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REVIEW

RNA interference in pain research

Thomas Röhl and Jens Kurreck

Institute of Chemistry and Biochemistry, Free University Berlin, Germany

Abstract

Within the course of only the last few years, RNA interference (RNAi) has been established as a standard technology for investigation of protein function and target validation. The present review summarizes recent progress made in the application of RNAi in neurosciences with special emphasis on pain research. RNAi is a straightforward method to generate loss-of-function phenotypes for any gene of interest. In mammals, silencing is induced by small interfering RNAs (siRNAs), which have been shown to surpass traditional antisense molecules. Due to its high specificity, RNAi has the potential for subtype selective silencing of even closely related genes. One of the major challenges for *in vivo* investigations of RNAi remains efficient delivery of siRNA molecules to the relevant tissues and cells, particularly to the central nervous system. Various examples will be given to demonstrate that intrathecal application of siRNAs is a suitable approach to analyse the function of receptors or other proteins that are hypothesized to play an important role in pain signalling. Intensive efforts are currently ongoing to solve remaining problems such as the risk of off-target effects, the stability of siRNA molecules and their efficient delivery to the CNS. RNAi has thus demonstrated that it is an extremely valuable tool for the development of new analgesic drugs.

Keywords: gene silencing, pain, RNA interference, small interfering RNA, target validation, viral delivery.

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Pain is an unpleasant but indispensable sensation that warns the body to protect from severe damage. Prolonged suffering from pain, however, can become a serious burden for the affected persons and is one of the major reasons for people to seek medical care and pharmacological treatment. More than 320 million individuals worldwide are affected from chronic pain (Ganju and Hall 2004). Despite the obvious need for pain medication and the great progress that has been made in recent years in elucidating the molecular and cellular mechanisms underlying nociception, pain drugs available to date are frequently unsatisfactory (Scholz and Woolf 2002). Current pharmacotherapy of pain is still mainly based on two classes of drugs that have been established for a long time: non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. Both classes of analgesic substances, however, have severe drawbacks, in that they frequently result in only partial and transient relief from pain. In addition, prolonged usage of these pain drugs produces severe side-effects in some patients, including gastric, kidney and liver toxicity in the case of NSAIDs and constipation, nausea, respiratory depression and sedation in the case of opioids. Thus, the need to develop new pharmacological strategies for pain relief is obvious and numerous potential targets for analgesic substances have been identified (Gillen and Maul 2002).

Some progress in the treatment of chronic and neuropathic pain has recently been achieved with new classes of drugs, including sodium and calcium channel inhibitors as well as serotonin/norepinephrine uptake blockers (for a recent review see Rice and Hill 2006). Interestingly, most of these

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Address correspondence and reprint requests to Dr Jens Kurreck, Institute of Chemistry and Biochemistry, Free University Berlin, Thielallee 63, D-14195 Berlin, Germany. E-mail: jkurreck@chemie.fu-berlin.de

Abbreviations used: AAV, adeno-associated virus; Ago2, Argonaute 2 protein; ALS, Amylothrophic Lateral Sclerosis; apoB, apolipoprotein B; ASO, antisense oligodeoxynucleotide; BBB, blood brain barrier; BDNF, brain-derived neurotrophic factor; CFA, complete Freud's adjuvant; DOR, delta opioid receptor; DRG, dorsal root ganglia; dsRNA, double-stranded RNA; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; FAF1, Fas-associated factor 1; GFP, green fluorescent protein; HIV, human immunodeficiency virus; INF, interferon; LNA, locked nucleic acids; NSAIDs, non-steroidal anti-inflammatory drugs; PEG, poly(ethylene glycol); PEI, polyethyleneimine; PI3K, phosphatidylinositol 3-kinase; polyQ, polyglutamine; PS, phosphorothioate; RISC, RNA-induced silencing complex; RLC, RISC loading complex; RNAi, RNA interference; RVM, rostral ventromedial medulla; shRNA, short hairpin RNA; siRNA, small interfering RNA; SNALP, stable nucleic acid lipid particles; TrkB, tyrosine kinase receptor B; TRPV1, transient receptor potential vanilloid 1; TTX, tetrodotoxin.

drugs were originally introduced for other therapeutic indications than pain like epilepsy and depression. Two examples for new pain killers that have been approved for the treatment of neuropathic and severe chronic pain in late 2004 are the N-type calcium channel blockers pregabalin (Lyrica) and ziconotide (Prialt), the venom of a Pacific Ocean cone snail (Garber 2005). But since most of the new drugs are used for special applications only and display certain side-effects, they cannot be considered to be the long awaited breakthrough for better treatment of pain.

In recent years, RNA interference (RNAi) has been developed as a powerful technique for functional investigation of protein function and target validation. RNAi is an evolutionary conserved mechanism by which double stranded small interfering RNAs (siRNAs) lead to the degradation of their cognate messenger RNAs, which results in a reduced content of the proteins they encode for. This mechanism was originally discovered in plants and named cosuppression (Napoli *et al*. 1990; van der Krol *et al*. 1990), later also in *Neurospora crassa* (Romano and Macino 1992), where it was dubbed quelling, and in *Caenorhabditis elegans* (Fire *et al*. 1998) and mammalian cells (Elbashir *et al*. 2001), where it was termed RNA interference. The process is induced either by longer double stranded RNA (dsRNA), which is specifically cleaved by the type III ribonuclease Dicer into short 21–23 nucleotide siRNA molecules with 2–3 nucleotides-3′-overhangs on both strands (Zamore *et al*. 2000; Bernstein *et al*. 2001; Elbashir *et al*. 2001), or by direct application of siRNA. Initially, the double stranded siRNA is assembled into the RISC complex (Fig. 1). The passenger strand is cleaved catalytically by Argonaute 2 (Ago2) (Matranga *et al*.; 2005; Rand *et al*. 2005), and after dissociation of the fragments, the single stranded guide within the complex can recognize and initiate multiple rounds of degradation of its complementary message. This mechanism might have an essential role in defending against exogenous RNA (like viral RNA) and in genome maintenance by sequestering repetitive sequences like transposons (Hannon 2002).

