this line (T29-1, Fig. 2A). Recombination activity was initiated in the third postnatal week [33], after the most crucial developmental period.

By crossing the CA1 specific Cre-expressing mice with the fNR1 mice, Tsien and co-workers succeeded in generating the first mice with a cell-type specific knockout of a neuronal gene - the knockout of all functional NMDA receptors in the CA1 region. This allowed the direct test of the role of NMDA receptors in hippocampal CA1 pyramidal neurons in learning. It was found that spatial learning in the Morris water maze was deficient in these mice [34], pointing to a pivotal role for CA1 NMDA receptors in spatial learning and memory. As indicated by this example and many further studies using similar approaches, the expression of Cre in specific neuronal cell types combined with gene floxing through homologous recombination to assure target specificity is an extremely powerful method to analyze the contribution of an individual gene to mouse behaviour. However, the transgenic expression of Cre is subject to the uncertainties of promoter specificity and integration site in the genome, as indicated above.

Cre recombinase can also be utilized directly in gene targeted ES cell clones to excise floxed elements. In fact, in the T286A αCaMKII example above, mutagenesis was by a ‘Pointlox’ procedure [20]. The neo gene used to select for cells carrying the site-directed mutant is itself floxed in the targeting plasmid, and then, after homologous recombinants were identified, transient transfection with a Cre-expressing plasmid allowed specific excision of the selective marker, which could otherwise affect local gene expression. While this left a single loxP site in the targeted region, expression controls showed the effect of the element was negligible.

These strategies for conditional genetic modification are critically dependent upon appropriate and controlled expression of Cre recombinase. Turning Cre activity on and off at particular time points may also minimize uncontrolled and perhaps deleterious recombination in the wild-type mouse genome [35], which can occur by Cre acting upon endogenous cryptic loxP sites of the mouse genome. These potential problems may in part be resolved through regulation of Cre activity.

One approach is to use a floxed version of the Cre gene itself, so its expression is abrogated by recombination after the Cre recombinase reaches an effective concentration. Another strategy has been to control the localization and thereby the enzymatic activity of the Cre recombinase, by fusing it to a mutant estrogen receptor fragment (CreERT2) [36, 37]. This Cre fusion is insensitive to endogenous estradiol, but sensitive to a synthetic ligand, tamoxifen. The expressed fusion protein is trapped in the cell cytoplasm by heat shock proteins, but addition of tamoxifen frees it, allowing it to enter the nucleus [37]. After attaining access to genomic DNA, the Cre recombinase activity of the fusion protein works efficiently on loxP sites. In combination with mice carrying a floxed gene, administration of tamoxifen would therefore produce a temporally controlled knockout. Tamoxifen also can be administered to lactating mothers to allow recombination in early postnatal development of floxed offspring [37]. Again, spatial and cell type specificity must arise from the interaction between vector promoters and genomic integration site driving CreERT2 transgene expression.

One way to increase the reproducibility of transgene expression and gain further control is to introduce the transgene in a site-specific manner in a targeted genomic locus. Transgene insertion can be targeted with Cre recombinase after knockin of loxP elements. This so-called ‘recombination-medi-
ated cassette exchange’ allows the creation of new transgenic mice carrying single integrants at pre-defined loci [38] to aid both comparative analyses [39] and also induction strategies [40].

**Regulation of gene expression (doxycycline, rtTA2/tetO)**

A different approach to gain temporal control of gene expression involves the application of a ligand-dependent transcription system. The basic idea is that an exogenous chemical ligand with no known mammalian targets would be able to cross the blood-brain barrier, entering cells to regulate transcription in an inducible and reversible manner (in contrast to the irreversible KO mediated by Cre recombinase). Such a system has been developed by Hermann Bujard and colleagues [41, 42] and is composed of two genetically encoded elements based on bacterial tetracycline resistance. The first element is a transcriptional repressor (TetR), and the second is its specific DNA binding motif (tetO). In bacteria, tetracycline binds the repressor protein, relieves transcriptional repression by preventing binding of the repressor to DNA, and thus induces resistance genes. Fusion of the repressor protein to a transcriptional activation domain (from a herpes simplex virus protein, VP16) allows binding at tetO elements to induce RNA transcription. This tetracycline-controlled trans-activator (tTA) has been shown to allow regulation of transcription over five orders of magnitude *in vitro* [41]. Variant factors have been further selected so that addition of ligand (tetracycline or the most commonly used analog, doxycycline - Dox) can not only turn gene expression off (tTA), but also on (reverse tTA or rtTA).

In order for Dox to regulate gene expression in the mouse, the promoter driving the gene of interest must include tetO sequences, usually in multiple copies, for binding of the specific transcriptional regulator (tTA or rtTA). These elements can be inserted into transgene constructs directly or into an endogenous locus *via* gene targeting in ES cells, as described above. An optimized rtTA, rTA2 [43, 44], schematically depicted in Fig. 2B, has now been tested using the αCaMKII promoter for forebrain specificity, and both inducibility and reversibility of transgene expression look promising [43, 45]. Although the *in vivo* induction was not as sensitive as would be predicted from the *in vitro* report [43], both the dose and duration of Dox treatment were found to correlate well with the amount of expression when analysed in tetO-lacZ reporter mice. The induction by Dox in food (6 mg/g) takes about six days to reach steady-state in this system, with the earliest βgal activity seen after four days of treatment [45]. One important benefit of this strategy is that a mouse can develop normally before inducing transgene expression. Alternatively, Dox can even be fed to the pregnant mother. Using this method, it is therefore possible to regulate the temporal expression pattern of a transgene.

Further layers of specificity in genetic control can be generated through combining Cre-loxP recombination and doxycycline regulation [46]. However, even with such combinatorial strategies, as before, spatial control and cell type specificity are dependent upon the interaction between transgene promoter sequences and integration sites. This may in part be remedied by site-specific transgene insertion as mentioned above.

