# Antisense and RNA interference approaches to target validation in pain research

Jens Kurreck

**Address**
Free University Berlin
Institute for Chemistry/Biochemistry
Thielallee 63
14195 Berlin
Germany
Email: jkurreck@chemie.fu-berlin.de

**Current Opinion in Drug Discovery & Development** 2004 **7**(2):179-187
 ISSN 1367-6733

*In recent years numerous potential new targets for the development of novel drugs to treat chronic pain have been identified. Antisense strategies provide a straightforward approach to validate and further analyze their function. Furthermore, they offer the possibility of investigating a single member of a closely related family of proteins that cannot be easily targeted using small-molecule pharmacological tools. The recent discovery that short double-stranded RNA molecules can be employed to induce RNA interference in mammalian systems has opened exciting new roads for functional genomics. Gene silencing by small interfering RNAs has been demonstrated in neurons, and several targets involved in pain perception have been addressed. Furthermore, promising in vivo results have recently been obtained with small interfering RNAs.*

***Keywords*** Antisense oligonucleotides, central nervous system, pain, RNA interference, small interfering RNA, target validation

## Abbreviations

| | |
|---|---|
| AS | Antisense |
| CNS | Central nervous system |
| LNA | Locked nucleic acid |
| ON | Oligonucleotide |
| PNA | Peptide nucleic acid |
| RNAi | RNA interference |
| shRNA | Short hairpin RNA |
| siRNA | Small interfering RNA |
| VR1 | Vanilloid receptor subtype 1 |

## Introduction

Persistent pain affects hundreds of millions of individuals worldwide and is one of the major reasons for people to seek medical care. Most analgesics prescribed today are based on two well-established principles: non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. For many patients, however, pharmacological treatment remains unsatisfactory due to only partial effectiveness of the drugs or because of distressing side effects [1]. Therefore, the need to improve the pharmacotherapy of pain is obvious. In recent years, numerous new potential targets for the development of novel drugs for pain therapy have been identified [2].

Validation and detailed characterization of new potential targets is currently a major challenge for researchers of pain. Generation of knockout animals is the most widely used strategy to study loss-of-function phenomena. This approach, however, has several drawbacks: it is time consuming, costly and labor intensive. In addition, it is non-informative if the knockout is lethal during embryogenesis. Antisense (AS) approaches offer an alternative, combining many desired properties, ie, they are broadly applicable and relatively fast at comparatively low costs. As well as being used to analyze the functional role of a potential new target, AS strategies can also be used to investigate potential unwanted side effects of the inhibition of a target, before further resources are employed to search for low-molecular-weight compounds that act at the protein level.

The potential of single-stranded AS oligonucleotides (ONs) to knockdown the expression of a targeted gene has been known for 25 years and has been widely exploited for research and target validation. Furthermore, several AS ONs are currently being investigated in clinical trials [3,4]. In contrast, RNA intereference (RNAi) is a rather new technique, but due to its high efficiency, researchers are convinced that it will help to overcome some of the problems that have been experienced with conventional AS strategies. This review will consider both approaches, with a special focus on their applicability for target validation. Furthermore, contributions of AS strategies to our knowledge of pain perception will be outlined and preliminary results of RNAi experiments in this research area will be presented.