Longer dsRNAs (> 30 bp) do not evoke severe side-effects in invertebrates and plants, but induce an interferon (IFN) response in mammalian cells as an unspecific viral defence (Stark *et al*. 1998) by activation of protein kinase R, which can shut down protein synthesis in the affected cell (Clemens and Elia 1997), and enzymes that generate 2′-5′ phosphodiester linked oligoadenylates (2–5 A), which results in a RNAse L mediated, relatively unspecific degradation of single stranded RNA (Player and Torrence 1998). To circumvent these side-effects, siRNAs have been used in mammalian cell culture (Elbashir *et al*. 2001), a method which is applied commonly in functional genomics and target validation these days. To achieve longer lasting effects, a variety of expression vectors have been generated to produce short hairpin RNAs (shRNAs) (Fig. 1; for a review see Shi 2002), some of which have proven to achieve efficient knockdown *in vivo*. There are several recent reports, however, that indicted that non-specific effects can be induced by siRNAs at the level of mRNAs as well as at the protein level (e.g. Bridge *et al*. 2003; Sledz *et al*. 2003; for a review see Jackson and Linsley 2004). This suggests that RNAi can regulate the expression of targets other than those against which they are directed.

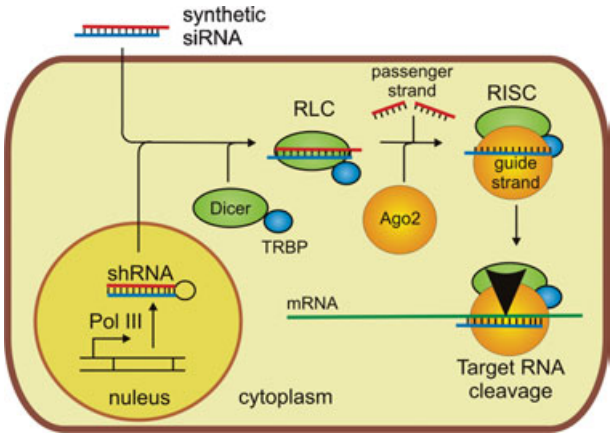

Fig. 1 Current model of the RNAi pathway. After cellular uptake of synthetic small interfering RNAs (siRNAs) the double stranded molecules are incorporated into the *R*ISC-*L*oading *C*omplex (RLC), which consists of the RNase Dicer and TRBP (HIV-*T*ransactivating *R*esponse RNA-*B*inding *P*rotein). During assembly of the *R*NA-*I*nduced *S*ilencing *C*omplex (RISC) one of the two siRNA strands, referred to as the passenger strand, is cleaved and released, whereas the other strand (guide strand) is incorporated into the Argoaute protein Ago2. The remaining strand then guides RISC to its complementary target RNA, which is finally cleaved by the RNase activity located in the Ago2 protein. For investigations of pain signalling in the spinal cord, siRNA molecules are usually delivered intrathecally. Long-term silencing of a targeted gene can be achieved by intracellular expression of a short hairpin RNA (shRNA) under control of an RNA polymerase III promoter. After being exported to the cytoplasm, the shRNA is processed to give an siRNA and follows the pathway described above.

As the RNAi mechanism potentially allows the specific knockdown of any deleterious protein, several pharmaceutical companies are currently trying to develop RNAi-based therapeutics targeting, for example, genes involved in age-related macula degeneration, cancer and asthma as well as viral RNAs (e.g. respiratory syncytial virus, influenza, hepatitis C virus and human immunodeficiency virus). RNAi is likely to emerge as the method of choice in knocking down deleterious genes of either viral or non-viral origin, e.g. mutated genes or variant alleles whose proteins are refractory to a specific treatment by conventional means such as small molecules.

Here, we give a general overview of RNAi with a special focus on *in vivo* delivery of siRNAs, particularly to the CNS. We will then summarize recent progress in the application of RNAi in neurosciences with special emphasis on pain

research. For further details on the RNAi mechanism and the functional relevance of small RNAs, the reader is referred to some excellent reviews e.g. Meister and Tuschl (2004) Eckstein (2005), Grünweller and Hartmann (2005) and Leung and Whittaker (2005).

Knockdown of pain-related genes in cell culture

Initial observations indicated that neurons might be resistant to the effects of RNAi, but this impression might have been due to a special strategy used to modulate RNAi in the nervous system of *C. elegans* (Kennedy *et al*. 2004): An RNase specific to neurons, ERI-1 (named after enhanced RNAi), which was identified in a genetic screen, specifically degrades siRNAs, thus rendering the nervous system refractory to RNAi. In the nematode worm, ERI-1 is mainly expressed in the gonad and a subset of neurons and predominantly located in the cytoplasm of these cell types, suggesting that ERI-1 siRNase suppresses the level of RNAi in these tissues. Thus, ERI-1 is a negative regulator that limits the half-life of siRNA, cell-type specificity or endogenous functions of siRNAs.

For mammals, Krichevsky and Kosik (2002) were the first to show that RNAi can work well in neuronal cells. Though primary cells from the brain are notoriously difficult to transfect, i.e. generally less than 10% of the cells take up plasmids (Trülzsch and Wood 2004), RNA duplexes are taken up relatively efficiently by postmitotic primary neuronal cells from the rat hippocampus and forebrain when applied with cationic lipids (Krichevsky and Kosik 2002). One of three targets under investigation (YB-1) was not affected by a specific siRNA, which might be explained by high protein stability of the extremely abundant YB-1 protein or by an intrinsic inaccessibility of the targeted sequence, but the expression of transfected enhanced green fluorescent protein (EGFP) and endogenous microtubule-associated protein 2 was effectively inhibited. This study clearly demonstrates that the RNAi pathway is operative in primary mammalian neurons and thus opened the road to employ this technique for pain research.

