Recent advances in the manipulation of murine gene expression and its utility for the study of human neurological disease.

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Abstract

Transgenic mouse models have vastly contributed to our knowledge of the genetic and molecular pathways underlying the pathogenesis of neurological disorders that affect millions of people worldwide. Not only they have allow the generation of disease models mimicking the human pathological state but they have also permit the exploration of the pathological role of specific genes through the generation of knock-out and knock-in models. Classical constitutive transgenic mice have several limitations however, due to behavioral adaptation process occurring and conditional mouse models are time-consuming and often lack of extensive spatial or temporal control of gene manipulation. These limitations could be overcome by means of innovative methods that are now available such as RNAi, viral vectors and large cloning DNA vectors. These tools have been extensively used for the generation of mouse models and are characterized by the superior control of transgene expression that has proved invaluable in the assessment of novel treatments for neurological diseases and to further investigate the molecular processes underlying the etiopathology of neurological disorders. Furthermore, in association with classical transgenic mouse models, they have allowed the validation of innovative therapeutic strategies for the treatment of human neurological disorders. This review describes how these tools have overcome the limitations of classical transgenic mouse models and how they have been of value for the study of human neurological diseases.

1. Introduction

Neurological disorders are a family of more than 600 different diseases that affect millions of people worldwide. Due to the extension of life expectancy and aging of population, the occurrence of these diseases in the future is likely to increase. Our knowledge of the molecular pathways underlying the pathogenesis of this wide class of disorders has been remarkably improved through the use of transgenic mouse models [1]. These animal models have played an important role in the study of neurological diseases thanks to the possibility to stably introduce normal or modified genes into, or indeed, eliminate genes from a living organism. They have allowed the generation of disease models that resemble the human pathological state and provided convincing tools to test and validate new possible therapeutic approaches [2]. They have also lead to the exploration of the pathological significance of a putative target gene in neurological disorders. Recently, thanks to the advent of several functional genomic technologies such as microarray-based analysis or proteomic platforms a wide number of putative genes, potentially involved in a specific disorder have been identified. However the effective patho-physiological significance of these genes requires in vivo validation. One of the methods most broadly used for in vivo target validation is the genetic manipulation of the gene of interest and the analysis of the resulting behavioral phenotype and/or of the physiological alterations. Traditionally transgenic animal models were created through homologous recombination in embryonic stem

(ES) cells such that the gene of interest was either stably knocked out or over-expressed in mice. However the traditional transgenic mouse models carrying constitutive mutations were often associated with several disadvantages such as genetic compensation and developmental adaptations that could mask a clear phenotype [3]. Therefore in the last decades an extensive research has been carried out to develop technical strategies that allow the introduction or suppression of a target protein in a spatial and temporal manner. In this context tissue-specific transgenic expression or the use of inducible systems to temporally control transgene expression have been successfully applied to generate mice models beneficial for deepening our knowledge of the etiology of neurological disorders [4]. Nowadays several innovative methods for gene manipulation such as RNA interference (RNAi), viral vectors and DNA vectors as yeast-derived artificial chromosomes (YACs) or bacterial-derived artificial chromosomes (BACs) have became available and have been successfully employed to create more sophisticated animal models which have been invaluable for the study of neurological disorders (Fig. 1) [5-7]. These modern technologies offer the main advantage of allowing rapid and specific genetic manipulation in several species compared with the classical transgenic methodology. This review describes the application of these innovative methods in developing mouse models for neurological disorders and how they have overcome some of the restrictions of embryonic genetic manipulation.

2. RNA interference

RNAi is an evolutionary conserved natural process of post-transcriptional gene silencing, first demonstrated by Fire and colleagues in 1998 in the worm Caenorhabditis elegans [8]. RNA interference is defined as the process by which double-stranded (ds)RNA molecule silences gene expression, either by inducing the degradation or destabilization of complementary mRNA or by inhibiting translation. Although it is only a recent discovery, RNAi has soon become an important tool in drug discovery and had opened several biological applications and new therapeutic strategies. Tuschl and colleagues in their studies of RNAi processes in Drosophila melanogaster embryo extracts showed that long dsRNA substrates could be cleaved into short interfering dsRNA species (siRNAs) of ~22 nucleotides (nt), able to anneal to the target mRNA driving its degradation. They also showed that the introduction of a chemically synthesized siRNA of 21-22nt to these extracts facilitated the degradation of the homologous target mammalian genes [9], thereby evading the interferon response. Another technical advance came from the demonstration that siRNAs could be endogenously expressed in the form of short hairpin RNAs (shRNAs) by a DNA vector transfected into a cell. The vector contains a shRNA cassette with a promoter that drives the transcription of a fold-back stem-loop RNA structure. This shRNA is then processed to siRNA by the RNAi machinery and is therefore able to induce target-gene silencing in mammalian cells. In

addition to its power as a tool for gene silencing in biology, RNAi is also a conserved endogenous system of gene expression regulation through micro RNAs (miRNAs) [10,11]. miRNAs have been cloned from various organisms and cells (the first identified was lin-4 from C.elegans) and in vertebrates miRNAs are currently estimated to comprise 1% of animal genes [12]. Through experiments of over-expression or knockdown of individual miRNA, it has been demonstrated that miRNAs affect the mRNA levels of many genes and regulate several cellular processes, such as the control of cellular state and the developmental process [13]. Recent evidences suggest that a large number of miRNAs are expressed in the mammalian brain in a spatio-temporal manner, suggesting a crucial role of these molecules in regulating brain development and neuronal cell fate [11,14]. In fact, conditional transgenic mice lacking the activity of Dicer, the enzyme necessary for the miRNA synthesis, die postnatal due to neural hypotrophy [15]. Evidence suggests that each miRNA potentially controls the expression of hundreds of mRNAs in the brain, suggesting the presence of feedback control loops or indicating moderate modification at the level of target proteins expression. Not surprisingly several miRNAs have been shown to be associated with different neurological and psychiatric disorders since even subtle modifications of neuronal physiology of the human central nervous system (CNS) may be associated with strong effects on cognitive or neurological functions [16,17].

2.1 RNAi machinery

The miRNAs have been shown to share much of the same machinery of siRNA-induced gene silencing that is characterized by evolutionary conserved enzymes. The siRNAs, characterized by 2nt 3' overhangs and 5'-phospate and 3'-hydroxyl groups, are incorporated in the multi-protein complex known as the RNA-induced silencing complex (RISC) that unwinds the duplex siRNA into single stranded siRNA, using an ATP-dependent RNA-helicase activity. The antisense strand of the duplex guides the RISC complex to the homologous mRNA, targeting it for degradation (Fig. 2a). As previously seen, the siRNAs can also be generated in the cytosol by the evolutionary-conserved Dicer enzyme, starting from longer dsRNA molecules in the form of a hairpin structure. These shRNAs can be produced from a DNA template under the control of RNA polymerase (pol) II or III promoters (Fig. 2b). The siRNA produced from the shRNA construct is then recognized by the endogenous RNAi machinery and is therefore able to induce target-gene silencing in mammalian cells. The miRNAs are endogenous short single stranded RNAs of roughly 22nt produced by cleavage of longer (~70nt) endogenous precursors with imperfect hairpin RNA structure (pre-miRNA) by the Dicer enzyme (Fig. 2c), which suppress the expression of partially complementary target mRNAs by translation inhibition or mRNA destabilization rather than mRNA degradation [10].