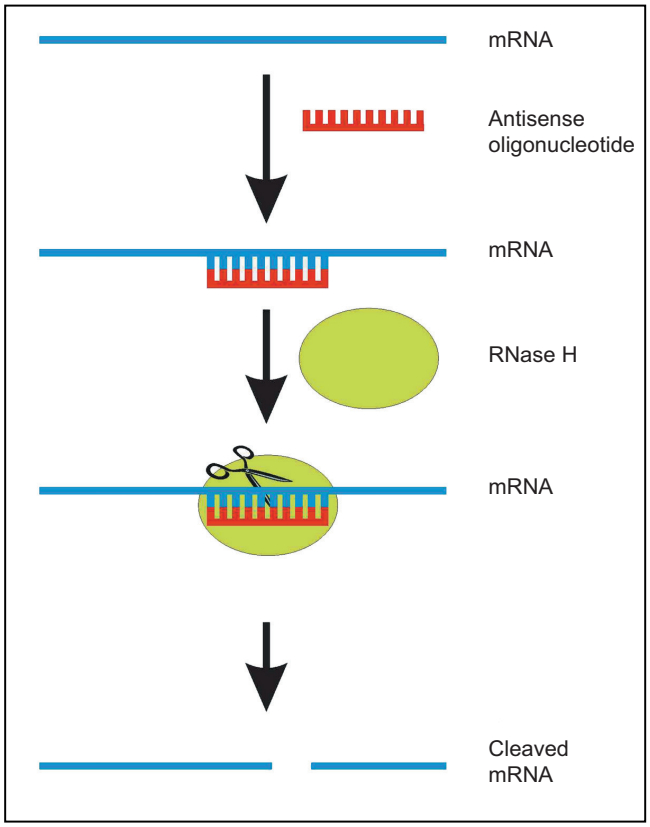
## Antisense oligonucleotides

AS approaches can be used to inhibit the expression of any gene of interest to study a loss-of-function phenotype and are therefore valuable tools for functional genomics. Furthermore, they can be used as therapeutic agents to treat diseases that are caused by the expression of deleterious genes, for example, viral infections, cancer or inflammatory diseases. AS ONs usually consist of 15 to 20 nucleotides that bind to their complementary target mRNA. Two major mechanisms contribute to their AS activity: firstly, most AS ONs activate RNase H, which cleaves the RNA moiety of a DNA/RNA heteroduplex (Figure 1). Secondly, the AS ON bound to an mRNA inhibits translation by a steric blockade of the ribosome.

Many potential target sites are inaccessible for complementary ONs due to the secondary and tertiary structures of the long RNA molecule. Furthermore, RNA binding proteins shield some regions of the mRNA. Therefore, the first task for the development of an efficient AS ON is to identify accessible target sites on the mRNA. Various methods have been developed for this purpose [5], including computer-based models of the RNA structure, systematic gene walking with numerous ONs, the use of randomized ON libraries, or sophisticated approaches such as the use of DNA arrays.

Based on long-term experiences in the AS field, guidelines have been formulated for the design of AS ONs to avoid

**Figure 1. Mechanism of action of antisense oligonucleotides.**




Antisense oligonucleotides (AS ONs) bind to their target via Watson-Crick base pairing and induce RNA cleavage by RNase H in the nucleus. Furthermore, AS ONs inhibit translation by steric blockade of the ribosome.

numerous pitfalls [6]. Contiguous stretches of guanosine residues should be excluded as they might form G-quartets via Hoogsten base pairing. In addition, CpG motifs should be avoided for *in vivo* experiments, since they are known to induce immune responses in mammalian systems. One of the most crucial requirements for an AS experiment is the design of suitable controls to prove that the observed effects are due to AS inhibition. Therefore, control ONs have to be employed that consist of a random sequence, a scrambled sequence with the same base composition as the AS ON, the inverted sequence of the AS ON or a sequence with several mismatches.

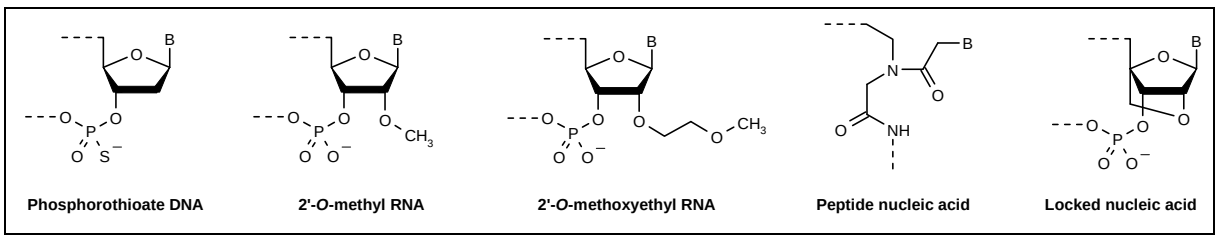
### *Modified nucleotides*

Another important pre-requisite for a successful AS experiment is the choice of suitable chemistry for the AS ON. Since unmodified ONs are rapidly degraded by nucleases in biological fluids, chemically modified nucleotides are used to stabilize them. To date, phosphorothioates, which contain a sulfur atom instead of a non-bridging oxygen atom in the phosphodiester bond (Figure 2), are the most widely used modification for AS ONs [7]. Phosphorothioates possess an enhanced resistance against nucleases compared to unmodified DNA, form regular Watson-Crick base pairs with complementary RNA molecules and activate RNase H. Their enhanced binding to certain proteins, however, may cause cellular toxicity [8]. Side effects elicited by phosphorothioates, including weight loss, brain tissue damage and behavioral abnormalities, are especially pronounced in the central nervous system (CNS) [9]. The use of phosphorothioate ONs in the CNS is therefore not recommended.