The $P2X_3$ receptor, a member of the family of P2X receptors that function as ligand-gated cation channels, attracts special attention due to its role in pain signalling (Julius and Basbaum 2001). This channel is highly localized to peripheral sensory neurons in dorsal root ganglia (DRG), where it functions as homo- or heterodimer with $P2X_2$ (Lewis *et al*. 1995). As there are no specific inhibitors for $P2X_3$ presently available, Hemmings-Mieszczak *et al*. (2003) developed an antisense approach to investigate if an effective specific inhibition could be achieved in cell culture. Indeed, siRNAs targeting different sequences of the gene regulated mRNA and protein levels down at much lower concentrations than the corresponding antisense molecules. Interestingly, the knockdown could be enhanced when siRNA was combined with non-homologous antisense oligodeoxynucleotides (ASOs), which targeted a distant region on the $P2X_3$ mRNA.

A similar superiority in potency of siRNAs compared to ASOs was observed by Grünweller *et al*. (2003) when optimizing an antisense strategy against the vanilloid receptor TRPV1, which is considered to be a key molecular integrator of diverse noxious stimuli (Cortright and Szallasi 2004; Tominaga and Tominaga 2005). siRNAs and different antisense molecules like phosphorothioates (PS), locked nucleic acids (LNA) as LNA-DNA-LNA gapmers as well as 2′-*O*-methyl RNA-DNA gapmers were analysed for their efficiency in cell culture. Reporter assays were performed with the TRPV1 cDNA fused to the green fluorescent protein (GFP) gene, and the efficiency of the knockdown was monitored by reduction of fluorescence, as well as reduced protein content. Clearly, the employed siRNAs were the most efficient molecules, being $\sim$ 7-fold more potent than the LNA gapmers and more than 1000-fold more potent than conventional PS.

Recently, the potential of RNAi for functional investigation of protein–protein interactions was demonstrated (Kim *et al*. 2006): The pharmacological response profiles between heterologously expressed TRPV1 and the native capsaicin receptors differ, i.e. the cloned receptor is more sensitive to capsaicin. These differences are likely to be due to interactions of TRPV1 with other proteins in the native environment. The Fas-associated factor 1 (FAF1) was found to be coexpressed with TRPV1 in sensory neurons and forms a complex with the receptor. Its regulatory function could be confirmed by RNAi-mediated knockdown of FAF1, which resulted in significantly increased capsaicin-sensitive currents in native sensory neurons.

Heterotrimeric GTP-binding proteins link a family of seven transmembrane receptors to intracellular effectors. Go proteins are expressed at a high level in neuronal and neuroendocrine cells and couple receptors for various neurotransmitters to neuronal calcium channels (Hescheler *et al*. 1987) and the release of hormones and neurotransmitters (Lang *et al*. 1995). Zhang *et al*. (2003a) investigated the function of Goα as a mediator of opioid receptor induced activation of extracellular signal-regulated kinase (ERK) in neuronal cells. Specific down regulation of Goα by siRNA in neuroblastoma × glioma cells lead to a decreased ERK phosphorylation and activity and thus blocked the opioid signalling pathway.

Delivery of siRNA molecules *in vivo*

For cell culture experiments, siRNAs are usually transfected with lipid-based vehicles. However, these methods cannot be directly adapted for *in vivo* applications: a rapid removal from the blood stream via the liver and the kidney has to be avoided and the delivery vehicle must not be toxic or induce

an interferon response. Furthermore, the siRNA has to be protected against nucleases in the blood stream. While enhanced stability can be achieved by chemical modifications of the sugar-phosphate backbone, like alternating 2′-*O*-methyl modifications (e.g. Czauderna *et al*. 2003a) or partial introduction of PS, efficient delivery of siRNAs represents the major obstacle for *in vivo* experiments and therapeutic applications. During the past years, various methods to overcome this hurdle have been developed. These approaches include direct application of naked siRNAs as well as the use of lipid-based delivery vehicles for siRNAs. In addition siRNA expression cassettes have been developed, which can be introduced into the target cells by viral or non-viral means.

For systemic delivery, most of the studies published to date were performed with hydrodynamic injections of naked siRNAs or shRNA expression vectors, which results in a target specific knockdown of the cognate message, primarily in the liver. However, this method does not allow delivery of siRNAs to the CNS and it is not applicable for clinical purposes due to the large volume of siRNA solution, which is injected into mice within only a few seconds. Thus, compatible strategies have to be developed for systemic delivery of siRNAs into humans.

Using siRNAs complexed with polyethyleneimine (PEI), a cationic polymer, the production of influenza A viruses in lungs of infected mice was reduced by injecting small volumes intravenously at low pressure (Ge *et al*. 2004), a method which is suitable for use in humans. With this formulation, DNA as well as siRNA was predominantly delivered to the lung.

Very recently, so called siRNA-lipoplexes were developed to deliver siRNA molecules to the endothelium after systemic administration (Santel *et al*. 2006a). This liposome formulation consists of cationic, fusogenic and PEGylated lipids, complexed with the negatively charged siRNA. PEG avoids carrier clearance by serum proteins or the complement system and may stabilize liposomes, thereby reducing macrophage clearance. The siRNA-lipoplexes were strongly taken up by the vascular endothelium in the liver, heart and lung, and knockdown of two endogenous genes, namely *Tie2* and *CD31*, correlated with this organ distribution. Strikingly, these lipoplexes did not show any toxicity, whereas non-PEGylated lipoplexes led to severe loss of weight in the animal experiments. This endothelial cell specific *in vivo* siRNA delivery technology provided the basis for antiangiogenic cancer therapies based on RNAi currently in development (Santel *et al*. 2006b).

Another sophisticated technique was described by Soutchek *et al*. (2004), who targeted the message of apolipoprotein B (apoB), a molecule involved in cholesterol metabolism. As the concentration of this protein in blood samples correlates with that of cholesterol, reducing of apoB level is expected to decrease the risk of coronary heart diseases. By covalently linking cholesterol to the hydroxyl end of the sense strand, efficient uptake of the chemically modified siRNA into the liver and jejunum was achieved after systemic administration. The treatment resulted in decreased plasma levels of apoB and, as a consequence, the cholesterol level was reduced.

In an alternative approach, lipid-encapsulated, chemically modified siRNAs, so called SNALPs (stable nucleic acid lipid particles) were injected intravenously (Morrissey *et al*. 2005). Encapsulation of the siRNA resulted in an increased half-life in plasma and liver, specifically reduced the replicative potential of the hepatitis B virus and did not induce interferon or inflammatory cytokines. The concentration of serum transaminases was not increased, which would indicate liver toxicity of the drug. The administration scheme in the animal experiments was close to a clinical dosing regime, and the prolonged activity emphasizes specificity of the mode of action and lack of systemic toxicity.