2.2 RNAi application for neurological disorders

Neurological disorders are often associated with mutations at the level of specific genes. In some cases the mutant genes are associated with an abnormal gain of function that leads to the pathological state. In this context, the advent of interfering processes naturally silencing the expression of specific gene could help in understanding the pathological process or investigating the therapeutic potential of the target gene. Moreover, conditional genetic manipulation techniques that allows the creation of mouse models with a spatial and temporal control over gene expression often lack of sufficient spatial control and are time consuming. In this context, RNAi can be used to generate localized gene knockdown in mouse brain for rapid production of new genetic disease models of interest for neurological disorders.

RNAi offers several advantages as tools for gene manipulation over other silencing technologies such as the conventional antisense or the ribozyme methods such as higher specificity, versatility (interfering RNA can be designed virtually against any gene), and efficiency (in many cases, genes can be silenced by over 90%). Moreover since it does not induce the complete silencing of the target gene, it allows it to mimic the pharmacological manipulation of the gene itself [18]. However the generation of neurological models using RNAi faces a major problem of in vivo brain delivery since the blood-brain-barrier limits the passive entry of materials

from the peripheral circulation. The methodologies for RNAi application in mouse brain are essentially two: direct pre-synthesized or chemically modified siRNAs delivery using several carriage systems or prolonged vector-mediated siRNA production. The simplest way to deliver siRNAs is to locally inject them in the desired region of the brain. Successful gene knockdown in mouse brainstem and hypothalamic nuclei has been reported with this method, but only a transient and highly restricted suppression around the injection site was achieved [19]. Transfection reagents such as nanoparticles, liposomes or other cationic carriers able to efficiently deliver siRNA in the mouse brain have been recently described [20,21]. As example, Peters and colleagues employed polyethyleneimine (PEI) to deliver siRNAs targeting cyclic AMP responsive element-binding protein (CREB) and protein phosphatase 1 (PP1) into the dorsal hippocampus of mice, in order to investigate the role of these genes in mediating contextual memory formation [22]. However these methods have been reported to be associated with neurotoxic effects [23]. Alternatively, electroporation can be used for siRNA-delivery and subsequent gene silencing [24], but very few reports in the literature describe the real efficacy of these approaches to study neurological disorders. Recently, Thakker and colleagues have successfully applied the osmotic mini pump technology to efficiently deliver siRNAs and mediate gene silencing throughout the whole mouse brain and without neurotoxic effects [25-27]. The siRNA released from the

pump by the osmotic pressure, is directed via a catheter to a stereotaxically placed cannula for infusion into the brain, resulting in prolonged siRNA administration and diffusion throughout the whole brain. The dopamine transporter (DAT) protein was reduced in the substantia nigra compacta and ventral tegmental area after infusion of an unmodified siRNA into the dorsal third ventricle of mice over a 2-week period. DAT suppression was associated with a time-dependent hyperlocomotor phenotype, similar to that obtained after infusing a pharmacologically-selective DAT inhibitor. DAT is the key modulator of dopamine release into the synapse and is therefore implicated in the control of dopamine action on locomotion, emotion, reward and cognition. Similarly, suppression of the serotonin transporter (SERT) gene, implicated in the etiology of anxiety and depression, was achieved after a 2-week siRNA infusion into the dorsal third ventricle. This decrease was reflected in an antidepressant-like behaviour in the mouse forced swim test, an effect identical to that obtained from mice receiving a pharmacologically-selective SERT inhibitor. Indeed, the application of RNAi to suppress SERT in adult mouse brain represents a major advantage over the classical SERT knock-out mouse model since these knock-out mice display a paradoxical depression-related behavior probably due to developmental adaptations deriving from early life absence of SERT [28]. Therefore RNAi-induced knockdown provides a superior model for investigating the role of SERT in the adult brain in an easier and less difficult time-

consuming manner compared to the generation of conditional knock-out animals. Similarly, osmotic mini pumps were used for siRNA-induced knockdown of the metabotropic glutamate receptor 7 (mGluR7) to investigate the role of this receptor in the extinction of aversive memories in two different amygdala-dependent tasks [27]. Overall these data showed the efficacy of osmotic mini pump in delivering siRNAs in the mouse brain to produce specific and bilateral knockdown of gene expression and this is particularly useful when the pattern of expression of the target gene is widespread in the brain or is not completely understood [29]. However a big disadvantage of this method is that it requires a large amount of chemically-modified siRNA in order to produce sustained silencing due to the relative instability of RNA molecules.

Direct delivery of siRNAs to the peripheral nervous system (PNS) is less demanding compared to the brain since the PNS is more amenable to deliver to than the CNS and has been successfully employed in the study of neuropathic and visceral pain. For example, siRNA targeting the vanilloid receptor 1 (TRPV1) was directly injected intrathechally into a mouse model of visceral pain producing a significant analgesic effect similar to the reduction of pain sensitivity observed after administration of an analgesic compound [30].

2.3 Vector-mediated RNAi

One of the major disadvantages of using pre-synthesized siRNA in vivo is the transient nature of gene knockdown since small RNA molecules are relatively unstable in the extracellular and intracellular environment due to the presence of RNA degrading enzymes (RNAses). Double stranded RNA is more stable than single strand RNA and even though chemical modifications increasing the stability of siRNA have been reported, these have, so far, not allow prolonged gene silencing in vivo. Consequently, DNA vectors with RNA pol III promoters such as the RNase P H1 or the U6 small nuclear promoter have been developed to constitutively express shRNAs in order to achieve a long-lasting siRNA production intracellularly. RNA pol III promoters efficiently direct the synthesis of small non-coding RNA with well defined ends but transfer RNA (tRNA) promoters and RNA pol II-based cytomegalovirus (CMV) promoters have been also successfully used [31]. Recently, several groups employed miRNA-based expression cassettes to express artificial miRNAs resembling the stem loop structure of endogenous precursor miRNA allowing these artificial miRNAs to enter the miRNA pathway and to be processed into very high level of mature miRNAs. These artificial miRNAs have been shown to effectively silence target gene expression and miRNA-expressing vectors have been developed based on an artificial miRNA scaffold derived from human miR genes and are now commercially available [32]. Few cases of the delivery of shRNA- or miRNA-plasmids using transfection agents or electroporation

have been reported as these have lead to neurotoxic effects. For example, Makimura and colleagues reported the transient silencing of agouti-related protein (AGRP) in the hypothalamic arcuate nucleus of adult mice with liposomal transfection of an H1 promoter-driven shRNA expression vector [19]. Osmotic mini pumps have been employed to deliver siRNA-expressing plasmids to neural neoplastic cells in order to investigate the effect of silencing potential oncogenic proteins in brain tumor growth and invasion. Lakka and colleagues employed osmotic mini pump to deliver a CMV-promoter plasmid expressing siRNAs against urokinase-type plasminogen activator receptor (uPAR) and matrix metalloproteinase-9 (MMP-9) in glioblastoma-bearing nude mice [33]. siRNA expression resulted in a total regression of the pre-established intracerebral tumor growth, demonstrating that RNAi could be an effective tool to target genes involved in tumor cell invasion. Recently, encapsulated and coated siRNA-expression vectors, able to cross the blood-brain-barrier, have been developed as a potential new gene therapy for silencing oncogenic genes in brain cancer [34]. These vector-encapsulating liposomes (PILs) are externally conjugated to polyethylene glycol (PEG) to extend their half-life in the bloodstream and bind receptor-specific peptidomimetic monoclonal antibodies that facilitate brain-specific delivery. Encapsulated plasmids expressing siRNA targeting the epidermal growth factor receptor (EGFR) were injected intravenously in adult severe combined immunodeficient mice

implanted with human U87 gliomas. The targeted gene was successfully silenced and the survival time of shRNA-expressing mice approximately doubled, suggesting the efficacy of this strategy for experimental as well as therapeutic knockdown of oncogenes in the brain tumours [35].