**Transduction vectors**

Remarkable progress has been made in the construction of specific and controllable transgenic or gene-targeted mice. A major limitation at this point in time is the specificity of available promoter sequences, and their sensitivity to integration site genomic context. Indeed, it is not clear whether single promoters will ever be available to, for example, specifically express in a given cell type in a given layer of a particular neocortical area. Furthermore, some promoters might work well in one context, but fail when transferred to a different mouse strain, for example [47–49]. In addition, different mouse strains behave differently (*e.g.* Refs. [50–52] to cite only a few examples), potentially complicating analyses. For instance, when crossing a transgenic mouse created by random insertion (*i.e.* a Creexpressing mouse line) with targeted mutants generated in ES cells (*i.e.* a mouse line carrying a floxed gene of interest), the genetic background of the cross-progeny might include contributions from up to four different mouse strains. Non-germline genetic manipulation could help mitigate such complications.
Another drawback of all the transgenic approaches is that they cannot be used for therapeutic purposes, since they require genetic manipulation at the germline level. There is therefore considerable interest in developing alternative strategies for controlled genetic manipulation, which might ultimately be used to cure diseases of the mature nervous system. Various transduction vectors have already provided promising results in a variety of systems, as will be described below.

The discovery of methods for selective down-regulation of gene expression (‘knockdown’) by RNA interference (RNAi) [53–55] has provided further motivation for exploring alternative strategies to germline genetic manipulation. Short sequences of double-stranded RNA, termed short interfering RNAs (siRNAs), sharing sequence identity with the targeted gene down-regulate expression of the endogenous gene by targeting its mRNA for destruction. The siRNAs can be produced in cells from plasmids expressing short hairpin sequences (shRNAs). RNAi can be induced through shRNA-expressing transgenic mice, but perhaps more importantly this strategy can be employed via molecular manipulations induced by transduction vectors without the need to affect the germline [56, 57].

A key aspect of transduction methods for stable gene delivery to brain cells is that they can provide spatial selectivity, for instance, through targeted injection into the mature nervous system.

**DNA for direct gene delivery**

Many methods have been developed and utilized for gene transfer into neurons, including utilization of ‘naked’ DNA [58], which can be taken up by cells after injection or can be directly electroporated into target cells [59, 60]. Encouraging recent studies in mouse liver, using plasmids encoding the cDNA for phenylalanine hydroxylase and a bacteriophage integrase [61], resulted in a long-term cure of a genetic disease, phenylketonuria. DNA can furthermore be coated with lipids and other proteins, like monoclonal antibodies, for specific targeting, even through the blood brain barrier [62]. These strategies are likely to advance over the coming years, but currently viral vectors are the most efficient tools for genetic manipulation in the adult mouse brain.

**Viral vectors to transduce neurons**

Viruses evolved to direct gene expression in infected hosts in order to replicate. The generic virus consists of a nucleic acid genome encapsidated within a viral particle (or virion). The virus on structural proteins surround and protect the genome, and enzymatic activities are sometimes also encapsidated within the virion, particularly if the genomic molecule cannot be directly translated in the host cell. Some viruses do not encode replicative functions in their genome, but such activities can be provided by another, ‘helper,’ virus. Certain viruses are surrounded by lipid derived from the infected host cells, and such ‘enveloped’ viruses generally require additional membrane bound molecules to allow binding to target cell surface receptors. Basic virology research has defined key mechanisms of how a virus recognizes a target cell, enters the target cell, transfers genetic information, and initiates a productive infection.

Through genetic engineering, the viral genome can be harnessed to transfer genes of interest into a variety of cell types. Viral packaging cell lines have been developed, which allow production of vector particles including the gene of interest but lacking replicative viral genomic material. Such cell lines can be created by expressing capsid and enzymatic functions from plasmids lacking sequences necessary for viral propagation. The gene of interest is expressed for vector production from a plasmid in the appropriate genetic context for encapsidation into vector particles and transduction into target cells. Many viruses have already been used for genetic manipulation of target cells, including retroviruses, Sindbis virus, herpes simplex virus, Semliki Forest virus, and adenovirus, some with therapeutic goals (as reviewed in Ref. [63]). The main issues of concern for viral transduction strategies are target cell specificity, target cell health, potential immune responses, controlled vector production and safety (for instance, potential generation of replication competent virus). Here we focus on recombinant adeno-associated virus (rAAV) and pseudotyped lentivirus (LV), which are currently some of the most efficient vectors with low intrinsic toxicity for transduction into many cell types.
Recombinant adeno-associated virus (rAAV)

Adeno-associated viruses (AAV) have stood out among viral vectors in the capacity to allow genetic modifications of large numbers of cells [63]. AAV is a parvovirus with a single-stranded DNA genome. Notably, it has not been associated with any known disease in animals, making it attractive for development as a therapeutic tool. Replication of AAV requires a helper virus. As indicated by the name, adenovirus is the classic helper, but herpes simplex virus and other packaging line or mutant helper constructs can be used for virus production [64, 65]. If replication competent recombinants, which can arise from nonhomologous recombination between AAV and helper virus, are generated, however, these cannot be readily eliminated [64]. Nonetheless, since about 80% of people carry circulating antibodies to wild-type AAV with 30% expressing neutralizing antibodies, without obvious negative effects, these vectors are considered safe. High levels of neutralizing antibodies can decrease transduction efficiency, but alterations of serotypes (see below) can potentially surmount such problems [66].

The AAV genome encodes Rep proteins (responsible for replication and packaging) and Cap proteins (structural capsid proteins). The genome is flanked by inverted terminal repeats (ITRs), which are required for packaging and replication of the genome. These ITR sequences are the only part of the AAV genome included in the recombinant vector (rAAV), with a gene of interest inserted between ITRs in the vector plasmid. AAV genomes are mainly extrachromosomal (or episomal) both during replication and long-term persistence. Therefore rapidly dividing cells are not generally good targets for transduction with this vector, since the AAV genome will be lost gradually. However, wild-type AAV can integrate inefficiently into the genome of infected cells [67] (for human DNA, usually at a preferred site of chromosome 19 [68], where it is apparently well-tolerated). Integration is dependent upon viral enzymatic activities of Rep proteins. Because Rep proteins are made de novo during productive infections and are not packaged into either AAV or rAAV particles, such directed integration into chromosome 19 should therefore not occur after transduction by rAAV vectors. Oncogenesis and insertional mutagenesis, as reviewed previously [67] and discussed further below, might further result from rare random integration, but this also is normally dependent upon Rep function.