To overcome the problems associated with phosphorothioate ONs, a second generation of nucleotides was developed that contain chemical modifications at the 2'-position of the ribose. 2'-*O*-methyl and 2'-*O*-methoxyethyl RNA building blocks (Figure 2) are the major representatives of this class of RNA analogs. These ONs are less toxic than phosphorothioates and possess an enhanced target affinity. One major disadvantage is their inability to activate RNase H. Therefore, so-called gapmers have been developed that consist of a central stretch of DNA or phosphorothioate monomers and modified nucleotides at each end in order to protect the ON from exonucleolytic degradation. An AS ON with 2'-*O*-methoxylethyl RNA endblocks has, for example, been used to demonstrate the involvement of protein kinase Cα in the phenomenon of morphine tolerance [10].

In recent years, major advances have been achieved by the development of novel, chemically modified nucleotides with improved properties, such as enhanced nuclease resistance, high-target affinity and low toxicity [4]. Peptide nucleic acids (PNAs) and locked nucleic acids (LNAs) are two of the most promising examples of this class of new building blocks (Figure 2). In PNAs, the deoxyribose phosphate backbone is replaced by polyamide linkages [11]. LNAs are characterized by a methylene bridge, which connects the oxygen at the 2'-position of the ribose with the carbon at the

**Figure 2. Selected modified nucleotides.**




**B** denotes one of the bases, either, adenine, guanine, cytosine or thymine.

4'-position [11,12]. LNA gapmers with four to five modified monomers at each end and a central stretch of eight to ten unmodified DNA nucleotides in the center were significantly more stable than unmodified ONs and had an AS potency exceeding that of isosequential phosphorothioates or 2'-*O*-methyl RNA gapmers [13,14].

Interestingly, the CNS represents a special case, in which unmodified ONs have been shown to produce an AS effect (see, eg, reference [15••]). It appears that nuclease activity is much lower in the CNS than in other body fluids. It is, however, necessary to apply repeated doses of DNA ONs to maintain the AS effect. Therefore, the use of modified ONs might be advantageous. It has, for example, been demonstrated that AS ONs containing LNAs have a much higher potency to knockdown the expression of the δ-opioid receptor than their unmodified counterparts [16••]. In addition, this study showed that unwanted side effects that were observed with phosphorothioate ONs in rat brains (tissue damage and elevated body temperature) were not detected after treatment with LNA ONs.

### *Delivery of oligonucleotides*

Phosphorothioates distribute to all peripheral tissues, but - like most larger substances - ONs are unable to cross the blood-brain barrier [17•]. Intrathecal delivery is therefore the most effective route to administer AS ONs for the study of pain-related phenomena in the CNS. Repeated bolus injections using an intrathecal catheter have been established as a suitable method for the delivery of AS ONs to the CNS [15••,18]. Alternatively, an osmotic minipump can be employed to guarantee a constant level of AS molecules is delivered. In a comparative study, slow infusion was demonstrated to be superior to bolus injections for AS ON delivery to dorsal root ganglion neurons [19].

Another important issue for AS experiments is cellular uptake of the ONs. In cell culture, transfection reagents (eg, cationic lipids) have to be used to achieve efficient internalization of ONs. Interestingly, *in vivo* AS ONs are taken up by neuronal cells, even in the absence of transfection reagents [18], but the underlying mechanism for this phenomenon remains to be elucidated. Nevertheless, the development of delivery systems that enhance cellular uptake of ONs would be useful to improve AS strategies. For example, efficient and long-term delivery of AS ONs targeting the metabotropic glutamate receptor mGluR1 has been achieved using a sophisticated gene transfer vector, which was obtained by incubating liposomes with inactivated hemagglutinating virus of Japan (HVJ) to allow fusion into a HVJ-liposome [20]. Subsequently, ONs were incorporated into the complex. This method enhanced cellular uptake of the AS ONs and prolonged their half-life. The knockdown lasted for at least 4 days and reduced nociceptive reactions in the early-sustained phase of the formalin model.

### *Confirmation of gene expression*

The proof of the specific suppression of the target gene is important for any type of AS experiment. The inhibition of gene expression can be confirmed at the mRNA level by Northern blotting, real-time reverse transcriptase-polymerization chain reaction (RT-PCR) or *in situ* hybridization. It is also advisable to analyze the protein level by Western blot or other immunochemical methods, since the mRNA level does not always correlate with the protein level. Interestingly, the observed biological effect sometimes exceeds changes of expression levels achieved by AS-knockdown. For example, a less than 50% knockdown of $Na_v1.8$ expression returned the paw withdrawal threshold of neuropathic rats back to the control level [18].