Undoubtedly the most investigated method to deliver and express siRNA or miRNA in the brain is by means of recombinant viral vectors. Viruses are naturally evolved gene vehicles that can be engineered for the delivery of the desired sequences into host cells. The several viruses available ensure broad tropism and efficient transduction of different mammalian cells (Table 1). In the main, the viral vectors that have been employed to mediate prolonged RNAi in the brain are derived from lentivirus (LV), adenovirus (Ad), adeno-associated-virus (AAV) and herpes simplex virus (HSV). Given their inability to transduce non-dividing or post-mitotic cells, the use of oncoretroviruses for targeting neuronal gene expression in vivo is limited. On the contrary, recombinant LV vectors based on both human immunodeficiency virus (HIV-1) and equine infectious anaemia virus (EIAV) have been successfully employed to obtain long-term expression of siRNAs and subsequent gene silencing in the brain [36]. In the first proof-of-concept study, an HIV-1-derived lentiviral vector expressing a shRNA targeting the enhanced green fluorescent protein (eGFP) under the control of a U6 promoter was injected into the striatum of adult mice [37]. One week after injection, eGFP expression from a co-injected eGFP-encoding

lentiviral vector was completely silenced. Recently, lentiviral-mediated RNAi in mice was employed to investigate the potential of a new therapeutic approach for refractory epilepsy through focal cell-mediated release of adenosine to effectively suppress seizures by local augmentation of the adenosine system. Lentiviral constructs were used for the expression of anti-adenosine kinase (ADK) targeting miRNAs to mediate downregulation of ADK, the major adenosine-removing enzyme, in human mesenchymal stem cells (hMSCs), which would be compatible with autologous cell grafting in patients [38]. Adult mice that underwent the implant of hMSCs in hippocampus were less susceptibility to seizures, demonstrating the efficacy of this approach for the generation of patient identical autologous adult stem cell grafts for the treatment of epilepsy. Similarly, lentiviral-mediated RNAi was employed to silence a neuronal cell adhesion molecule, L1CAM, in glioma cells before injection into immunocompromised mice or directly in the established tumor. The L1CAM-siRNA expression resulted in suppression of tumor growth and an increase in the survival of tumor-bearing animals thus confirming the potential of both viral and non-viral RNAi gene therapy for the treatment of human brain cancer [39]. Adenoviruses and adeno-associated viruses (AAV) are single and double-stranded DNA viruses, respectively, which exhibit comparable transduction efficiencies in neuronal cells to lentiviruses. Local injections of recombinant adenoviral shRNA constructs have been shown to induce substantial knockdown of

eGFP expression in the striatum of adult eGFP-expressing mice [40]. AAV-based vectors have been also extensively used to mediate neuronal RNAi in mice for the study of neurological disorders. Hommel and colleagues investigated the effect of tyrosine hydroxylase (TH) silencing in the substantia nigra compacta and ventral tegmental area of adult mice [41]. TH is a key enzyme for the production of dopamine and the degeneration of dopaminergic neurons in the midbrain substantia nigra compacta is the primary cause of Parkinson disease. Hommel et al developed AAV-shRNA vectors targeting TH mRNA and reported shRNA-mediated downregulation of TH that persisted for a period of up to 50 days post-transduction and rostrocaudally spread across the substantia nigra compacta. This localized gene knockdown was coupled to a modified behaviour, in specific a motor-deficit and a reduced response to psychostimulant.

2.4 Viral-vector mediated RNAi for neurodegenerative disorders

Despite the useful application of RNAi in investigating the functional role of target genes in a specific behaviour or a physiological process, a major application of viral vector-mediated RNAi in the brain has been the exploration of the potential therapeutic efficacy of RNA interference for neurodegenerative disorders. Thanks to the high specificity of RNAi-mediated gene silencing, RNA interference has shown therapeutic efficacy in several mouse models of human neurological conditions [42].

Moreover, in combination with the long-lasting expression of siRNAs achieved by viral vectors, it represents a promising therapeutic option for treating neurodegenerative diseases [43]. Several groups have employed RNAi to target dominantly-inherited genes associated with neurodegenerative disorders such as spinocerebellar ataxia (SCA), Huntington's disease (HD) and Amyotrophic Lateral Sclerosis (ALS). Dominant mutations in the human Cu/Zn superoxide dismutase (SOD1) gene, one of the causes of familial ALS, lead to progressive death of motoneurons through a gain-of-function mechanism. The efficacy of RNAi to silence the pathogenic SOD1 mutant gene in ALS mice has been reported by three independent groups. In mice expressing a mutated form of SOD1 (SOD1G93A), increase in the survival and function of motor neurons and a marked reduction in the muscular atrophy was observed after intraspinal injection of SOD1-shRNA expressing lentiviral vector [44]. Similarly, an EIAV-based lentiviral vector [45] and an AAV2 vector [46], both expressing a shRNA against SOD1 mutant, when injected into the muscle of mouse models of ALS, have producing similar therapeutic effects. These results show that intramuscle or intraspinal delivery of a viral vector silencing SOD1G93A provided a considerable therapeutic benefit in ALS mouse models by delaying beginning of motor neuron impairment and prolonging neuron survival. Nonetheless, a major hurdle of viral vector-mediated RNAi therapy for humans is the invasive delivery of viral vectors in the target neuronal population which would

require surgical intervention. Recently AAV6 vectors expressing shRNA targeting SOD1 was delivered intravenously (i.v.) in SOD1G93A mice, inducing a significant reduction in SOD1 protein in the muscles but without altering the time course of the disease. This demonstrated the limited efficacy of viral vector-mediated RNAi for treating neurodegenerative disorders if the viral vectors are delivered i.v. [47]. Similarly, several studies reported efficacy of RNAi in treating animal models of polyglutamine disorders, such as Huntington's disease and SCA1. These disorders are induced by expanded CAG repeat mutations in the genes for the huntingtin or ataxin-1 proteins, resulting in abnormal polyglutamine stretches which confer a disabling neurotoxic gain-of-function in HD or SCA1 respectively. Recombinant AAV-shRNA vectors were reported to efficiently silence the mutant huntingtin in the striata or cerebellar lobules of murine models of HD and the successful protein suppression was associated with a significant reduction in the HD neuropathology [48-50]. AAV vectors have also been used to express siRNAs targeting a specific isoform of acid-sensing ion channel (ASIC) in a mouse model of HD. The RNAi-mediated ASIC silencing resulted in a decreased aggregation of the mutated huntingtin in the striatum of HD mice [51]. Thus, these studies provide evidence of the potential efficacy of viral vector-mediated RNAi for post-symptomatic HD therapy. Similarly, recombinant AAV vectors, expressing human ataxin-1-targeting shRNA from the H1 promoter, have been injected at multiple sites in the midline