In fact, the first study to investigate systemic delivery for RNAi-mediated silencing in non-human primates was performed with SNALP-encapsulated siRNAs against apoB (Zimmermann *et al*. 2006). Intravenous injection of the nucleic acid lipid particles to cynomolgus monkeys resulted in up to 90% reduced mRNA levels in the liver. Significant reductions in apoB protein, serum cholesterol and low-density lipoprotein levels were observed for up to 11 days. Interestingly, the SNALP encapsulation of siRNAs led to a 100-fold improvement in potency compared with systemic administration of a cholesterol-conjugated siRNA against ApoB. No side-effects were observed, suggesting that this method is suitable for systemic delivery of siRNAs to hepatocytes in primates.

A further very promising strategy for cell-type specific delivery of siRNAs was developed by Song *et al*. (2005). These authors employed a fusion protein of protamine and the heavy chain Fab fragment of an antibody that binds to extracellulary displayed envelope-glycoprotein of the human immunodeficiency virus-1 (HIV-1). An siRNA was attached non-covalently to the nucleic acid-binding protamine. This strategy enables specific delivery of the siRNA into cells expressing the antigen, e.g. the HIV envelope protein. Additionally, the reversible binding of the siRNA to protamine facilitates release of the cargo after cellular uptake. Several targets were efficiently down-regulated with this approach and neither an interferon response nor any toxicity was observed. This approach demonstrates the possibility of cell-type specific, ligand-mediated delivery of siRNAs after systemic administration.

Furthermore, various systems for either constitutive or regulated expression of shRNAs have been developed. In transgenic mice, ubiquitous knockdown of gene expression can be achieved by integration of a shRNA expression cassette with a RNA polymerase III (pol III) H1 or U6

promoter at the *rosa*26 locus (Seibler *et al*. 2005). The pol III expression has also been modified to an inducible version, e.g. based on the tetracycline system. This approach allows conditional RNAi upon addition of doxycycline (van de Wetering *et al*. 2003). It has, for example, been employed to study the function of two catalytic subunits of the phosphatidylinositol 3-kinase (PI3K) in an orthotopic metastatic mouse model with human prostate cancer cells (PC-3) stably transfected with a doxycycline-inducible shRNA expression system (Czauderna *et al*. 2003b).

The various approaches to achieve efficient delivery of siRNAs and shRNA expression vectors described above point to the directions that can be followed for applications of RNAi in the CNS. Strategies to encapsulate siRNAs into liposomes and to conjugate antibodies for improved delivery have already been implemented as will be outlined in the next section. In addition, examples for viral delivery of shRNA expression cassettes will be given.

Delivery of siRNAs to neuronal cells

Prior to RNAi becoming a common research tool, ASO were employed to study the function of various pain related proteins (for review articles, see Stone and Vulchanova 2003; Kurreck 2004). One example is the tetrodotoxin (TTX)-resistant sodium channel NaV1.8, which is involved in the nociception of neuropathic pain that often results from injuries to peripheral nerves. As the expression of this channel is restricted to the periphery in sensory neurons of DRG, it represents a promising target to achieve relief from neuropathic pain. Existing sodium channel antagonists have a limited selectivity for individual members of the NaV family, so therefore Lai *et al*. (2002) developed a method to specifically inhibit target gene expression by intrathecal injection of ASOs into rats. The treatment led to a time-dependent, selective and specific knockdown of NaV1.8 and resulted in a diminished TTX-resistant sodium channel conductance leading to an antiallodynic and antihyperalgesic effect in a neuropathic pain animal model. This study clearly shows that ASOs are taken up by neurons even without delivery agents. NaV1.8 knock-out mice show up-regulation of the TTX-sensitive sodium channel NaV1.7 (PN1) in C-fibers in compensation for the missing NaV1.8, which can be misinterpreted as a specific consequence of the knockout. Comparable compensatory effects were not observed after ASO treatment of juvenile rats (Porreca *et al*. 1999). This demonstrates that, at least in this case, a knockdown approach more closely resembles the phenotype of the disease the model is intended to reflect.

Employing RNAi in pain models has the advantage that a highly efficient cellular mechanism is used, which allows to utilize significantly lower concentrations of oligonucleotides compared to the dosage needed for traditional antisense experiments (e.g. Grünweller *et al*. 2003). For the application of nucleic acids in the CNS, however, some specific features have to be considered. Firstly, naked siRNAs do not cross the blood brain barrier (BBB), and therefore neither oral nor simple intravenous administration is suitable. Secondly, siRNAs are not efficiently internalised by brain cells *in situ*, although neuronal cells can easily be transfected in cell culture (Krichevsky and Kosik 2002; see above): When siRNA was injected directly into the brain parenchyma of rats, neither a knockdown of the dopamine D1 receptor mRNA nor a decrease of the protein level were detected (Isacson *et al*. 2003), while the same siRNA proved to be efficient in cell culture when applied to mouse fibroblasts (LTK(–) cells) stably transfected with the rat dopamine D1 cDNA. In another study, however, Thakker *et al*. (2004) successfully silenced target gene expression by continuous intracerebroventricular infusion of naked siRNA at a high concentration (400 μg/day). Extensive knockdown of transgenic EGFP expression was observed in regions adjacent or dorsoventrally and mediolaterally distant to the infusion site (dorsal third ventricle), while silencing was less pronounced in more distal regions. The endogenous dopamine transporter, which is expressed within the ventral midbrain neurons, i.e. far away from the siRNA infusion site, was also knocked down to a significant extent. The relative ease of this approach clearly accelerates target validation of neuropsychiatric disorders when compared to viral delivery of siRNAs or the generation of transgenic animals.