Domains within the AAV capsid proteins encode the determinants for host cell entry, and thus define the serotype of the AAV [69]. For instance, rAAV-2 (serotype 2), one of the most commonly used serotypes of the vector, binds heparin sulfate proteoglycan, fibroblast growth factor receptors and integrins, while rAAV-5 binds sialic acid (and likely other components of a receptor complex) found on airway epithelial cells for viral entry [70]. Serotypes can be manipulated by genetic modification of the capsid protein. Since a high-resolution structure of the capsid protein has been described, exposed positions can be selected for insertion of peptide determinants. For instance, both neuronal target cell specificity and retrograde transport were enhanced through elegant engineering of rAAV capsid proteins with, respectively, a peptide from an NMDA receptor antagonist and a peptide that mimics binding domains of dynein [71]. With this engineered capsid, infection of a peripheral site, even via intramuscular injections, can result in transduction into neurons [71]. This highlights another aspect of AAV infection: both anterograde and retrograde transport have been documented (for instance, see Ref. [72]). Because rAAV does not replicate, expression of transduced genes is limited to directly infected cells. Long term transduction and long range tracing of axonal trajectories of infected neurons with little or no inflammation in the brain are both possible after stereotaxic injections of rAAV [73, 74].

rAAV has already been used for a variety of successful neurobiological research applications. Imaging axons and synaptic boutons in adult animals by two photon microscopy in macaque visual cortex after rAAV-eGFP expression was the focus of one recent study [75], showing that ongoing processes of synaptogenesis and synaptic elimination occur in primary visual cortex. Furthermore, rAAV has been used for Cre recombinase expression and was shown to induce conditional genetic modifications within seven days after stereotaxic injection of vector in brains of reporter mice [76]. Targeting of the adenosine A1 receptor in the hippocampus of floxed adenosine A1 receptor transgenic mice with such a vector resulted in focal deletion of the receptor and demonstrated that the A1 receptor acts presynaptically in CA3 neurons of the hippocampus [77].
While this local KO abolished the response to adenosine, infected neurons retained otherwise normal physiological responses. Similar focal KOs with rAAV-Cre in fNR1 mice recently demonstrated a specific role for CA3 NMDARs in learning from new experience, using a paired associate task [78].

rAAV vectors are also progressing towards clinical applications. A canine model of childhood blindness has been successfully treated through retinal injection of a single dose of rAAV, expressing the RPE65 gene, which encodes a retinal pigment epithelium protein [79]. Many different genetic mutations in retinal proteins contribute to a high prevalence of inherited retinopathies (numbering about one in 2000 individuals worldwide) [80]. The rAAV transduction vector strategy offers hope for some of these retinal diseases. In a rat model of Parkinson Disease, a combination of rAAV vectors has been used to temporally control dopamine production [81]. One vector expressed the regulated CreERT2 and the other carried a floxed gene for a dopamine synthetic enzyme, allowing selective KO of the synthetic enzyme, tyrosine hydroxylase, upon treatment with tamoxifen. This can prevent adverse effects due to over-expression of the synthetic enzyme, which might interfere with therapeutic effects of L-DOPA in patients. Finally, rAAV has been used for RNAi, for instance to deliver shRNAs to inhibit mutant ataxin-1 in a mouse model for polyglutamine-induced dominant neurodegenerative diseases [82]. rAAV therefore appears a useful candidate transduction vector to manipulate neuronal molecular genetics both for research and for therapy.

Lentivirus (LV)

Lentiviruses are RNA viruses, of which HIV is the best-known example. They can be considered complex retroviruses. Thus, reverse transcription of the RNA genome to DNA and integration into the genome of infected host cells is essential to the lentiviral life cycle. In addition to the retroviral genes, gag (structural proteins including capsid), pol (polymerase, reverse transcriptase) and env (envelope glycoprotein), the lentivirus encodes accessory proteins, which help maintain persistent infection. Many of these accessory proteins were removed as the lentivirus was developed into a transduction vector [83, 84]. Fig. 3A summarizes the four plasmids required for LV vector production by transient transfection of transformed human fibroblast cells. This is a so-called ‘third generation’ self-inactivating (SIN) virus system [85], lacking sequence elements from the unique 3’ (U3) region of the transfer vector, which otherwise might allow expression from the long terminal repeats (LTRs) formed after reverse transcription and integration in the infected cell. One plasmid encodes virion components and an accessory protein, Tat (a transactivator). Another plasmid encodes Rev (an RNA export factor). The third plasmid encodes VSV-G (an envelope glycoprotein from the Vesicular Stomatitis Virus). These LV vectors do not use the natural lentiviral envelope gene product for cell entry, but are pseudotyped by VSV-G, which is broadly infective [86].

A gene of interest is cloned into the transfer vector plasmid, in this example GFP. The transfer vector plasmid contains all the viral elements necessary for transduction: a packaging sequence (Ψ), a promoter to drive gene expression, and LTRs, the second carrying the SIN U3 deletion (ΔU3). Transducing LV particles contain RNA derived from the transfer vector plasmid, whose expression is enhanced by Tat acting on the LTR. The transfer vector RNA is packaged in the vector particle along with the reverse transcriptase and integrase proteins (encoded by pol). Notably, the RNA packaging signal (Ψ) is deleted (ΔΨ) from the packaging constructs encoding viral components and only the transfer vector includes this element. Therefore, viral RNA sequences are excluded from the vector, whereas the transfer vector RNA is specifically packaged. Expression of the gene of interest in transduced cells will be driven by the internal promoter (PGK in this example) after reverse transcription and integration into the target cell genome.

A schematic depiction of a vector particle is shown in Fig. 3B. Such LV particles bud from producer cells [87] and are released into the cell culture medium. Vector stocks are prepared by collecting supernatants from the transfected cells and resuspended for direct injection into the brain after purification by ultracentrifugation. This simple purification is made possible by the stability of the VSV-G glycoprotein, which also retains activity after a freeze/thaw cycle. The natural lentiviral
envelope glycoprotein does not retain function upon such treatment. Another potential benefit from using VSV-G is that these pseudotyped LV vectors are not subject to superinfection interference, a mechanism by which viruses can limit infection of a host cell to a single integrated genome. Thus, multiple vector particles can enter, integrate, and be expressed. This means that the sum of gene expression by transfer vector sequences in the host cell reflects that of the given promoter, exhibiting little influence due to integration site, unlike a standard transgene. Alternatively, if desired, dilution of the vector stock can allow transduction via primarily single copy integration.