cerebellar lobules of SCA1 mice, expressing the human ataxin-1 disease allele in cerebellar Purkinje cells [52]. Using this approach, successful target gene suppression was achieved, together with a significant improvement in motor performance even if only a small percentage of all the cerebellar Purkinje cells were transduced with the shRNA-expressing AAV. Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting the elderly and characterized by the accumulation of amyloid-β protein (Aβ) in the CNS. A great deal of effort has been made recently to develop treatments reducing the accumulation of amyloid-β in the brain of AD patients that leads to neuronal dysfunction and eventually to neuronal death. Several independent groups have reported the efficacy of viral vector-mediated RNAi to directly reduce expression of amyloid precursor protein (APP) or silence genes involved in APP processing. Hong and colleagues developed HSV vectors inhibiting the accumulation of Aβ by expressing APP-targeting shRNA. These workers initially over-expressed the APP protein through injection of LV-APP vectors in hippocampus and then observed a significant inhibition of Aβ deposits in the mouse brain after treatment with the HSV-APP/shRNA vectors but not with the control vector [53]. However since the functional role of APP in normal adult brain is not clear, silencing APP function could represent a uncertain therapeutic strategy as it could lead to physiological alterations in the treated brain. Abnormal activity of the β-secretase enzyme BACE1 has been associated with the

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accumulation of Aβ and with the neurodegenerative process in the brain of AD patients. Injection of lentiviral vector expressing a siRNA against BACE1 reduced both APP cleavage and the neurodegenerative process in the brain of APP transgenic mice [54]. Using this approach the BACE1 activity was reduced but not completely abolished. Similarly, lentiviral-mediated RNAi has been employed to investigate the effect of angiotensin II (AT2) receptors silencing in hippocampus of a mouse model of Alzheimer disease. AT2 silencing was associated with a decreased Tau phosphorylation and Aβ aggregation, demonstrating the important role of Aβ-induced AT2 oligomers as a previously unrecognised sign of ongoing neurodegeneration in AD [55]. Parkinson's disease (PD) is one of the most common neurological disorders, caused by the death of dopaminergic neurons in the substantia nigra (SN) that provide dopamine input to the striatum. Since the localized area of intervention and the discrete number of neurons that have to be targeted, RNAi could represent a valid therapeutic approach for this disorder [56]. Overall results confirm the ability of siRNA-expressing viral vectors to mediate sustained and prolonged suppression of target gene in specific brain area which can be invaluable either to study or to treat neurodegenerative disorders. However, the potential benefit of RNAi as therapeutic strategy for human patients of neurological diseases could be limited by safety factors. Recently, Grimm and colleagues reported fatal side effects in mice induced by AAV-mediated shRNAs expression after low-

pressure i.v. vector injection [57]. Lethal effects were observed only with certain shRNA constructs and were not related to the target gene of the shRNA itself. In the past RNA interference were shown to induce immune response and off-target effects under certain conditions but the proper design of the siRNA or shRNA sequences can avoid or limit these side outcomes. However, the fatal effects reported by Grimm and colleagues seem to be probably induced by a competition with the endogenous microRNA pathway for post-transcriptional gene regulation [58]. Similarly, McBride and colleagues reported severe neurotoxic effects in mouse striatum induced by certain shRNA constructs, not related to the silencing efficacy or to the target gene of the shRNA itself. This shRNA neurotoxicity, presumably induced by the high levels of antisense RNA generated in mouse brain only by certain shRNA constructs was mitigated when the same sequences were placed into artificial miRNA expression systems [59]. Moreover, the difficulties of delivery of the siRNA-expressing vectors to the brain and the safety concerns associated with the use of viral vectors could limit the application of RNAi-based therapy in humans. Nonetheless, RNAi still remains a promising therapeutic approach for the treatment of neurodegenerative disorders and recent progress in non-viral siRNA delivery could represent a solution in overcoming the blood-brain-barrier and successfully delivering the siRNA into the brain.

2.5 Transgenic RNAi

In addition RNAi has been successfully employed for the generation of prolonged, stable, loss-of-function phenotypes through shRNA-mediated transgenic RNAi. shRNA expressing vectors have been transfected into mouse ES cells using electroporation to generate stable integrants with target gene knockdown that could be then injected into blastocysts. Furthermore, stable passage of the shRNA-expressing vector from the ES cells to adult mice and germline transmission to the F1 progeny has been demonstrated [60]. Since lentiviral vectors can infect mouse ES cells and are believed to be resistant to proviral silencing during development, they have been used for transducing ES cells with the shRNA-expressing constructs [61,62]. To overcome the limitations of the constitutive and widespread gene knockdown of transgenic RNAi animals, the Cre–loxP recombination system has been employed to control the spatial-temporal expression of shRNAs. In this system, the shRNA-expressing vector is modified in order to incorporate a specific sequence that prevents transcription from the vector, flanked by loxP sites. Since these sites are recognized by the Cre recombinase, the original open reading frame of the vector is restored only by a Cre-mediated recombination event. Using inducible Cre expression systems, such as those controlled by tetracycline or tamoxifen, along with transgenic RNAi, it could be possible to investigate the phenotypical outcome for brain-specific genes loss of function with temporal and spatial specific control. This approach possesses several advantages over gene knockout

approach by homologous recombination since is faster, cheaper and is applicable to several mammalian species [63]. Zhou and colleagues employed transgenic RNAi to investigate the effect of silencing a Parkinson disease-associated gene, the PTEN-induced putative kinase 1 (PINK1) in the mouse brain. They developed RNAi transgenic mice with inducible expression of a PINK1-targeting shRNA using the Cre-loxP inducible U6 promoter. The expression of PINK1 was effectively silenced by more than 95% in mouse brain after shRNA induction but this did not result in loss of dopaminergic neurons, suggesting that loss of PINK1 gene expression in mice is not sufficient to induce Parkinson's disease phenotypes [64]. The results showed also the validity of the Cre-loxP regulated U6 promoter for conditional gene silencing in vivo, since shRNA expression was widespread in all tissues of the transgenic mice only after Cre induction.

3. Viral vectors

Viral vectors are commonly used in a wide number of applications, such as gene therapy to restore normal function or for gene expression modulation to investigate the function of a target gene or to confirm its involvement in a disease mechanism [65]. They offer the advantage of manipulating gene expression in a wide range of mitotic and post-mitotic cells and to mediate persistent expression of the delivered genetic sequences (Table 1). Among the different viral types, retroviruses are RNA viruses which reverse-

transcribe their RNA viral genome into double stranded DNA that is then stably inserted into the host genome. They mediate high gene transfer efficiency, low immunogenicity and mediate persistent expression of the transgene. However, due to their ability to integrate into host DNA they could be associated with risk of insertional mutagenesis. DNA viruses contain a single- or double-stranded DNA genome. The most predominant viruses used are the Adenovirus, AAV and HSV thanks to their broad cell tropism and efficient infection and gene transfer. Viral vectors based on adenovirus possess the advantages of having large insert capacity and relative ease of manipulation. On the other hand, their in vivo use has been limited by vector-associated immunotoxicity, inflammation, and transient transgene expression [66]. AAV-based vectors are attractive for their low immunogenicity and the ability to persist as stable episomal form in the infected cells, allowing lasting transgene expression without risk of insertional mutagenesis. Moreover, their small size makes them ideal for application requiring diffusion into wide brain area. A disadvantage is their limited insert capacity, which can be an obstacle if it is required the expression of large transgenic insert. HSV is of interest because of its large insertional capacity for foreign DNA, its natural tropism toward neurons, its relative stability and long-term expression of transgenes, particularly in neurons. However HSV vectors have associated with toxicity after viral infection. More recently, numerous new viral vectors have been developed based on vaccinia

virus, human cytomegalovirus, Epstein-Barr virus and others but their used in vivo is still limited [65].