### *Targets for pain therapy*

AS approaches have been widely used to study pain perception *in vivo*. Target molecules can be broadly classified into the following groups: ion channels, G protein-coupled receptors, G proteins and other targets. A few examples of these studies will be discussed to demonstrate the opportunities and the borders of the AS strategy. Due to space restraints, however, only a limited number of reports can be mentioned. For further details, the interested reader is referred to the recently published, comprehensive review by Stone and Vulchanova [21].

One major advantage of AS approaches became obvious when studying the functional importance of single subunits of the P2X purinergic receptor family, which consists of ATP-gated ion channels. The close relationship between members of this family made the analysis of the role of single members, such as the $P2X_3$ receptor, difficult due to the lack of pharmacological tools that are selective antagonists for a family member. This problem has been overcome by continuous intrathecal administration of AS ONs, which specifically knockdown the expression of the $P2X_3$ receptor [19,22]. Behavioral effects observed in various animal models after AS treatment demonstrated an important role of the $P2X_3$ receptor for chronic inflammation and neuropathic pain states, and suggested that a blockade of this receptor might help to achieve relief from certain forms of chronic pain.

Studies by Porreca and co-workers [15••,18] contributed to the increase of knowledge about the function of the tetrodotoxin-resistant sodium channel $Na_v1.8$. Behavioral studies following AS knockdown of $Na_v1.8$ revealed its involvement in the development and maintenance of thermal hyperalgesia and mechanical allodynia in neuropathic rats. Moreover, knockdown of $Na_v1.8$ abolished the mechanical hypernociception induced by intrathecal administration of *N*-methyl-D-aspartate [23]. In addition, it has been demonstrated that upregulation of the tetrodoxin-sensitive sodium channel $Na_v1.3$ after spinal cord injury can be decreased by intrathecal application of AS ONs targeting $Na_v1.3$ mRNA [24]. This treatment reduced hyperexcitability of multi-receptive dorsal horn neurons and attenuated mechanical allodynia and thermal hyperalgesia.

One of the most promising new targets for pain therapy is the vanilloid receptor subtype 1 (VR1, TRPV1), which is activated by heat and capsaicin (the hot component of chilli peppers), and is modulated by protons [25••]. Tactile allodynia of mononeuropathic rats was reduced after intrathecal injection of AS ONs targeting VR1 [Christoph T, Kurreck J, Grünweller A, Mika J, Schäfer M, Weihe E, Erdmann VA, Gillen C, manuscript in preparation].

Opioid receptors, which belong to the class of G protein-coupled receptors, are among the major targets of currently used analgesics. It is, therefore, not surprising that AS approaches have been widely used to elucidate their function in more detail. AS studies were particularly valuable before knockout mice were available to analyze the antinociceptive effect mediated by the various subtypes and splice variants of opioid receptors and the G proteins they are coupled to (these studies are summarized in references [21] and [26]). For example, AS suppression of the δ-opioid receptor by an ON consisting of LNAs inhibited the antinociceptive response to deltorphin II in the warm water tail-flick test in rats [16••]. In addition, desensitization of opioid receptors has been investigated by AS approaches. Knockdown of β-arrestin, which is involved in this process, has been demonstrated to inhibit the development of morphine tolerance and to antagonize allodynia in neuropathic rat models [27].