As outlined in the previous section, conjugation of carrier molecules is a promising strategy to improve efficient and specific uptake of siRNAs. This strategy has also been employed to deliver siRNAs to neuronal cells. Rapid and efficient delivery to primary mammalian hippocampal and sympathetic neurons was achieved in cell culture by conjugating an siRNA with Penetratin 1, a 16 amino acid peptide corresponding to the third helix of the *Antennapedia* homeodomain protein (Davidson *et al*. 2004). The siRNA was coupled to the peptide via a disulfide bridge, which is cleaved when exposed to the reducing environment of the cytoplasm after cellular uptake and thereby liberates the functional siRNA. This technique allowed specific knockdown of the target gene without the toxic side-effects that are frequently observed after lipid-mediated transfection.

Since naked nucleic acids do not traverse the BBB, a sophisticated approach had to be developed to deliver an shRNA expression system to the CNS after peripheral administration (Zhang *et al*. 2003b; Pardridge 2004). A plasmid encoding the shRNA expression cassette was encapsulated in the interior of PEGylated immunoliposomes. A monoclonal antibody against the transferrin receptor was tethered to the tips of the PEG strands that were conjugated to the surface of the liposome. The antibody allowed receptor-mediated transcytosis across the BBB, resulting in an up to 90% knockdown of a target gene in an intracranial brain cancer for at least 5 days after a single intravenous

injection of plasmid DNA. When the PEGylated immunoliposomes were tested for toxic side-effects, neither histological abnormalities nor differences regarding body weight or serum parameters were observed during chronic weekly intravenous administration (Zhang *et al*. 2003c).

Viral delivery of shRNA expression cassettes also allows efficient transduction of tissues such as brain and liver. In one of the first examples, Xia *et al*. (2002) employed a recombinant adenoviral construct containing an expression cassette with a modified cytomegalovirus promoter, which belongs to the class of pol II-promotors, and a minimal poly A cassette since functional siRNAs require minimal overhangs. After direct injection of the adenoviral vector encoding an shRNA against GFP into the striatal region of the brain, transgenic GFP expression was reduced at the injection site. A gene endogenously expressed in the liver, β-glucoronidase, was successfully knocked down after tail vein injection of a corresponding construct, and in neuronal PC12 cells the amount of polyglutamine (polyQ) proteins was reduced in a dose-dependent manner. As a consequence, less aggregation of polyQ proteins was observed. Repeats of polyQ are involved in the etiopathology of diseases like myotonic dystrophy and Huntington's disease and this study implies that polyQ diseases can be addressed by RNAi-based approaches.

Indeed, spinocerebellar ataxia type I, another neurodegenerative polyQ disorder that is caused by mutations in the ataxin-1 gene, was treated in a mouse model (Xia *et al*. 2004). Recombinant adeno-associated virus (AAV) vectors expressing shRNA were employed to knock down the mutant gene. Intracerebellar injection of the AAV vectors profoundly improved motor coordination, restored cerebellar morphology and resolved characteristic ataxin-1 inclusions in Purkinje cells in mice.

A lentiviral shRNA expression system has been developed as a new therapeutic strategy for the treatment of Amylotrophic Lateral Sclerosis (ALS), an inheritable neurodegenerative disease that is caused by mutations in the SOD-1 gene, encoding the Cu/Zn superoxide dismutase (Ralph *et al*. 2005; Raoul *et al*. 2005). RNAi-mediated knockdown of the mutated SOD-variant in spinal motor neurons rescued mice with dominant familial ALS and increased the life span of the animals.

RNA interference in pain-related animal models

In the first proof-of-concept study to employ RNAi in pain research, Dorn *et al*. (2004) developed a method to silence the ATP-gated cation-channel $P2X_3$, which is known to be involved in inflammatory pain signalling, by intrathecal application of naked siRNAs. The siRNAs, whose sequences were based on earlier results obtained with ASOs (Dorn *et al*. 2001), were continuously infused via a mini-pump into naïve and neuropathic rats at a comparatively high intrathecal dose of 400 μg/day for up to 7 days. Target gene expression in the dorsal horn of the spinal cord was diminished significantly without any apparent toxicity or non-specific side-effects. The siRNA treatment was found to block pathophysiological pain responses (hyperalgesia and tactile allodynia) and to provide relief from neuropathic pain in a rat disease model. In line with previous studies discussed above, siRNAs were found to be more efficacious when compared to ASOs at equivalent doses. Furthermore, the observed effects correlated very well with those published for low molecular weight inhibitors of $P2X_3$ and $P2X_{2/3}$ (Jarvis *et al*. 2002; McGaraughty *et al*. 2003).

In a subsequent study, a cationic lipid was used as a vehicle to enhance cellular uptake of the siRNAs (Luo *et al*. 2005). An siRNAs directed against the delta opioid receptor (DOR) mixed with the transfection reagent i-Fect™ was delivered via an implanted intrathecal catheter to the lumbar spinal cord of rats. The presence of a transfection agent permitted a significant reduction of the siRNA dosage as compared to the above mentioned study (Dorn *et al*. 2004) and allowed repeated daily bolus injections instead of continuous infusion. The dose was 23 times lower than the amount of ASOs required in a previous study (Blisky *et al*. 1996). Cellular uptake of the siRNAs in lumbar DRG and spinal cord was confirmed with fluorescently labelled siRNA molecules. Notably, uptake of the tagged siRNA after application without the delivery agent was extremely poor. 24 h after the last injection of siRNAs approximately 70% knockdown of the DOR density was achieved. Silencing was transient and returned to normal after 72 h. Inhibition of DOR expression blocked anti-nociception evoked by the DOR selective agonist [D-Ala^2, Glu^4] deltorphin II. RNAi-mediated silencing of DOR was specific, since the anti-nociceptive effect of an agonist to the related mu opioid receptor was not altered by the treatment.

NMDA receptors are another class of important mediators of pain signaling that are implicated in polysynaptic spinal pathways and chronic nociceptive responses. As NMDA antagonists used as analgesics provoke severe side-effects, the NR2B subunit of the NMDA receptor was silenced by means of RNAi (Tan *et al*. 2005). The siRNA was complexed with polyethyleneimine and delivered by intrathecal injection. This transfection reagent is thought to form a 'proton sponge' due to its buffering capacity, which enables it to buffer endosomes and potentially induce their rupture to release the siRNA into the cytoplasm (Godbey *et al*. 1999). Reduced mRNA and protein levels were demonstrated and decreased receptor density was further confirmed by immunohistochemical staining of the spinal cord and abolished formalin-induced pain behaviours in the rat model. Interestingly, the reduction of mRNA content was maximal 3 days after injection and lasted at least until day 14, whereas the protein content reached its minimum after 7 days, reflecting a relatively long half-life of the NR2B subunit. Both, mRNA

and protein reached starting levels after 21 days, underlining the transient character of the treatment.