3.1 Viral vector applications for neurological disorders

In recent years, viral vectors have been extensively used for the generation of mouse models of interest in the study of neurological disorders. As previously described, viral vectors have been effectively employed to mediate persistent RNAi in the brain for the generation of animal models with selective target gene silencing or to validate RNAi-based therapeutic approaches in mouse model of gain-of-function neurodegenerative disorders. In addition, they have been employed for the study and for the treatment of those disorders characterized by a loss-of-function mechanism and for the generation of mouse models for gain-of-function disorders, through persistent delivery of the therapeutic gene. They can be employed for either inducing persistent genetic manipulation in the whole body through transduction of the mouse germ line or in specific area of the central nervous system through intracerebral injection. Several routes for viral vectors delivery in the brain are available: intracerebral stereotaxic injection, intrathecal and intraventricular injection and intravascular infusion with or without modification of the blood-brain-barrier. The choice of route for viral vectors administration needs to be carefully considered since it affects neuronal cells transduction efficiency and spatial distribution, as well as the level of transgene expression in the

infected cells [67]. Intracerebral injection offers the advantages of low toxicity, high local vector concentrations and localized transgene delivery but it does not allow wide viral vector distribution and requires invasive surgical intervention. Ubiquitous distribution of viral vectors in the CNS could be achieved by intrathecal or intraventricular injection but these methods do not permit spatial selectivity of delivery and require a large amount of vectors. Finally, intravascular viral vector applications do not require invasive surgical intervention but necessitate the use of high vector concentrations due to losses in peripheral organs such as the liver.

Viral vectors represent a valid alternative for the generation of mouse models characterized by target gene over-expression over the classical transgene knock-in models. They allow transgene expression in a precise spatial-temporal manner in the desired animal strain and in a less time consuming manner. Viral vectors have been used to over-express alpha-synuclein (α-Syn), a Parkinson's disease related protein, in the substantia nigra pars compacta of mice to generate an innovative PD mouse model. Viral vector-mediated α-Syn over-expression resulted in a rapid degeneration of nigrostriatal neurons, which was not previously observed by genetic mutations in mice [68]. Lentiviral-mediated over-expression of a mutated APP form in mouse hippocampus resulted in rapid Aβ accumulation and was employed to quickly investigate the therapeutic potential of Aβ protein suppression by

RNAi in the brain as novel treatment for AD [53]. However, the major drawbacks of viral-mediated gene transfer compared with gene knock-in in transgenic mice are the labor-intensive nature of the stereotactic injections, and the difficulty in obtaining widespread and controlled level of transgene expression. Viral vector-mediated over-expression can be employed also to investigate the effect of target gene expression on the behaviors or in a specific molecular pathway of knock-out mice. As example, an HSV1-based vector was used to analyze the effect of restoring the expression of Kv1.1 protein in the hippocampus of Kv1.1 knock-out mice [69]. The functional role of α-synuclein in the dopamine synthesis was investigated though LV-α-Syn injection in dopaminergic neurons of α-Syn knockout mouse (ASKO) olfactory bulb. The LV-mediated over-expression of wild type α-Syn in the ASKO mice revealed the important role of this protein on TH regulation, dopamine synthesis, and cell viability [70].

Gene therapy holds great promise for the treatment of human disorders and the numerous applications of viral vectors in the last years have involved the validation of potential therapeutic approaches reversing the loss-of-function phenotype associated with numerous neurological diseases [71]. Several studies have been carried out in mouse models of neurological disorders to explore the efficacy of over-expressing potential therapeutic genes, in particular for the treatment of neurodegenerative disorders such as HD, PD and ALS. Many articles have been published in recent years

describing viral vector-mediated over-expression of various neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF) for the potential treatment of Huntigton's and Parkinson's disease. AAV2-mediated GDNF over-expression in striatum of a pre-symptomatic mouse model of HD ameliorated behavioral deficits on the accelerating rotorod and hind limb clasping tests and decreased the number of neurons containing mutant huntingtin-stained inclusion bodies, a characteristic of HD pathology [72]. Similarly, AAV-mediated over-expression of BDNF and GDNF has been shown to ameliorate the symptoms of an acute mouse model of HD [73]. These results open the possibility of using viral vector-mediated delivery of neurotrophic factors as viable treatment for patients suffering from HD. The neuroprotective effect of GDNF in a murine model of Parkinson's disease has also been demonstrated. In this study a lentiviral vector was used to over-express GDNF in the substancia nigra of a 6-hydroxydopamine (6-OHDA) model of Parkinson's disease [74]. The same group employed lentiviral vector to over-express the antioxidant enzyme glutathione peroxidase (GPX) in nigral dopaminergic neurons prior to intrastriatal injection of the toxin 6-OHDA in mice. The authors reported a small but significant neuroprotection effect of GPX in these cells against drug-induced toxicity [75]. An alternative approach to delivering neurotrophic factors in the brain affected by neurodegenerative disorders is to directly activate the intracellular signaling pathways

responsible for their effects. Ries and colleagues used an AAV1 vector to over-express the myristoylated, constitutively active form of the oncoprotein Akt/PKB in the substantia nigra of a murine model of Parkinson's disease [76]. The Akt/PKB over-expression caused a pronounced neurotrophic and neuroprotective effect in the dopaminergic neurons of affected mice. The potential therapeutic effect of insulin-like growth factor 1 (IGF1) in retarding the progression of the fatal neurodegenerative disease ALS was investigated in symptomatic ALS mice. The injection of AAV-IGF1 vectors in the deep cerebellar nuclei reduced ALS neuropathology and significantly extended life span in ALS mice [77]. Interestingly, systemic delivery of AAV-IGF1 vectors was also shown to improve motor function in a mouse model of diabetic peripheral neuropathy [78]. Viral vectors have also been successfully applied to develop novel treatments for muscular dystrophies (MDs) that are a heterogeneous group of inherited disorders characterized by progressive skeletal muscle degeneration. Several gene replacement and repair strategies mediated by viral vectors have been developed in mouse models of MDs, demonstrating the efficacy of this therapeutic strategy for the treatment of this class of disorders [79]. As example, the therapeutic potential of alpha-sarcoglycan gene (alpha-SG) replacement for patients suffering of limb-girdle muscular dystrophy type 2D (LGMD 2D) was investigated through AAV8-mediated alpha-SG expression in alpha-SG-deficient mice. A single intramuscular injection in the knock-out mice resulted in a

reversal of the muscle pathology and improvement in the contractile force [80].