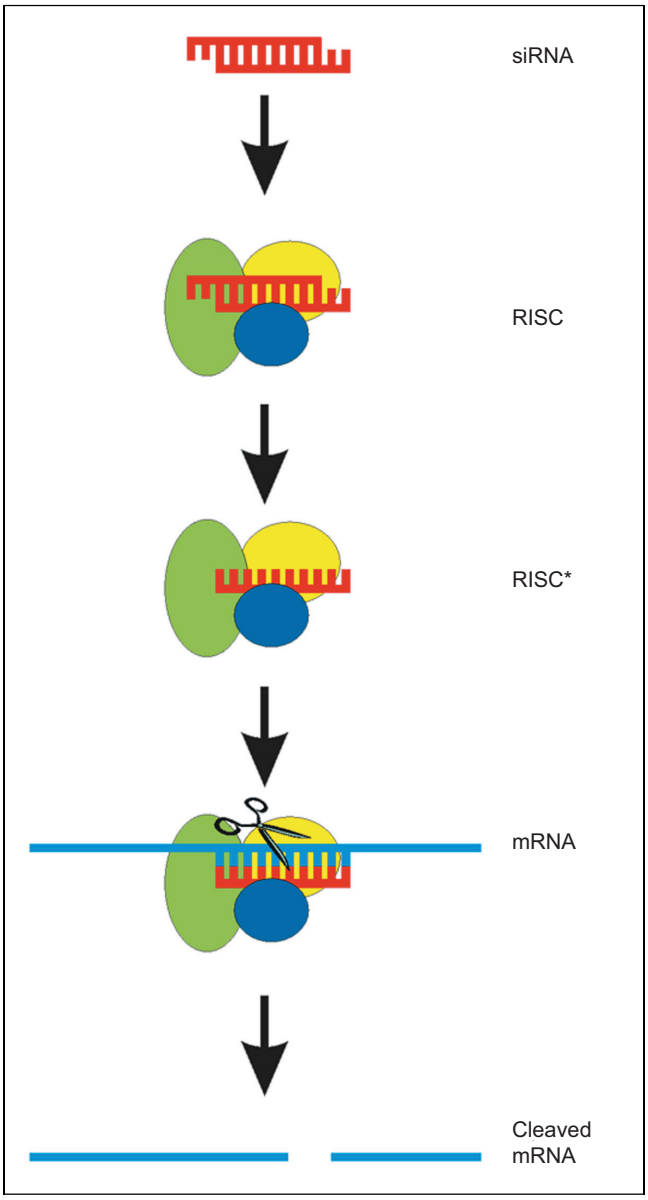
These examples indicate that AS approaches have already made significant contributions to our understanding of pain-related phenomena. Several problems that AS approaches are currently facing are expected to be overcome by: (i) appropriate design of future studies; (ii) improved methods for ON delivery to the target cells; and (iii) new chemical modifications that lead to ONs having desirable properties for *in vivo* AS experiments.

## RNA interference

The phenomenon of RNA interference (RNAi) as a post transcriptional gene silencing mechanism in response to injection of long double-stranded RNA was first recognized in the nematode *Caenorhabditis elegans* [28•]. The use of RNAi approaches in mammalian cells had been hampered by the fact that long double-stranded RNA molecules induce an unspecific interferon response in these cells. This limitation, however, has been overcome by the discovery that short double-stranded RNAs, termed small interfering RNAs (siRNAs), can be used for sequence-specific gene silencing in mammalian cells without induction of cytotoxic processes [29••]. This finding triggered an enormous number of studies that have been published in the last few years. A detailed description of the RNAi mechanism and a summary of related publications have been presented in numerous excellent review articles recently (see, eg, references [30-32]). In this review, only a brief general description of the RNAi phenomenon will be given, and subsequently the most important studies that demonstrate the potential of this new tool for target validation in pain research will be summarized.

RNAi is initiated by an enzyme called Dicer, which cuts long double-stranded RNA molecules into siRNA duplexes consisting of 21 to 23 nucleotides with 3'-overhangs of two or three nucleotides. These molecules are subsequently incorporated into the RNA-induced silencing complex (RISC), which has to be activated from a latent form to the activated state RISC*. This activation comprises an ATP-dependent unwinding step of the siRNA. The single-stranded AS strand then presumably guides RISC* to its target RNA, which is finally endonucleolytically cleaved (Figure 3).

**Figure 3. The principle of RNA interference.**




In mammalian cells, RNAi is initiated by small interfering RNAs (siRNA), which are incorporated into the multicomponent RNA-induced silencing complex (RISC), a ribonucleoparticle. RISC is activated to the RISC* form by ATP-dependent unwinding of the siRNA. The single-stranded RNA guides the complex to its target mRNA, which is cleaved by RISC* and degraded. In human cells, RNAi seems to be limited mostly to the cytoplasm.

Since its discovery, the phenomenon of RNAi has widely been used to knockdown the expression of a target gene in order to study its functional role by analyzing the resulting loss-of-function phenotype. In many cases RNAi is a highly efficient process, exceeding the potency of conventional AS approaches [14,33,34]. Despite the impressive efficacy of siRNAs in inhibiting gene expression by 80 to > 90%, however, complete elimination of the gene product is usually not achieved. The same is true for AS ONs. These methods must therefore be considered as 'knockdown' rather than 'knockout' approaches. It should be noted that small-molecule drugs are also incapable of

completely knocking out the function of their target genes. Thus, an incomplete inhibition of gene function, combined with the possibility of dose-dependence, makes AS approaches and siRNA superior to knockouts for target validation.