LV vectors, like rAAV, can be stereotaxically injected directly into the central nervous system, for
example, into the mouse somatosensory barrel cortex as shown in Fig. 3C. Under anesthesia, a small craniotomy is made and usually about 20–100 nl of the viral suspension is injected with glass capillaries pulled to about 7 μm inner diameter. Brain tissue analyzed from 4 days to 8 months after injection appears normal, indicating that there is no obvious toxicity associated with lentiviral injection. Injection of lentivirus encoding GFP under the PGK promoter reveals brightly fluorescent cell bodies, spiny dendritic processes, and both local and long-range axonal projections. Fig. 3D shows an example of a cluster of L5 cortical cells, expressing eGFP after lentiviral infection. Depending upon titer and injection conditions, cells within 200 μm of the injection site are infected, as previously described [88]. Sparsely infected cells can also be obtained, as shown in Fig. 3E, which depicts an isolated GFP-positive pyramidal neuron. Fig. 3F shows a close up of dendritic spines from the same cell. As indicated by these confocal micrographs, high-resolution imaging is possible in neurons expressing GFP transduced by LV. The morphology of infected neurons appears normal and healthy by light microscopy. Ultrastructural analysis using serial section electron microscopy [89] further confirms normal synaptic structure of transduced neurons. The lentivirus is therefore a useful tool for studying neuronal morphology, allowing both the study of synapses and of long-range axonal projections. In this context, it is important to reiterate that neurons are transduced almost exclusively in the immediate vicinity of the lentivirus injection site, allowing unambiguous tracing of axonal projections (not complicated by mixed retrograde/anterograde transport of commonly used anatomical tracers).

Critically, the use of GFP to label transduced cells allows analysis of living neurons both in vitro (in brain slice preparations) and in vivo (through two photon microscopy) [88]. Fig. 4A shows two photon images of a cluster of infected cells in a living brain slice. The GFP expression in these layer 2/3 cortical neurons is shown alone in the left panel, while the right panel overlays an image of the whole-cell patch-clamp recording electrode filled with a red dye (Alexa-594), which diffuses into the recorded neuron. Transduced cells respond normally to depolarizing steps, firing action potentials after reaching

**Fig. 4** Electrophysiological analysis of living neurons infected with lentivirus. A. Two photon laser scanning microscope images of an *in vitro* brain slice prepared from a mouse injected with lentivirus in the barrel cortex. The left panel shows the GFP signal in transduced neocortical L2/3 neurons, and the right image overlays the red fluorescence (Alexa 594) of the whole-cell pipette and the recorded layer 2/3 pyramidal neuron. B. Membrane potential responses to current injection (-300pA to +300pA). Lentiviral transduced neurons have indistinguishable action potential discharge properties compared to uninfected neurons. C. Excitatory postsynaptic potential (EPSP) evoked by extracellular field stimulus delivered in L4. Lentiviral transduced neurons have indistinguishable EPSPs compared to uninfected neurons.
threshold (Fig. 4B). Extracellular field stimuli delivered in cortical layer 4, evoke EPSPs in the recorded layer 2/3 pyramidal neuron, which are indistinguishable from control untransfected neurons (Fig. 4C). Neurons transduced with lentivirus are therefore both morphologically and physiologically normal and remain healthy for many months after infection, likely for the lifetime of the animal. Lentiviral-mediated transduction of molecules to alter neuronal function should therefore be a reliable tool, since infection per se has no apparent deleterious effect upon transduced neurons [90].

In a recent example of how LV transduction can be applied to basic questions in neuroscience, Maskos et al. [91] investigated the role of nicotinic acetylcholine receptors specifically in the ventral tegmental area (VTA), an area of the brain that is believed to mediate reinforcement properties of addictive drugs. Targeted LV transduction of the VTA with vector expressing the β2 subunit of the nicotinic acetylcholine receptor (nAChRβ2), into transgenic mice lacking this receptor, allowed ‘re-expression’ of the subunit in dopaminergic cells of the VTA. The PGK promoter drove expression in this study, with an internal ribosomal entry site (ires2) for a bi-cistronic transfer vector also expressing eGFP to label infected cells. Electrophysiological responses to nicotine were rescued, and nicotine self-administration and nicotine-induced dopamine release were reinstated in the knockout mice expressing the introduced nAChRβ2 subunit. This elegant study thus defined both an anatomical focus (neurons of VTA) and the molecules (nAChRs containing the β2 subunit) mediating complex behaviour. The in vivo molecular dissection of specific neuronal contributions to many brain functions is thus clearly possible using such tools.

Vector technology and use

Transduction vector approaches, in summary, offer simple methods to express genetic activities in localized and targeted cells, in particular neurons. Direct comparisons between the two vector systems have begun [92], but which vector is best for a particular application is still open to debate. Lentiviral vectors may have advantages for long-term transduction, since they integrate stably into the genome, and the transferred genes are then carried and expressed as normal cellular genes, unlike genes transduced by the rAAV vectors. However, rAAV expression has been shown to be stable for at least 6 months in neurons (see for example, Ref. [74]). Other features, like coding capacity, infectivity, and promoters for expression of such vector systems, have been subjects of several reviews (for instance, Refs. [63, 93, 94]) and can help determine which is best to utilize. Nonetheless, even potential limitations of a particular system can be overcome. For example, whereas rAAV has a more limited coding capacity than LV, the use of split or trans-splicing AAV vectors can double coding capacity [95]. Both vector systems can allow targeted knockout via transduced Cre recombinase in transgenic mice carrying floxed genes of interest, or knockdown of gene expression via RNAi in transduced wild-type mouse strains. Cell-specific promoters are possible in both systems, to target different neuronal subtypes and developmental stages (see for instance, Ref. [88]), although success has been obtained in both systems also with very general promoters, like the PGK promoter. Additionally, similar to alterations in the AAV capsid described above allowing distal infection sites to result in neuronal infection of the CNS [71], pseudotyping LV with other envelope proteins can also change its infective capacity (i.e. also analogous to rabies-pseudotyped EIAVs [96]). It should furthermore be noted that the VSV-G pseudotyped lentivirus can also be used as a simple way to generate transgenic animals by zygote infection rather than DNA pronuclear injection [97, 98].

A viral vector strategy may be well-suited to gene therapy where local transgene expression could be used to compensate known molecular deficits. Obviously enormous caution is necessary when considering how to apply these technologies therapeutically. The most basic safety issues relate to replication competence and integration site. Most viral transduction vectors are engineered to prevent the generation of replication competent variants. However, recombination could perhaps generate these at low frequency, so both the design of vector plasmids and the production stage for generating transduction vectors are crucial. Limiting homology between vector plasmids helps prevent recombination when the plasmids are introduced into the producer cells by transfection. The ‘SIN’ LV plasmids described above require at least four recombination events for the vector to attain replication
competence from the input DNA plasmids, which is considered highly unlikely to occur. For AAV, not only helper sequences, but also common exogenous virus might provide substrates for recombination, and homology is not a prerequisite for recombination in this system. Since wild-type AAV is considered non-pathogenic, risks are believed to be low.