Viral vector-mediated enzymatic complementation represents a promising treatment for those rare inherited neurodegenerative disorders characterized by the lack of single specific enzymes that lead to premature death. The efficacy of these novel genetic therapeutic approaches using viral vectors has been investigated in mouse models of the rare neurological inherited disorders Mucopolysaccharidosis (MPS) IIIB disease, Tay-Sachs (TS) disease, Niemann–Pick type A disease (NPA) and others. MPS IIIB is a lysosomal storage disease, caused by the deficiency of α-N-acetylglucosaminidase (NaGlu) enzyme that leads to severe progressive neurological deficits and to premature death. Intravenous and intracisternal injection of AAV vector over-expressing the NaGlu enzyme in young adult mice affected by MPS IIIB was shown to significantly prolong the lifespan and improved behavioral performances [81]. Tay-Sachs-related diseases are characterized by lysosomal storage of GM2 ganglioside caused by deficiency of α-hexosaminidase A, a heterodimeric protein. Intracranial inoculation of recombinant AAV vectors encoding the human β-hexosaminidase subunit genes, has been shown to increase the survival and improved the behavioral performance in a mouse model of Tay-Sachs disease [82]. Similarly, HSV-mediated expression of the hexosaminidase A alpha-subunit in the cerebellum and spinal cord of a TS animal model, through injection in the

internal capsule, restored the enzyme activity and abolished the GM2 ganglioside storage, without signs of viral vector toxicity [83]. AAV vectors were also used to express the therapeutic gene acid sphingomyelinase (ASM), which activity is lacking in the human Niemann–Pick type A disease, in the deep cerebellar nuclei of ASM knock-out mice, resulting in the reduction of storage pathology and correction of the behavioral deficits [84]. Taken all together, these studies indicate that viral vector-mediated expression of therapeutic genes has realistic potential for treating these rare human neurological genetic diseases that currently lack effective treatment.

Viral vectors have been of huge value to investigate potential therapeutic approaches for the treatment of brain tumors [85]. The effect of different tumor suppressor genes in inhibiting growth of tumor xenografts in nude mice has been investigated by using both retrovirus- and adenovirus-mediated gene transfer. Adenovirus has been used to express the tumor suppressor gene phosphatase and tensin homologue (PTEN) in models of malignant astrocytoma bearing mutations in this gene. PTEN expression resulted in the blockade of both tumor invasiveness and angiogenesis [86]. Both retroviral and adenoviral vectors have been employed to mediate the expression of growth arrest specific-1 (GAS1) gene in astrocytoma xenografts in nude mice and both showed efficacy in inhibiting tumor growth [87,88]. Similarly, the effect of anti-angiogenic genes on tumor growth has been investigated by means of viral vector-mediated over-expression. As example, AAV8-mediated expression

of an inhibitor of vascular endothelial growth factor (VEGF) reduced the tumor volume and increased the survival after injection in different mouse models of glioblastoma multiforme [89]. Recently, the potential of oncolytic replication-selective HSV as a new platform for brain cancer therapy, has been evaluated in nude mice bearing xenografts. These HSV-based vectors are characterized by mutations that bestow upon the virus the ability to selectively replicate in and lyse the dividing tumor cells. For example, the oncolytic HSV vector G47Δ efficiently induced regression of different forms of schwannoma tumor implanted subcutaneously in nude mice [90]. The efficacy and the safety of several oncolytic HSV vectors in human brain tumors have been already studied in several clinical trials but more efforts are needed to increase the therapeutic benefit of the treatment. Liu and colleagues investigate the potential of expressing onco-suppressor genes by oncolytic HSV to increase the therapeutic efficacy. The dominant-negative fibroblast growth factor (FGF) receptor (dnFGFR) has been expressed using an oncolytic HSV in glioma xenografts and has demonstrated increased efficacy in inhibiting tumor growth and angiogenesis compared to the standard oncolytic HSV [91]. Several gene therapy clinical trials are currently ongoing using viral vectors for the treatment of nervous system tumors and the possibility to combine viral vector-mediated gene therapy with classical treatments such as surgical and chemotherapeutic or

radiation treatments could significantly improve survival and the quality of life of human patients with brain tumors.

3.2 Viral vector applications for transgenic mice

Viral vectors can be used for the generation of more sophisticated conditional transgenic mice, using the Cre-loxP recombination strategy [92]. Exploring the functional role of a putative target gene is routinely carried out by means of transgenic mouse models, in which the target gene is either knock-down or knock-in. Brain-specific gene manipulation or modulation of the adult animal is likely to be required if the target gene is of neurological interest or if it is involved in the early developmental stages. Moreover, since the same gene may have different effects in different brain regions, the effect of the gene in a specific brain area may need to be shielded from effects in other areas. Several strategies have been developed over the past years to create conditional mouse models with specific temporal and spatial patterns of transgene expression such as combination of the Cre-loxP system with tissue-specific or inducible promoters. However, these strategies do not allow transgene manipulation in a specific localized brain area. Infection of somatic tissues of loxP transgenic mice with a viral vector expressing Cre recombinase is a means of allowing flexible temporal-spatial control [93]. Since chronic antidepressant treatment was reported to increase BDNF expression in dentate gyrus (DG) but not in CA1 region of hippocampus,

Adachi and colleagues employed AAV-mediated Cre expression to investigate the role of BDNF in subregions of the hippocampus on the etiology of depression and anxiety [94]. They developed floxed mice in which BDNF was only deleted in the presence of Cre recombinase and delivered AAV-Cre virus specifically in dentate gyrus or CA1 regions of these transgenic mice. With this approach, they reported that selective loss of BDNF in either DG or CA1 did not induce depression-like behavior but that selective loss of BDNF in the DG but not the CA1 region reduced the response to antidepressant drugs. Similarly, the effect of NMDA receptors on seizure profile in hippocampal CA3 region was investigated by viral vector expression of Cre recombinase in loxP-GluRγ1 mice, obtained by inserting the loxP sites in the GluRγ1 gene [95]. The inducible ablation of NMDA receptors in the hippocampal CA3 region of adult brain induced EEG spikes with larger amplitude compare to control and allowed to investigate the role of hippocampal CA3 NMDA receptors in controlling the excitability of the neural recurrent network oscillation. Moreover, viral vector-mediated Cre expression was employed to develop a mouse model of Friedreich's ataxia (FA), the most common of the hereditary ataxias characterized by mutations in the frataxin gene (frda). HSV1-Cre viruses were injected in the brainstem of loxP-frda mice to generate localized suppression of frataxin protein. These conditional knock-out mice developed a motor coordination deficit

that was recovered after injection with HSV-1 vectors expressing human frataxin gene [96].