Very recently, an important role of the brain-derived neurotrophic factor (BDNF) and its receptor, the tyrosine kinase receptor B (TrkB), in the development of persistent pain has been discovered. The BDNF/TrkB signalling was already known to play a critical role in activity-dependent synaptic plasticity underlying learning and memory. Guo *et al*. (2006) further analysed the function of this system by RNAi-mediated silencing of TrkB in the rostral ventromedial medulla (RVM), a relay between the periaqueductal gray and the spinal cord. To this end, siRNAs were injected into the RVM followed by local *in vivo* electroporation to achieve cellular uptake. When siRNAs were injected 4 d before hind paw injection of complete Freud's adjuvant (CFA), enhancement of TrkB expression as a result of the inflammation was completely blocked, and the animals exhibited a significant and dose-dependent attenuation of thermal hyperalgesia from 30 min to at least 6 h after hind paw injection of CFA. Sequestration of BDNF by microinjection of anti-BDNF antibodys also attenuated inflammatory hyperalgesia, whereas microinjection of BDNF facilitated nociception. The discovery of this signalling pathway raises the possibility that application of BDNF to speed recovery from CNS disorders could lead to undesirable central pain.

As outlined above, the vanilloid receptor, TRPV1, is a central molecular integrator of diverse noxious stimuli including heat, tissue injury, pollutants and endogenous pro-inflammatory substances (Cortright and Szallasi 2004; Tominaga and Tominaga 2005). Knockout animals lacking TRPV1 did not respond to the receptor agonist capsaicin and revealed diminished heat response and perturbed micturition. We recently used an RNAi approach to investigate the relevance of TRPV1 in neuropathic pain. Intrathecal injection of siRNAs against TRPV1 resulted in reduced cold allodynia of mononeuropathic rats (Christoph, Grünweller, Mika *et al.* manuscript in preparation). This study demonstrates that transient inhibition of gene expression by intrathecally delivered siRNAs can be employed as a rapid method to investigate the functional role of targets for analgesic substances.

Finally, it should be noted that not only the protein content, but also the subcellular protein localization is of significance for neurochemical processes. For example, Zhang *et al*. (2005) have shown that TRPV1 is phosphorylated after stimulation with the nerve growth factor (NGF) in a PI3K-dependent manner. The phosphorylation event enhances the fraction TRPV1 inserted into the plasma membrane. This observation opens up the possibility of employing RNAi to target proteins that are either important in the responsible signalling cascade or involved in the vesicle flow from intracellular vesicles to the plasma membrane to prevent sensitisation of TRPV1.

Outlook

Only a few years after the discovery of siRNAs, RNAi already represents an invaluable tool for researchers working in the field of functional genomics and target validation. Inhibition of target gene expression by means of RNAi has proven to be much faster than the generation of knockout animals by homologous recombination. In addition, the phenotype obtained after partial knockdown by RNAi might resemble the situation of protein inhibition with conventional low-molecular weight drugs more closely than the complete knockout does. RNAi provides the possibility to selectively

Table 1 Applications of RNA interference in pain-related animal models

Target	Delivery of siRNAs	Animal model	Biological effect	Reference
$P2X_3$	Continuous intrathecal infusion of siRNAs via minipump	Pressure stimulus with rats after partial nerve ligation	Reduced mechanical hyperalgesia and allodynia in models of chronic neuropathic pain	Dorn *et al.* (2004)
DOR	Repeated daily intrathecal injections of siRNAs with transfection reagent	Radiant heat paw withdrawal test with rats	Block of antinociceptive effect of DOR agonist	Luo *et al.* (2005)
NMDA receptor NR2B subunit	Intrathecal injection of siRNA with a PEI-based delivery system	Induction of persistent pain by formalin injection into the hindpaw of rats	Reduction of formalin-induced pain	Tan *et al.* (2005)
TrkB	*In vivo* electroporation after injection of siRNAs into the RVM	Radiant heat stimulus after hindpaw injection of CFA into rats	Attenuation of inflammatory pain	Guo *et al.* (2006)
TRPV1	Intrathecal injection of siRNA	Cold plate assay with rats after partial sciatic nerve ligation	Reduced cold allodynia of mononeuropathic rats	Christoph *et al.* manuscript in preparation

silence a member of a family of closely related genes or to inhibit the expression of several genes simultaneously. In recent years, several methods for delivery of siRNAs to the CNS have been developed and the function of pain-related proteins has successfully been studied by means of RNAi-mediated gene silencing (Table 1). RNAi, however, is not only considered to be a valuable tool the development of new drugs, but is currently being developed for therapeutic applications as well. Several clinical trials with siRNAs have already been started or are expected to commence soon and siRNA-based drugs are likely to become an exciting perspective for new therapeutic strategies for many diseases in the near future.

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References

Bernstein E., Caudy A. A., Hammond S. M. and Hannon G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.

Blisky E. J., Bernstein R. N., Hruby V. J., Rothman R. B., Lai J. and Porreca F. (1996) Characterization of antinociception to opioid receptor selective agonists after antisense oligodeoxynucleotide-mediated 'knock-down' of opioid receptor in vivo. *J. Pharmacol. Exp. Ther.* **220**, 491–501.

Bridge A. J., Pebernard S., Ducraux A., Nicoulaz A. L. and Iggo R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* **34**, 263–264.

Clemens M. J. and Elia A. (1997) The double-stranded RNA-dependent protein kinase PKR: structure and function. *J. Interferon Cytokine Res.* **17**, 503–524.

Cortright D. N. and Szallasi A. (2004) Biochemical pharmacology of the vanilloid receptor TRPV1. *Eur. J. Biochem.* **271**, 1814–1819.