Preventing molecules that might provide replicative or pathogenic functions from getting into vector particles is very important. ITRs of rAAV are required for nucleic acid incorporation into vector particles, and therefore no other genetic material is incorporated. AAV capsid proteins can be expressed from plasmids to make viral particles entirely lacking nucleic acid [99]. In contrast, for retroviruses, other RNAs can be found in virions, besides those which are specifically encapsidated due to the presence of a viral packaging sequence, Ψ [100]. Indeed inclusion of Ψ is sufficient for specific encapsidation of even non-viral RNA into some retroviruses [100–102], although lentivirus packaging is more complex [103]. Additional RNAs, for instance, the GAPDH mRNA, which is efficiently incorporated into avian retroviruses, may be packaged because a part of the sequence folds into a similar conformation to Ψ. However, because they are unlikely to also contain sequence elements that will form LTRs upon reverse transcription, they would not be readily integrated or expressed, and such RNAs will generally have no effect upon the transduced cell. Rare recombination or integration of DNA reverse transcribed from such additional RNAs could be problematic, particularly if endogenous retroviral-related genes are involved. About 10% of the mammalian genome encodes retroviral-related sequences, and some of these can even express functional reverse transcriptase [104, 105]. Engineered endogenous elements have even been shown to ‘jump’ in neuronal precursor cells [106], leading to potential somatic genetic changes. Some retroviral-related RNAs and other endogenous mRNAs have been shown not only to be incorporated into retroviral virions [100], but also reverse transcribed and integrated in infected cells [107, 108]. The evolutionary distance between human endogenous retroviruses and the lentivirus is such that the risk of homologous recombination resulting in replication competence is thought to be negligible. However, other qualities (for example, transmission pathway and pathogenicity) could be acquired through recombination.

Another potentially serious problem relates to readthrough of a LV transfer vector plasmid DNA during virus production. If readthrough occurs, additional unknown sequences could be specifically packaged together with the actual transfer vector. In the worst case, readthrough might occur into an oncogene or might generate a replication competent virus. The SIN vector described in Fig. 3A does not readily allow readthrough, unlike some others [109], because a so-called ‘up-stream element,’ important to maintain the strength of the polyadenylation signal, is retained in the vector after the SIN deletion from the viral U3 region of the LTR.

The other major safety concern, for all transduction vectors, is the lack of control over integration site in the genome. Integration sites not only contribute to regulation of expression, but can potentially act to disrupt or induce expression of nearby genes [110]. Insertional activation of the same proto-oncogene [111] in independent trials to attempt to cure severe combined immunodeficiency with retroviral vectors caused leukaemia in patients. Thus, efficient strategies to sequence integration sites in cells marked for clinical trials have been proposed [112]. Because AAV replication is primarily episomal, the risk of insertional activation is reduced, in comparison to LV. The specificity of target sites for the natural LV integrase seems minimal. For instance, bent DNA has been shown to be a preferred target for both the HIV and MLV integrases [113]. However, integration site choice can be influenced by altering integrase. For instance, when HIV integrase is fused to lambda repressor, integration is found to occur near the repressor binding site [114]. Addition of designed DNA binding domains can also allow site specific integration by a modified integrase [115, 116]. Ideally, integration of transduction vectors should be directed towards a ‘safe’ region of the genome.

Resolving these safety issues is of great importance, since current research suggests that transduction vectors may be able to provide successful therapies for brain diseases such as ALS [53], Parkinson’s [117–119], Huntington’s [120–122] and other CNS disease models [72, 123–125]. Additionally, since functional enhancement of brain function will also likely become a more interesting issue as more is learned [126, 127], discussions about the neuroethics of such transduction vector use should be continued [128].
Concluding remarks

Extraordinary progress has been made over the last decades in developing specific and controlled genetic manipulations, which in mice allow defined changes in gene function in specific brain areas. However, the lack of highly specific promoters for most brain regions has prevented these strategies alone from becoming generally useful for delineating roles of specific genes in behaviour. Transduction vectors like recombinant adeno-associated virus and lentivirus offer interesting approaches for controlled and localized delivery of genetic activities into neurons, particularly those of gene-targeted transgenic mice. Additionally, these vector systems may provide the molecular and spatial specificity necessary for safe and well-controlled therapy of the diseased brain.

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References

1. Mello CC, Kramer JM, Stinchcomb D, Ambros V. Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 1991; 10: 3959–70.
2. Rubin GM, Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science 1982; 218: 348–53.
3. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the mouse embryo: a laboratory manual. 3rd Edition ed, ed. A.e.a. Nagy. 2003, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
4. Auerbach AB. Production of functional transgenic mice by DNA pronuclear microinjection. Acta Biochim Pol. 2004; 51: 9–31.
5. Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 2000; 28: 41–51.
6. Vidal M, Morris R, Grosveld F, Spanopoulou E. Tissue-specific control elements of the Thy-1 gene. EMBO J. 1990; 9: 833–40.
24. Barria A, Malinow R. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 2005; 48: 289–301.

25. Lisman JE. A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. Proc Natl Acad Sci USA 1985; 82: 3055–7.

26. Lisman J, Schulman H, Cline H. The molecular basis of CaMKII function in synaptic and behavior memory. Nat Rev Neurosci. 2002; 3: 175–90.

27. Petersen CC, Malenka RC, Nicoll RA, Hopfield JJ. All-or-none potentiation at CA3-CA1 synapses. Proc Natl Acad Sci USA. 1998; 95: 4732–7.

28. O’Connor DH, Wittenberg GM, Wang SS. Graded bidirectional synaptic plasticity is composed of switch-like unitary events. Proc Natl Acad Sci USA. 2005; 102: 9679–84.

29. Bagal AA, Kao JP, Tang CM, Thompson SM. Long-term potentiation of exogenous glutamate responses at single dendritic spines. Proc Natl Acad Sci USA. 2005; 102: 14434–9.

30. Hoess RH, Ziese M, Sternberg N. The role of the loxP spacer region in P1 site-specific recombination. Nucleic Acids Res. 1986; 14: 2287–300.

31. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci USA. 1988; 85: 5166–70.

32. Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, Tonegawa S. Subregion- and cell type-restricted gene knockout in mouse brain. Cell 1996; 87: 1317–26.