4. YAC, BAC, PAC and other vectors

Transgenic animals expressing the gene of interest are commonly applied to the study of neurological disorders and a large effort has been made in recent last years to generate animal models resembling the pathological or physiological state in vivo as closely as possible [5]. One of the main problems encountered during the creation of transgenic mice is to control the expression of the transgene in order to mimic the in vivo state. In case of stochastic integration of the transgenic construct, the surrounding sequences in the host genome can modify the transgene expression pattern. In addition, most of the mammalian genes possess several regulatory sequences determining the final level of gene expression. The chromosomal position effect due by stochastic transgene integration can be overcome by targeting the site of integration by homologous recombination in ES cells. Moreover by adding all the regulatory sequences in the transgenic construct it is possible to obtain optimal transgene expression regardless of its position of integration. Since classical transgenic technologies are unable to introduce standard preparations of genomic fragments >40-50 kb, the ability to deliver vectors of large cloning capacity is required. These characteristics are present in artificial-chromosome type vectors such as yeast artificial chromosomes or YACs, bacterial artificial chromosomes or

BACs and P1-derived artificial chromosomes or PACs [97]. Thanks to their properties, these vectors have been extensively used over the past years to generate transgenic animals of use in the field of neurological disorders (Table 2). YACs are yeast artificial chromosomes with a DNA capacity of >2 Mb that are stably propagated in yeast as artificial chromosomes. Thanks to their large cloning capacity and the high versatility to genetic manipulation by homologous recombination in yeast, they are very attractive for gene transfer experiments. The main advantage that they present over standard cloning vectors is the possibility to ensure an optimal level of transgene expression, without positioning effect through providing all the regulatory elements of the transgene. Because of their large size, however, such cloning vectors are associated with several disadvantages such as the difficulty in handling, the insert instability and the complex process for advisable isolation of YAC from yeast chromosome. The bacterial artificial chromosomes are circular plasmid DNA molecules hosted in E.coli which can accommodate up to 300 Kb of foreign DNA while PACs are derived from the E.coli bacteriophage P1. Compared to YACs, BACs and PACs can be manipulated more easily but rearrangements and transgene fragmentations are still possible [98].

4.1 YACs, BACs and PACs for neurological transgenic mice

Because of the potential to express transgenes in an appropriate spatial- and temporal-specific manner, large vectors

such as YACs, BACs and PACs have been extensively used to generate transgenic mice with the aim of generating improved animal models of human neurological disorders. In particular, both YACs and BACs have been extensively used to generate enhanced animal models of neurodegenerative disorders such as Huntington, Parkinson, SCA, Alzheimer and others (Table 2). Several independent groups applied YAC and BAC vectors to generate suitable mice models for examining the mechanism of neurodegeneration occurring in Huntington disease. As previously stated, human HD is caused by a trinucleotide CAG repeat expansion in the gene coding for htt translated into a polyglutamine stretch in the protein and the size of CAG expansion in the gene is directly proportional to the severity of the disease. YACs were used to express the human mutated form of huntingtin (htt). All the regulatory endogenous regions were included in the vectors together with the genomic sequence coding the mutated htt containing different numbers of copies of the CAG repeats. As in human disease, the severity of the disease increases with the size of CAG expansion in the YAC vector and in particular YAC128 mice (with 128 polyglutamine repeats) present motor and cognitive deficits comparable with the human disease [99]. These animal models have been widely investigated to explore the mechanism underling the selective neurodegenerative process that resembles the process occurring in human HD patients [100]. For example, increased calpain activity in striatal medium-sized spiny neurons (MSNs) was

observed in YAC128 mice, suggesting that calpain could be involved in the NMDA-induced apoptosis occurring in MSNs of Huntington patients [101]. Similarly, BAC-mediated transgenic mouse models of Huntington disorder have been developed that express the human mutant form of huntingtin with 97 glutamine repeats under the control of the endogenous regulatory machinery [102]. YACs and BACs have been utilized to create transgenic mice models of spinocerebellar ataxia by using them to express the mutated form of the ataxin protein under control of its endogenous promoter and regulatory sequences [103,104]. These transgenic mice therefore represent an optimal model to investigate the degenerative process and possible therapeutic approaches. Superior genetic models of Alzheimer's disease have been created using the YAC approach. Entire genomic copies of the human mutated APP or the mutated presenilin-1 (PS-1) genes, together with their regulatory elements have been introduced into transgenic mice. These animals were shown to develop many unique features characteristic of human AD that were not observed in other transgenic mouse models of AD [105]. Similarly, BAC transgenic mouse model of Parkinson disease was created by expressing a C-terminal truncated human mutant parkin (Parkin-Q311X) in dopaminergic (DA) neurons under a dopamine transporter promoter [106]. This mouse model showed progressive hypokinetic motor deficits and degeneration of DA neurons in substantia nigra, demonstrating the toxic gain of function of parkin mutants. The

possibility to clone large fragment of genomic DNA has been extremely useful in cases in which the sequence of a particular gene is not known or when is necessary to investigate the effect of multiple genes as in the case of aneuploidy syndromes such as Down syndrome (DS). Several transgenic mice with YACs expressing part of the sequence of human chromosome 21 (HSA21) have been generated and their behavioral profile analyzed [107]. BAC containing the human DYRK1A gene located on human chromosome 21q22.2 was used to create a transgenic mouse model for the study of DS mental retardation. The transgenic mice exhibited significant impairment in spatial learning and memory tasks and alteration in hippocampal synaptic plasticity [108].

Taken together these studies demonstrate the validity of using YACs, BACs and other large cloning vectors to develop transgenic mice with a high level of transgene expression control, that represent invaluable animal models for the study of the pathogenesis and of the possible therapeutic strategies for neurological disorders.

5. Mice versus other mammals

Using mice to create suitable animal models for the study of specific disorders or to investigate the role of candidate genes has obvious advantages. First of all manipulation of the mouse genome to create specific genetic changes by microinjection of DNA into fertilized eggs or by homologous recombination in embryonic stem

cells is relatively easy compared to other mammalian species such as rats. Another advantage is the availability of "inbred strains" of mice that are genetically identical, obtained by breeding sibling mice over 20 generations. Since these animals present the same genetic background, they can be compared for the response to a treatment or a genetic modification between different laboratories [109]. However the choice of the genetic background in creating the animal model is a critical point that requires extensive consideration since different genetic traits could contribute in several ways to the mutant phenotype [110]. As described in this review, innovative technologies for gene expression manipulation, such as RNAi and/or viral vectors and DNA vectors such as YACs and BACs have been extensively applied in mice in order to create sophisticated animal models of great value in the study of human neurological disorders. Indeed, the human genome shares to a great extent the same genes as that of the mouse. Therefore, by engineering a genetic defect that causes a human neurological disease, into the mouse genome, is likely to result in the same disease in mice. Undeniably, numerous mice models of neurological disease that have been produced over recent years have developed many of the same symptoms and behavioral traits of human patients of neurological disorders [111]. In this context, RNAi and viral vectors have been applied to create even more sophisticated genetic manipulation in mice. It has been possible to analyze the effect of transgene expression modulation in specific spatial- and temporal-manner and to investigate the

potential of therapeutic approaches in pre-established mouse models of neurological disorders. For these reasons, mice represent one of the most exploited laboratory animal species in the field of neurological disease research. However, it has to be considered that genetic manipulation in mice could be associated with several disadvantages that could make it difficult to elucidate the phenotype associated with a specific genetic change [112]. As previously seen, one of these drawbacks is the variations of the behavioral phenotype deriving by the same genetic manipulation in different strain of mice. Moreover, the presence of a rich diversity of inbred mouse strains could complicate the analysis of the effects of target gene mutation. In addition, recent experimental evidences suggest that epigenetic changes can derive from the transmission of RNA or protein in the oocyte or sperm from transgenic parents to non-transgenic offspring [112]. The presence of this phenomenon limits the use of non-transgenic littermates as controls only after careful comparison with wild-type mice. Moreover, from a physiological and psychological point of view, rats have been the organism of choice for decades and the first ever laboratory animal models used since their physiological processes are similar to humans and because their larger dimension compared to mice make them more suited to the application of invasive techniques such as cannula implantation, and carrying out physiological tests. In this context, the recent tools for genetic manipulation, RNA interference and

viral-vector transgene delivery, present the obvious advantage of being applicable not only to mice but also rats and other species.