### *Expression of siRNAs*

Exogenously delivered siRNAs only result in a transient silencing of targeted genes. Therefore, vectors have been developed that constitutively express siRNAs [35]. In most of these studies, the siRNA is transcribed under control of RNA polymerase III promoters that normally regulate the expression of the small nuclear RNA U6 or the H1 RNA component of RNase P. Two different approaches have been described for the intracellular expression of short double-stranded RNA molecules: in the first case, two promoters transcribe the sense and AS strand separately, whereas in the second case only one stem-loop structure, a so-called short hairpin RNA (shRNA), is transcribed that is subsequently processed to an siRNA by the Dicer enzyme. Vectors that encode an antibiotic-resistance gene in addition to the siRNA expression cassette can furthermore be employed to generate cells that permanently produce siRNA: target gene silencing was observed in stably transfected cells, even 2 months after vector delivery [36].

An interesting new development is the generation of plasmids that allow inducible expression of shRNA. For this purpose, the tetracycline operator was introduced between the TATA box and the transcription start site of the H1 [37•] or the U6 and 7SK promoter [38•]. Additional expression of the tetracycline repressor prevented the expression of the shRNA. Only after addition of tetracycline or doxycycline was expression of shRNA induced, leading to silencing of the targeted gene. Czauderna *et al* also demonstrated that suppression of the two catalytic subunits of the phosphatidylinositol 3-kinase significantly reduced the formation of lymph node metastases, but not the size of an implanted prostate tumor in nude mice [38•]. The inducible systems allow the comparative analysis of loss-of-function phenotypes in isogenic cell populations in the induced and non-induced state. Furthermore, compensatory responses caused by constitutive shRNA expression will be avoided with an inducible system.

### *In vivo studies*

Several lines of evidence have already been presented which show that RNAi is a suitable method for *in vivo* studies. In the first siRNA experiments in animals, reporter genes were targeted that were either encoded on co-transfected plasmids or in transgenic mouse strains. In addition, RNAs of potential medical relevance have been targeted *in vivo*. For example, silencing of the endogenous *Fas* gene protected mice from fulminant hepatitis induced by injection of an agonistic Fas-specific antibody [39••]. More than 80% of the siRNA-treated animals survived a 10-day period of observation, whereas all untreated control animals died. In a similar approach, caspase-8 inhibition in the liver by siRNA prevented Fas-mediated apoptosis, which is involved in acute liver failure [40]. In the latter study, improvement of survival was achieved, even when siRNA treatment began after the onset of acute liver failure - a scenario that is more likely to reflect a clinical situation than pretreatment with siRNA before liver failure is induced. Furthermore, replication of the hepatitis B virus was successfully inhibited by RNAi in mice [41•].

### *siRNA delivery*

A high-pressure, tail-vein injection technique has been used for most siRNA *in vivo* experiments published to date. This relatively harsh method might influence results, and cannot be used for therapeutic applications in humans. Therefore, viral vectors have been developed as delivery vehicles for gene therapy approaches. For example, an oncoretroviral vector was employed to silence the carcinogenic *K-ras* allele in human pancreatic carcinoma cells [42]. Lentiviruses are a distinct class of retrovirus with the ability to transfect cycling as well as non-cycling cells. In addition, they allow long-term expression of a transgene. The expression of an enhanced green fluorescent protein was suppressed in stably transfected cells for at least 25 days after infection with a lentiviral vector expressing siRNA [43]. Lentivirus-based systems have also been demonstrated to be suitable for silencing genes by RNAi in cycling and non-cycling mammalian cells, stem cells, zygotes and their differentiated progeny [44].

In contrast to retroviruses, adenoviruses do not integrate their genetic material into the host genome, thus preventing the risk of insertion mutagenesis. Adenoviruses have therefore been used for gene therapy and are promising vehicles for siRNA delivery; they have been demonstrated to be suitable to silence target gene expression in cell culture [45] and in brain and liver tissue *in vivo* [46]. Advantages of adenoviruses include their high transfection efficiency and the wide variety of cell types that they can transfect. However, they are known to induce a strong immune response, limiting their repeated application. Adeno-associated viruses are an attractive alternative, since they are not associated with disease, do not induce an immune response and allow long-term expression of a gene. An adeno-associated viral vector has been used to deliver siRNAs against p53 and caspase-8 into mammalian cells [47], and subsequently adeno-associated viruses were employed to knockdown tyrosine hydroxylase within neurons of the mid-brain in mice [48], resulting in behavioral changes (motor performance deficit and reduced response to amphetamines). This study underscores the potential of virus-mediated RNAi for the rapid production and testing of new genetic disease models.