33. Tsien JZ, Huerta PT, Tonegawa S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 1996; 87: 1327–38.

34. Silver DP, Livingston DM. Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediating cellular toxicity. Mol Cell 2001; 8: 233–43.

35. Metzger D, Chambon P. Site- and time-specific gene targeting in the mouse. Methods 2001; 24: 71–80.

36. Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, Tonegawa S. Subregion- and cell type-restricted gene knockout in mouse brain. Cell 1996; 87: 1317–26.

37. Tsien JZ, Huerta PT, Tonegawa S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 1996; 87: 1317–26.

38. Long Q, Shelton KD, Lindner J, Jones JR, Magnuson MA. Efficient DNA cassette exchange in mouse embryonic stem cells by staggered positive-negative selection. Genesis 2004; 39: 256–62.

39. Le Y, Gagneten S, Larson T, Santha E, Dobi A, v Agoston D, Sauer B. Far-upstream elements are dispensable for tissue-specific proenkephalin expression using a Cre-mediated knock-in strategy. J Neurochem. 2003; 84: 689–97.

40. Wong ET, Kolman JL, Li YC, Mesner LD, Hillen W, Berens C, Wahl GM. Reproducible doxycycline-inducible transgene expression at specific loci generated by Cre-recombinase mediated cassette exchange. Nucleic Acids Res. 2005; 33: e147.

41. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA. 1992; 89: 5547–51.

42. Furth PA, St Onge L, Boger H, Gruss P, Gossen M, Kistner A, Bujard H, Hennighausen L. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc Natl Acad Sci USA. 1994; 91: 9302–6.

43. Uralinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci USA. 2000; 97: 7963–8.

44. Mansuy IM, Winder DG, Moalem TM, Osman M, Mayford M, Hawkins RD, Kandel ER. Inducible and reversible gene expression with the rtTA system for the study of memory. Neuron 1998; 21: 257–65.

45. Michalon A, Koshibu K, Baumgartel K, Spirig DH, Mansuy IM. Inducible and neuron-specific gene expression in the adult mouse brain with the rTa2S-M2 system. Genesis 2005; 43: 205–12.

46. Yu HM, Liu B, Chiu SY, Costantini F, Hsu W. Development of a unique system for spatiotemporal and lineage-specific gene expression in mice. Proc Natl Acad Sci USA. 2005; 102: 8615–20.

47. Raineri I, Carlson EJ, Gacayan R, Carra S, Oberley TD, Huang TT, Epstein CJ. Strain-dependent high-level expression of a transgene for manganese superoxide dismutase is associated with growth rate and increased fertility. Free Radic Biol Med. 2001; 31: 1018–30.

48. Everett RS, Evans HK, Hodges BL, Ding EY, Serra DM, Amalfitano A. Strain-specific rate of shutdown of CMV enhancer activity in murine liver confirmed by use of persistent [E1(-), E2b(-)] adenoviral vectors. Virology 2004; 325: 96–105.

49. Schumacher A, Koetsier PA, Hertz J, Doerfler W. Epigenetic and genotype-specific effects on the stability of de novo imposed methylation patterns in transgenic mice. J Biol Chem. 2000; 275: 37915–21.

50. Brooks SP, Pask T, Jones L, Dunnett SB. Behavioural profiles of inbred mouse strains used as transgenic backgrounds. II: cognitive tests. Genes Brain Behav. 2005; 4: 307–17.

51. Kalueff AV, Tuohimaa P. Contrasting grooming phenotypes in three mouse strains markedly different in anxiety and activity (129S1, BALB/c and NMRI). Behav Brain Res. 2005; 160: 1–10.

52. Brooks SP, Pask T, Jones L, Dunnett SB. Behavioural profiles of inbred mouse strains used as transgenic backgrounds. I: motor tests. Genes Brain Behav. 2005; 4: 206–15.

53. Raoul C, Abbas-Terki T, Bensadoun JC, Guillot S, Haase G, Szulc J, Henderson CE, Aebischer P. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nat Med. 2005; 11: 423–8.

54. Gene S, Koroglu TF, Gene K. RNA interference in neuroscience. Brain Res Mol Brain Res. 2004; 132: 260–70.
60. Rathenberg J, Nevian T, Witzemann V
58. Tiscornia G, Tergaonkar V, Galimi F, Verma IM. CRE recombinase-inducible RNA interference mediated by lentiviral vectors. Proc Natl Acad Sci USA. 2004; 101: 7347–51.

59. Szulc J, Wiznerowicz M, Sauvain MO, Trono D, Aebischer P. A versatile tool for conditional gene expression and knockdown. Nat Methods. 2006; 3: 109–16.

61. Wolff JA, Budker V. The mechanism of naked DNA uptake and expression. Adv Genet. 2005; 54: 3–20.

62. Rathenberg J, Nevian T, Witzemann V. High-efficiency transfection of individual neurons using modified electrophysiology techniques. J Neurosci Methods. 2003; 126: 91–8.

63. Verma IM, Weitzman MD. Gene therapy: twenty-first century medicine. Annu Rev Biochem. 2005; 74: 711–38.

64. Merten OW, Geny-Fiamma C, Douar AM. Current issues in adeno-associated viral vector production. Gene Ther. 2005; 12: S51–61.

65. Li C, Samulski RJ. Serotype-specific replicating AAV helper constructs increase recombinant AAV type 2 vector production. Virology 2005; 335: 10–21.

66. Peden CS, Burger C, Muzyczka N, Mandel RJ. Circulating anti-wild-type adeno-associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. J Virol. 2004; 78: 6344–59.

67. McCarty DM, Young SM, Jr., Samulski RJ. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. Annu Rev Genet. 2004; 38: 819–45.

68. Weitzman MD, Kyostio SR, Kotin RM, Owens RA. Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. Proc Natl Acad Sci USA. 1994; 91: 5808–12.

69. Gao G, Vandenberghhe LH, Wilson JM. New recombinant serotypes of AAV vectors. Curr Gene Ther. 2005; 5: 285–97.

70. Walters RW, Yi SM, Keshavjee S, Brown KE, Welsh MJ, Chiorini JA, Zabner J. Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. J Biol Chem. 2001; 276: 20610–6.

71. Xu J, Ma C, Bass C, Terwilliger EF. A combination of mutations enhances the neurotropism of AAV-2. Virology. 2005; 341: 203–14.