6. Conclusions and perspective

Extensive research has been carried out in recent years to develop and apply innovative technologies for targeted gene manipulation. Since the first application of homologous recombination and DNA microinjection in mice, many attempts have been made to create more sophisticated models resembling the human pathological state or for further investigating the functional role of target genes. Transgenic mouse models have contributed largely to the understanding of the genetics of neurological disorders and to the development of therapeutic strategies. However, more robust and rapid methods for precise and specialized assessment of phenotype are required. Undoubtedly RNA interference has represented an innovative method for analysis of gene function through selective gene silencing and has been applied extensively to the study of neurological disorders. Besides its physiological role in controlling gene expression through miRNAs, it has provided an efficient tool for functional genomics and target validation processes. Using osmotic mini-pumps or in association with viral vectors, it has allowed us to investigate the role played by several genes in neurological disorders. At the same time, considerable effort has been made recently to investigate the therapeutic potential of RNAi in human neurological disorders. Novel therapeutic

approaches have been opened up for this wide class of disorders since RNAi has demonstrated therapeutic efficacy in several mouse models of human neurological states and clinical trials are already ongoing for other classes of disease. Several challenges face translation of RNAi to clinic, however, namely delivery and safety. Studies in mouse models will help in clarifying the effective potential of RNAi as a therapeutic strategy and to investigate possible side effects.

Viral vectors represent another method of manipulating gene expression in adult mice and have been applied both in target gene validation and in the investigation of potential therapeutic strategies. Viral vector-based approaches differ from the standard transgenic method since they can mediate target gene manipulation in well defined regions of the adult CNS and they have been successfully used to generate advanced mice models of neurological disorders through over-expression of the disease-causing protein in specific brain regions or neuron populations. More significantly, several reports have recently been published describing efficacy of viral vector-mediated transgene expression in the treatment of neurological disorders in several mouse models. However the efficacy of viral vector-mediated gene manipulation to treat human neurological disorders needs intensive investigation due to the safety concerns associated even if it could represent a successful approach for progressive neurodegenerative conditions disorders that currently lack any effective treatments. Finally, large DNA

vectors such as YACs, BACs and PACs represent an innovative means of creating animal disease models that resemble the in vivo pathological state more closely, invaluable for investigating the efficacy and safety of novel therapeutic treatments.

In conclusion, the advent of innovative methods for genetic manipulation has allowed the generation of superior mouse models that represent a considerable resource to investigate the pathogenesis and the therapeutic approaches in neurological disorders.

Fig. 1. Tools available for genetic manipulation in the mouse nervous system and their applications in the field of neurological disorders.

Fig. 2. The RNA interference pathway. a) Short interfering RNA: the siRNA is characterized by 5' phosphorylated ends, a 19nt duplexed region and 2nt unpaired and unphosphorylated 3' ends. This double stranded RNA molecule is recognized by the multiprotein complex RISC, which unwinds the siRNA duplex and incorporates the single stranded antisense strand. The antisense strand guides RISC to the target mRNA, driving its endonucleolytic cleavage. b). Short hairpin RNA: the siRNA is produced by the Dicer-mediated cleavage of longer dsRNA precursor molecules as those in the form of a hairpin structure. In the shRNA molecule the sense and antisense strands are separated by a loop sequence. This shRNA can be produced by the transcription of the shRNA construct mediated by Pol II or Pol III promoters. c) The microRNA pathway. Dicer cans also cleavage the ≈70nt hairpin miRNA precursor to produce the ≈22nt miRNA which is then incorporated in the miRNA-protein complex (miRNP). Unlike siRNAs, the miRNAs pair with partial sequence complementarity to the target mRNA and lead to translational repression or decrease mRNA stability.

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Table 1

Viral vector	Advantages	Disadvantages
Retroviral vectors	Large insert capacity for transgene (<7-8kb). Broad cell tropism. Stable and high transgene expression. Relatively easy generation of high recombinant virus titers.	No infection of non dividing cells. Possible insertional mutagenesis for random integration into host genome. Vector instability.
Lentiviral vectors	Large insert capacity for transgene (10kb). Broad cell tropism. Infect dividing and non-dividing cells. Stable and high transgene expression.	Possible insertional mutagenesis for random integration into host genome.
Adenoviral vectors	Large insert capacity for transgene (7-8kb). Broad cell tropism. Infect dividing and non-dividing cells. High transgene expression. Relatively easy generation of high recombinant virus titers.	Immune response to viral proteins. Transient transgene expression.
Adeno-associated viral vectors	Broad cell tropism. Infect dividing and non-dividing cells. Prolonged and high transgene expression. Low immunogenicity and nonpathogenic.	Limited capacity for transgene (4kb). Difficult generation of high virus titers. Requirement of helper virus for replication.
Herpes simplex viral vectors	Large insert capacity for transgene (up to 50kb). Broad cell tropism. Infect dividing and non-dividing cells. Generation of high recombinant virus titers. Natural tropism to neuronal cells.	Possible toxicities. Risk of recombination. Transient transgene expression (prolonged in neurons).

Advantages and disadvantages of the different viral vectors most used for gene transfer in the nervous system.

Table 2

Human transgene	Vector	Human neurological disease	Reference
Huntingtin with 46, 72 and 120 CAG repeats	YAC	Huntington disease	[99-101]
Huntingtin with 97 CAG repeats	BAC	Huntington disease	[102]
Ataxin 8 with [(CTA)$_3$(CTG)$_5$CCG(CTG)$_7$(CCG)(CTG)$_{104}$] configuration	BAC	Spinocerebellar ataxia type 8 (SCA8)	[103]
Ataxin 3 with 15 and 84 CAG repeats	YAC	Spinocerebellar ataxia type 3 (SCA3)	[104]
APP	YAC	Alzheimer disease	[105]
Presenilin-1	YAC	Alzheimer disease	[113]
Tau	PAC	Alzheimer disease	[114]
Parkin-Q311X	BAC	Parkinson disease	[106]
Chromosome 21 region 21q22.2	YAC	Down's syndrome	[107]
DYRK1A	BAC	Down's syndrome	[108]

Transgenic mouse models of human neurological diseases generated with YACs, BACs or PACs.
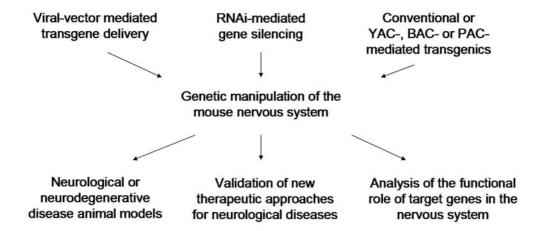
Fig. 1

Fig.2