### *Genome-wide analysis of gene function*

RNAi has been demonstrated to be suitable for genome-wide analysis of gene function in *C elegans* [49•]. More than 85% of the predicted genes were inhibited and phenotypic consequences were observed for over 1700 genes. This approach was subsequently employed to analyze the genes involved in fat metabolism [50]. Numerous genes were identified whose suppression resulted in either reduced or increased body-fat content. Although these studies were performed with a lower model organism and the approach cannot simply be transferred to mammals, they clearly demonstrate the potential of RNAi as a tool for genome-wide target identification and validation. In fact, a 'large-scale loss-of-function genetic screen' in mammalian cells was performed to analyze the role of 50 de-ubiquitinating

enzymes [51]. Inhibition of the familial cylindromatosis tumor suppression gene (*CYLD*) by shRNA-expressing vectors enhanced activation of the transcription factor NFκB. As a consequence, cellular resistance to apoptosis was increased, suggesting an oncogenic potential of *CYLD*. Further approaches to knockdown approximately 35,000 genes in the human genome by RNAi in cell culture have been announced recently [52].

### *Neuronal targets*

Some studies on *C elegans* indicated that neuronal cells are more resistant to RNAi than other cell types. To use this method in pain research, it was important to show that RNAi functions in mammalian neurons. Krichevsky and Kosik convincingly demonstrated that siRNAs introduced into primary mammalian neuronal cells effectively inhibit the expression of exogenous and endogenous genes [53•]. Surprisingly, long double-stranded RNAs were reported to induce specific inhibition of target genes in partially and fully differentiated mouse neuroblastoma cells [54]. In contrast to other types of mammalian cells, no unspecific inhibition of protein synthesis was observed in the neuronal cells. In other experiments, vector-mediated expression of shRNA was shown to be suitable to silence genes in primary post-mitotic mammalian neurons [55]. These results suggest that RNAi is likely to be of utility in the study of the nervous system in intact mammals. Examples of the knockdown of neuronal targets by RNAi are summarized in Table 1.

In experiments exploring RNAi in neuronal cells, a Cy3-labeled siRNA that was almost as efficient as its unmodified counterpart has been used to localize siRNA in the neuronal hybridoma cell line F-11 [56]. The siRNA was rapidly taken up by more than 90% of the cells after lipofection and a dotted pattern of the labeled siRNAs was observed, reflecting an endosomal uptake mechanism. Fluorescence was almost exclusively found in the cytoplasm, predominantly accumulated around the nucleus. A closer inspection of the F-11 cells revealed that the Cy3-labeled siRNA was not only localized in the somatic compartment, but can also be found in neurite-like structures formed by the cells. It is, therefore, likely that gene expression can also be suppressed in dendrites and axons.

### *Strategies for pain therapy*

As mentioned above, the study of the P2X receptor family is hampered by the fact that no small-molecule compounds are available for differentiating between these closely related receptors. AS and RNAi approaches are therefore a suitable strategy to address these medically relevant pain-related genes. An siRNA that efficiently inhibits the mRNA of the purinergic $P2X_3$ receptor was generated [57]. Interestingly, a cooperative effect was observed when siRNA was combined with non-homologous AS ONs. Researchers from the Novartis Institute for Medical Sciences in London, UK, reported that silencing of $P2X_3$ by siRNA reduced pain perception in an animal model. The siRNA was infused into the spinal cord of rats that were hypersensitive to pain following surgery to their sciatic nerve. Knockdown of $P2X_3$ reversed the increase in sensitivity by an average of 50%, compared to rats that did not receive siRNA [Ganju P, personal communication].

G proteins are important mediators of opioid receptor-induced signaling, and the $G_o\alpha$ subunit is involved in extracellular signal-regulated kinase activity that is induced by the δ-opioid receptor [58]. Decreasing $G_o\alpha$ expression by RNAi significantly blocks this signaling pathway in mouse neural cells. Kinase activity could subsequently be restored by exogenously expressed human $G_o\alpha_1$ and $G_o\alpha_1$ subunits that were not silenced by the siRNA.