72. Passini MA, Macauley SL, Huff MR, Taksir TV, Bu J, Wu IH, Piepenhagen PA, Dodge JC, Shihabuddin LS, O’Riordan CR, Schuchman EH, Stewart GR. AAV vector-mediated correction of brain pathology in a mouse model of Niemann-Pick A disease. Mol Ther. 2005; 11: 754–62.

73. Chamberlin NL, Du B, de Lacalle S, Saper CB. Recombinant adeno-associated virus vector: use for transgene expression and anterograde tract tracing in the CNS. Brain Res. 1998; 793: 169–75.

74. Kirik D, Rosenblad C, Bjorklund A, Mandel RJ. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson’s model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostrial system. J Neurosci. 2000; 20: 4686–700.

75. Stettler DD, Yamahachi H, Li W, Denk W, Gilbert CD. Axons and synaptic boutons are highly dynamic in adult visual cortex. Neuron 2006; 49: 877–87.

76. Kaspar BK, Vissel B, Bengoechea T, Crane S, Randolph-Moore L, Muller R, Brandon EP, Schaffer D, Verma IM, Lee KF, Heinemann SF, Gage FH. Adeno-associated virus effectively mediates conditional gene modification in the brain. Proc Natl Acad Sci USA. 2002; 99: 2320–5.

77. Scammell TE, Arrigoni E, Thompson MA, Ronan PJ, Saper CB, Greene RW. Focal deletion of the adenosine A1 receptor in adult mice using an adeno-associated viral vector. J Neurosci. 2003; 23: 5762–70.

78. Rajji T, Chapman D, Eichenbaum H, Greene R. The role of CA3 hippocampal NMDA receptors in paired associate learning. J Neurosci. 2006; 26: 908–15.

79. Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J, Dejnkea NS, Pearce-Kelling SE, Maguire AM, Paleczewski K, Hauswirth WW, Jacobson SG. Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. Mol Ther. 2005; 12: 1072–82.

80. Sohocki MM, Daiger SP, Bowne SJ, Rodriguez JA, Northrup H, Heckenlively JR, Birch DG, Mintz-Hittner H, Ruiz RS, Lewis RA, Saperstein DA, Sullivan LS. Prevalence of mutations causing retinitis pigmentosa and other inherited retinopathies. Hum Mutat. 2001; 17: 42–51.

81. Li XG, Okada T, Kodera M, Nara Y, Takino N, Muramatsu C, Ikeguchi K, Urano F, Ichinose H, Metzger D, Chambon P, Nakano I, Ozawa K, Muramatsu SI. Viral-mediated temporally controlled dopamine production in a rat model of Parkinson disease. Mol Ther. 2005.

82. Xia H, Mao Q, Eliaison SL, Harper SQ, Martins IH, Orr HT, Paulson HL, Yang L, Kotin RM, Davidson BL. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat Med. 2004; 10: 816–20.

83. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 1996; 272: 263–7.
84. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol. 1998; 72: 9873–80.

85. Deglon N, Tseng JL, Bensadoun JC, Zurn AD, Arsenijevic Y, Pereira de Almeida L, Zufferey R, Trono D, Aebischer P. Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson’s disease. Hum Gene Ther. 2000; 11: 179–90.

86. Jakobsson J, Ericson C, Jansson M, Bjork E, Lundberg C. Targeted transgene expression in rat brain using lentiviral vectors. J Neurosci Res. 2003; 73: 876–85.

87. Perletti G, Osti D, Marras E, Tettamanti G, de Eguileor M. Generation of VSV-G pseudotyped lentiviral particles in 293T cells. J Cell Mol Med. 2004; 8: 142–3.

88. Dittgen T, Nimmerjahn A, Komai S, Lizienski P, Waters J, Margrie TW, Helmcen F, Denk W, Brecht M, Osten P. Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring in vivo. Proc Natl Acad Sci USA. 2004; 101: 18206–11.

89. Aronoff R, Knott GW, Welker E, Zufferey R, Aebischer P, Petersen CC. Genetic manipulation of mouse barrel cortex using lentiviral vectors. in Society for Neuroscience. 2004. San Diego, CA. Society for Neuroscience, Washington, DC.

90. Brecht M, Fee MS, Garaschuk O, Helmcen F, Margrie TW, Svoboda K, Osten P. Novel approaches to monitor and manipulate single neurons in vivo. J Neurosci. 2004; 24: 9223–7.

91. Maskos U, Molles BE, Pons S, Besson M, Guiard BP, Guilloux JP, Eyvrad A, Cazala P, Cormier A, Mameli-Engvall M, Dufour N, Cloez-Tayarani I, Bemelmans AP, Mallet J, Gardier AM, David V, Faure P, Granon S, Changeux JP. Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. Nature 2005; 435: 903–10.

92. Ahmed BY, Chakravarthi S, Eggers R, Hermens WT, Zhang JY, Nioulo SP, Levent C, Sablitzky F, Anderson PN, Lieberman AR, Verhaagen J. Efficient delivery of Cre-recombinase to neurons in vivo and stable transduction of neurons using adeno-associated and lentiviral vectors. BMC Neurosci. 2004; 5: p. 4.

93. Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med. 2001; 7: 33–40.

94. Palu G, Parolin C, Takeuchi Y, Pizzato M. Progress with retroviral gene vectors. Rev Med Virol. 2000; 10: 185–202.

95. Ghosh A, Yue Y, Duan D. Viral serotype and the transgene sequence influence overlapping adeno-associated viral (AAV) vector-mediated gene transfer in skeletal muscle. J Gene Med. 2006; 8: 298–305.

96. Mazarakis ND, Azzouz M, Rohll JB, Ellard FM, Wilkes FJ, Olsen AL, Carter EE, Barber RD, Baban DF, Kingsman SM, Kingsman AJ, O’Malley K, Mitrophanous KA, Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. Hum Mol Genet. 2001; 10: 2109–21.

97. Pfeifer A. Lentiviral transgenesis. Transgenic Res. 2004; 13: 513–22.

98. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 2002; 295: 686–72.

99. Kronenberg S, Kleinschmidt JA, Bottcher B. Electron cryo-microscopy and image reconstruction of adeno-associated virus type 2 empty capsids. EMBO Rep. 2001; 2: 997–1002.

100. Aronoff R, Linial M. Specificity of retroviral RNA packaging. J Virol. 1991; 65: 71–80.

101. Adam MA, Miller AD. Identification of a signal in a murine retrovirus that is sufficient for packaging of non-retroviral RNA into virions. J Virol. 1988; 62: 3802–6.