Czauderna F., Fechtner M., Dames S., Aygün H., Klippel A., Pronk G. J., Giese K. and Kaufmann J. (2003a) Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucl Acids Res.* **31**, 2705–2716.

Czauderna F., Santel A., Hinz M. *et al.* (2003b) Inducible shRNA expression for application in a prostate cancer mouse model. *Nucleic Acids Res.* **31**, e127.

Davidson T. J., Harel S., Arboleda V. A., Prunell G. F., Shelanski M. L., Greene L. A. and Troy C. M. (2004) Highly efficient small interfering RNA delivery to primary mammalian neurons induces MicroRNA-like effects before mRNA degradation. *J. Neurosci.* **24**, 10 040–10 046.

Dorn G., Abdel'Al S., Natt F. J., Weiler J., Hall J., Meigel I., Mosbacher J. and Wishart W. (2001) Specific inhibition of the rat ligand-gated ion channel P2X3 function via methoxyethoxy-modified phosphorothioated antisense oligonucleotides. *Antisense Nucl. Acid Drug Dev.* **11**, 165–174.

Dorn G., Patel S., Wotherspoon G. *et al.* (2004) siRNA relieves chronic neuropathic pain. *Nucleic Acids Res.* **32**, e49.

Eckstein F. (2005) Small non-coding RNAs as magic bullets. *Trends Biochem. Sci.* **30**, 445–452.

Elbashir S. M., Harborth J., Lendeckel W., Yalcin A., Weber K. and Tuschl T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498.

Fire A., Xu S., Montgomery M. K., Kostas S. A., Driver S. E. and Mello C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.

Ganju P. and Hall J. (2004) Potential applications of siRNA for pain therapy. *Exp. Opin. Biol. Ther.* **4**, 531–542.

Garber K. (2005) Peptide leads new class of chronic pain drugs. *Nat. Biotechnol.* **23**, 399.

Ge Q., Filip L., Bai A., Nguyen T., Eisen H. N. and Chen J. (2004) Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl Acad. Sci. USA* **101**, 8676–8681.

Gillen C. and Maul C. (2002) New pharmacological strategies for pain relief. *Expert Rev. Neurotherapeutics* **2**, 89–101.

Godbey W. T., Wu K. K. and Mikos A. G. (1999) Tracking the intracellular path of poly (ethylenimine) /DNA complexes for gene delivery. *Proc. Natl Acad. Sci. USA* **96**, 5177–5181.

Grünweller A. and Hartmann R. K. (2005) RNA interference as a gene-specific approach for molecular medicine. *Curr. Med. Chem.* **12**, 3143–3163.

Grünweller A., Wyszko E., Bieber B., Jahnel R., Erdmann V. A. and Kurreck J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-*O*-methyl RNA, phosphorothioates and small interfering RNA. *Nucleic Acids Res.* **31**, 3185–3193.

Guo W., Robbins M. T., Wei F., Zou S., Dubner R. and Ren K. (2006) Supraspinal brain-derived neurotrophic factor signaling: a novel mechanism for descending pain facilitation. *J. Neurosci.* **26**, 126–137.

Hannon G. J. (2002) RNA interference. *Nature* **418**, 244–251.

Hemmings-Mieszczak M., Dorn G., Natt F. J., Hall J. and Wishart W. L. (2003) Independent combinatorial effect of antisense oligonucleotides and RNAi-mediated specific inhibition of the recombinant rat $P2X_3$ receptor. *Nucleic Acids Res.* **31**, 2117–2126.

Hescheler J., Rosenthal W., Trautwein W. and Schultz G. (1987) The GTP-binding protein, Go, regulates neuronal calcium channels. *Nature* **325**, 445–447.

Isacson R., Kull B., Salmi P. and Wahlestedt C. (2003) Lack of efficacy of 'naked' small interfering RNA applied directly to rat brain. *Acta Physiol. Scand.* **179**, 173–177.

Jackson A. L. and Linsley P. S. (2004) Noise amidst the silence: off-target effects of siRNAs? *Trends Genet.* **20**, 521–524.

Jarvis M. F., Burgard E. C., McGaraughty S. *et al.* (2002) A-317491, a novel potent and selective non-nucleotide antagonist of $P2X_3$ and $P2X_{2/3}$ receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proc. Natl Acad. Sci. USA* **99**, 17 179–17 184.

Julius D. and Basbaum A. I. (2001) Molecular mechanisms of nociception. *Nature* **413**, 203–210.

Kennedy S., Wang D. and Ruvkun G. (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427**, 645–649.

Kim S., Kang C., Shin C. Y. *et al.* (2006) TRPV1 recapitulates native capsaicin receptor in sensory neurons in association with Fas-associated factor 1. *J. Neurosci.* **26**, 2403–2412.

Krichevsky A. M. and Kosik K. S. (2002) RNAi functions in cultured mammalian neurons. *Proc. Natl Acad. Sci. USA* **99**, 11 926–11 929.

van der Krol A. R., Mur L. A., Beld M., Mol J. N. and Stuitje A. R. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell.* **2**, 291–299.

Kurreck J. (2004) Antisense and RNA interference approaches to target validation in pain research. *Cur. Opinion Drug Discov. Dev.* **7**, 179–187.

Lai J., Gold M. S., Kim C.-S., Bian D., Ossipov M. H., Hunter J. C. and Porreca F. (2002) Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, NaV1.8. *Pain* **95**, 143–152.

Lang J., Nishimoto I., Okamoto T., Regazzi R., Kiraly C., Weller U. and Wollheim C. B. (1995) Direct control of exocytosis by receptor-mediated activation of the heterotrimeric GTPases Gi and Go or by the expression of their active G alpha subunits. *EMBO J.* **14**, 3635–3644. Erratum in. *EMBO J.* **14**, 4639.

Leung R. K. and Whittaker P. A. (2005) RNA interference: from gene silencing to gene-specific therapeutics. *Pharmacol. Ther.* **107**, 222–239.

Lewis C., Neidhart S., Holy C., North R. A., Buell G. and Surprenant A. (1995) Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* **377**, 432–435.