102. Sullenger BA, Cech TR. Tethering ribosomes to a retroviral packaging signal for destruction of viral RNA. Science 1993; 262: 1566–9.

103. Luban J, Goff SP. Mutational analysis of cis-acting packaging signals in human immunodeficiency virus type 1 RNA. J Virol. 1994; 68: 3784–93.

104. Nakamura A, Okazaki Y, Sugimoto J, Oda T, Jinno Y. Human endogenous retroviruses with transcriptional potential in the brain. J Hum Genet. 2003; 48: 575–81.

105. Sinibaldi-Vallebona P, Lavia P, Garaci E, Spadafora C. A role for endogenous reverse transcriptase in tumorigenesis and as a target in differentiating cancer therapy. Genes Chromosomes Cancer. 2006; 45: 1–10.

106. Muotri AR, Chu VT, Marchetto MC, Deng W, Moran JV, Gage FH. Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. Nature 2005; 435: 903–10.

107. Levine KL, Steiner B, Johnson K, Aronoff R, Quinton P, Raff M, Kiem HP, Karlsson S. Associated clonal T cell proliferation in two patients after lentiviral transgenesis. Transplantation. 2003; 75: 2109–21.

108. Carlson MB, Colledge WH, Evans MJ. Generation of a pseudogene during retroviral infection. Mamm Genome. 1995; 6: 90–5.

109. Zaiq AS, Son S, Chang LJ. RNA 3’ readthrough of oncoretrovirus and lentivirus: implications for vector safety and efficacy. J Virol. 2002; 76: 7209–19.

110. Wu X, Burgess SM. Integration target site selection for retroviruses and transposable elements. Cell Mol Life Sci. 2004; 61: 2588–96.

111. Hacein-Bey-Ahina S, Von Kalle C, Schmidt M, McCormack MP, Wulfraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabessee E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 2003; 302: 415–9.

112. Schmidt M, Glimm H, Wissler M, Hoffmann G, Obshon K, Sellers S, Carbonaro D, Tisdale JF, Leurs C, Hanenberg H, Dunbar CE, Kiern HP, Karlsson S, Kohn DB, Williams D, Von Kalle C. Efficient character-
ization of retro-, lenti-, and foamy vector-transduced cell populations by high-accuracy insertion site sequencing. *Ann N Y Acad Sci.* 2003; 996: 112–21.

113. Muller HP, Varmus HE. DNA bending creates favored sites for retroviral integration: an explanation for preferred insertion sites in nucleosomes. *EMBO J.* 1994; 13: 4704–14.

114. Bushman FD. Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences. *Proc Natl Acad Sci USA.* 1994; 91: 9233–7.

115. Tan W, Zhu K, Segal DJ, Barbas CF, 3rd, Chow SA. Fusion proteins consisting of human immunodeficiency virus type 1 integrase and the designed polydactyl zinc finger protein E2C direct integration of viral DNA into specific sites. *J Virol.* 2004; 78: 1301–13.

116. Kolb AF, Coates CJ, Kaminski JM, Summers JB, Miller AD, Segal DJ. Site-directed genome modification: nucleic acid and protein modules for targeted integration and gene correction. *Trends Biotechnol.* 2005; 23: 399–406.

117. Lo Bianco C, Schneider BL, Bauer M, Sajadi A, Brice A, Iwatsubo T, Aeberscher P. Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an alpha-synuclein rat model of Parkinson’s disease. *Proc Natl Acad Sci USA.* 2004; 101: 17510–5.

118. Zhou C, Wen ZX, Wang ZP, Guo X, Shi DM, Zuo HC, Xie ZP. Green fluorescent protein-labeled mapping of neural stem cells migrating towards damaged areas in the adult central nervous system. *Cell Biol Int.* 2003; 27: 943–5.

119. Doroudchi MM, Liauw J, Heaton K, Zhen Z, Forsayeth JR. Adeno-associated virus-mediated gene transfer of human aromatic L-amino acid decarboxylase protects mixed striatal primary cultures from L-DOPA toxicity. *J Neurochem.* 2003; 89: 634–40.

120. Zala D, Bensadoun JC, Pereira de Almeida L, Leavitt BR, Gutekunst CA, Aeberscher P, Hayden MR, Deglon N. Long-term lentiviral-mediated expression of ciliary neurotrophic factor in the striatum of Huntington’s disease transgenic mice. *Exp Neurol.* 2004; 185: 26–35.

121. Kells AP, Fong DM, Dragunow M, During MJ, Young D, Connor B. AAV-mediated gene delivery of BDNF or GDNF is neuroprotective in a model of Huntington disease. *Mol Ther.* 2004; 9: 682–8.

122. Chen ZJ, Kren BT, Wong PY, Low WC, Steer CJ. Sleeping Beauty-mediated down-regulation of huntingtin expression by RNA interference. *Biochem Biophys Res Commun.* 2005; 329: 646–52.

123. Deglon N, Aeberscher P. Lentiviruses as vectors for CNS diseases. *Curr Top Microbiol Immunol.* 2002; 261: 191–209.

124. Richichi C, Lin EJ, Stefanin D, Colella D, Ravizza T, Grignaschi G, Veglione P, Spier G, During MJ, Vezzani A. Anticonvulsant and antiepileptogenic effects mediated by adeno-associated virus vector neuropeptide Y expression in the rat hippocampus. *J Neurosci.* 2004; 24: 3051–9.

125. Klugmann M, Wymond Symes C, Leichtlein CB, Claussenner BK, Dunning J, Fong D, Young D, During MJ. AAV-mediated hippocampal expression of short and long Homer 1 proteins differentially affects cognition and seizure activity in adult rats. *Mol Cell Neurosci.* 2005; 28: 347–60.

126. Malleret G, Haditsch U, Genoux D, Jones MW, Bliss TV, Vanhoose AM, Weltauf C, Kandel ER, Winder DG, Mansuy IM. Inducible and reversible enhancement of learning, memory, and long-term potentiation by genetic inhibition of calcineurin. *Cell* 2001; 104: 675–86.

127. Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, Liu G, Tsien JZ. Genetic enhancement of learning and memory in mice. *Nature* 1999; 401: 63–9.

128. Farah MJ, Illes J, Cook-Deegan R, Gardner H, Kandel E, King P, Pares E, Sahakian B, Wolpe PR. Neurocognitive enhancement: what can we do and what should we do? *Nat Rev Neurosci.* 2004; 5: 421–5.