Luo M.-C., Zhang D.-Q., Ma S.-W., Huang Y.-Y., Shuster S. J., Porreca F. and Lai J. (2005) An efficient intrathecal delivery of small interfering RNA to the spinal cord and peripheral neurons. *Mol. Pain* **1**, 29.

Matranga C., Tomari Y., Shin C., Bartel D. P. and Zamore P. D. (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**, 607–620.

McGaraughty S., Wismer C. T., Zhu C. Z., Mikusa J., Honore P., Chu K. L., Lee C. H., Faltynek C. R. and Jarvis M. F. (2003) Effects of A-317491, a novel and selective $P2X_3$/$P2X_{2/3}$ receptor antagonist, on neuropathic, inflammatory and chemogenic nociception following intrathecal and intraplantar administration. *Br. J. Pharmacol.* **140**, 1381–1388.

Meister G. and Tuschl T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343–349.

Morrissey D. V., Lockridge J. A., Shaw L. *et al.* (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol.* **23**, 1002–1007.

Napoli C., Lemieux C. and Jorgensen R. (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* **2**, 279–289.

Pardridge W. M. (2004) Intravenous, non-viral RNAi gene therapy of brain cancer. *Expert Opin. Biol. Ther.* **4**, 1103–1113.

Player M. R. and Torrence P. F. (1998) The 2–5A system: modulation of viral and cellular processes through acceleration of RNA degradation. *Pharmacol. Ther.* **78**, 55–113.

Porreca F., Lai J., Bian D. *et al.* (1999) A comparison of the potential role of the tetrodotoxin-insensitive sodium channels, PN3/SNS and NaN/SNS2, in rat models of chronic pain. *Proc. Natl Acad. Sci. USA* 96, 7640–7644. Erratum in *Proc. Natl Acad. Sci. USA* **96**, 10 548.

Ralph G. S., Radcliffe P. A., Day D. M. *et al.* (2005) Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat. Med.* **11**, 429–422.

Rand T. A., Petersen S., Du F. and Wang X. (2005) Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**, 621–629.

Raoul C., Abbas-Terki T. Bensadoun J. C. Guillot S. Haase G. Szulc J. Henderson C. E. and Aebischer P. (2005) Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat. Med.* **11**, 423–428.

Rice A. S. C. and Hill R. G. (2006) New treatments for neuropathic pain. *Annu. Rev. Med.* **57**, 535–551.

Romano N. and Macino G. (1992) Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol. Microbiol.* **6**, 3343–3453.

Santel A. Aleku M. Keil O. *et al.* (2006a) A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium. Gene Ther., E-pub ahead of print 20 April 2006, doi 10.1038/sj.gt.3302777.

Santel A., Aleku M., Keil O. *et al.* (2006b) RNA interference in the mouse vascular endothelium by systemic administration of siRNA-lipoplexes for cancer therapy. Gene Ther., E-pub ahead of print 20 April 2006. doi: 10.1038/sj.gt.3302778.

Scholz. J. and Woolf C. J. (2002) Can we conquer pain? *Nat. Neurosci.* **5**, 1062–1067.

Seibler J., Kuter-Luks B., Kern H., Streu S., Plum L., Mauer J., Kuhn R., Bruning J. C. and Schwenk F. (2005) Single copy shRNA configuration for ubiquitous gene knockdown in mice. *Nucleic Acids Res*. **33**, e67.

Shi Y. (2002) Mammalian RNAi for the masses. *Trends Genet.* **19**, 9–12.

Sledz C. A., Holko M., de Veer M. J., Silverman R. H. and Williams B. R. (2003) Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**, 834–839.

Song E., Zhu P., Lee S. K. *et al.* (2005) Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* **23**, 709–717.

Soutchek J., Akinc A., Bramlage B. *et al.* (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178.

Stark G. R., Kerr I. M., Williams B. R., Silverman R. H. and Schreiber R. D. (1998) How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264.

Stone L. S. and Vulchanova L. (2003) The pain of antisense: in vivo application of antisense oligonucleotides for functional genomics in pain and analgesia. *Advanced Drug Dev. Rev.* **55**, 1081–1112.

Tan P.-H., Yang L.-C., Shih H.-C., Lan K.-C. and Cheng J.-T. (2005) Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. *Gene Ther.* **12**, 59–66.

Thakker D. R., Natt F., Hüsken D., Maier R., Müller M., van der Putten H., Hoyer D. and Cryan J. F. (2004) Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proc. Natl Acad. Sci. USA* **101**, 17 270–17 275.

Tominaga M. and Tominaga T. (2005) Structure and function of TRPV1. *Pflugers Arch.* **451**, 143–150.

Trülzsch B. and Wood M. (2004) Applications of nucleic acid technology in the CNS. *J. Neurochem.* **88**, 257–265.

van de Wetering M., Oving I., Muncan V. *et al.* (2003) Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Report* **6**, 609–615.

Xia H., Mao Q., Eliason S. L. *et al.* (2004) RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat. Med.* **10**, 816–820.

Xia H., Mao Q., Paulson H. L. and Davidson B. L. (2002) siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nat. Biotechnol.* **20**, 1006–1010.

Zamore P. D., Tuschl T., Sharp P. A. and Bartel D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21–23 nucleotide intervals. *Cell* **101**, 25–33.

Zhang Y. F., Boado R. J. and Pardridge W. M. (2003b) *In vivo* knockdown of gene expression in brain cancer with intravenous RNAi in adult rats. *J. Gene Med.* **5**, 1039–1045.

Zhang Y. F., Boado R. J. and Pardridge W. M. (2003c) Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. *Pharm. Res.* **20**, 1779–1785.

Zhang X., Huang J. and McNaughton P. A. (2005) NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J.* **24**, 4211–4223.

Zhang W. B., Zhang Z., Ni Y. X., Wu Y. L. and Pei G. (2003a) A novel function of Goalpha: mediation of extracellular signal-regulated kinase activation by opioid receptors in neural cells. *J. Neurochem.* **86**, 1213–1222.

Zimmermann T. S., Lee A. C. H., Akinc A. *et al.* (2006) RNAi-mediated gene silencing in non-human primates. *Nature* **441**, 111–114.