The vanilloid receptor VR1 has already been introduced in the previous section as one of the most interesting new targets for therapeutic approaches against chronic pain. Our comparative study revealed that an siRNA targeting VR1 mRNA was the most potent AS molecule under

**Table 1. Examples for RNAi knockdown of neuronal targets.**

| Target | System | Functional consequences | Reference |
|---|---|---|---|
| Microtubule-associated protein 2 (MAP2) | Rat primary cortical and hippocampal neurons | Reduced filopodial elaboration | [53•] |
| $P2X_3$ | Cell culture | | [57] |
| | Intrathecal administration with osmotic minipump in a rat neuropathic pain model | Reduction of mechanical hyperalgesia and tactile allodynia | [Ganju P, personal communication] |
| Poly-ADP-ribose polymerase (PARP) | Mouse neuroblastoma cells | Resistance to oxygen-glucose deprivation | [54] |
| Myocyte enhancer Factor 2A | Rat primary cerebellar granule neurons | Inhibits activity-dependent neuronal survival | [55] |
| Tyrosine hydroxylase | Adeno-associated virus delivery to mid-brain neurons of mice | Behavioral changes (motor performance deficit, reduced response to amphetamines) | [48] |
| VR1, TRPV1 | Cell culture | | [14] |
| | Intrathecal administration in a neuropathic pain model | Reduction of cold allodynia | [Christoph T *et al*, unpublished data] |
| G protein subunit $G_o\alpha$ | Neuroblastoma X glioma NG108-15 hybrid cells | Reduction of δ-opioid receptor-induced extracellular signal-regulated kinase activity | [58] |

investigation, being 1000-fold more efficient than a phosphorothioate AS ON and 6-fold more active than an LNA gapmer [14]. When siRNA was repeatedly injected intrathecally into rats, however, severe side effects were observed. Therefore, the effect of a single intrathecal bolus injection of a lower dose of siRNA for the reduction of cold allodynia in mononeuropathic rats was investigated and found to be sufficient; this finding was subsequently confirmed for a second pain target [Christoph T, Mika J, Grünweller A, Schäfer M, Weihe E, Kurreck J, Erdmann VA,Gillen C, unpublished data].

A strategy has been reported to induce RNAi in the brain after intravenous injection of an shRNA-expressing vector [59•]. The plasmid was encapsulated in the interior of PEGylated immunoliposomes with a monoclonal antibody tethered to their surface. The antibody to the transferrin receptor mediated transcytosis across the blood-brain barrier, allowing silencing of a reporter gene in brain cancer cells. This new delivery system enables the knockdown of gene expression by RNAi in the CNS after intravenous injection of plasmid DNA.

### *Unspecific effects*

Despite the enthusiasm that has been triggered by the enormous potential of RNAi technology, some anomalies have been reported. The specificity of target gene suppression by siRNA has been analyzed using DNA arrays. In contrast to Chi *et al*, who did not observe any unspecific effects elicited by siRNA treatment [60], Semizarov *et al* observed the induction of stress and apoptosis genes after transfection of cells with higher concentrations of siRNAs [61]. In another study, expression profiling after siRNA transfection into HeLa cells revealed off-target gene regulation at low siRNA concentrations [62]. The unspecific effects had a similar dose-dependency to that of the silencing of the intended gene, and were most likely due to partial sequence similarities. In addition, a sequence-specific induction of an interferon response was observed when expressing shRNA by RNAi vectors in mammalian cells [63]. Furthermore, Sledz *et al* demonstrated an activation of the interferon system after introducing siRNAs into a human glioblastoma or a renal cell carcinoma cell line [64]. This effect was mediated by the double-stranded RNA-dependent protein kinase PKR. Taken together, these findings clearly demonstrate that it is essential to use low doses of efficient siRNAs for RNAi approaches and to carefully analyze unspecific effects when interpreting the results.

## Therapeutic perspectives

One important advantage of AS approaches is the opportunity to use molecules for therapeutic purposes, which have been proven successful in animal models for target validation. Numerous AS ONs are currently being tested in clinical trials and one AS drug has been approved for the treatment of cytomegalovirus-induced retinitis [3,4]. The clinical studies address a broad range of diseases, including viral infections, cancer and inflammatory diseases. Due to the enormous potential of the RNAi method, several researchers and companies (eg, Sirna Therapeutics, Avocel and Alnylam Pharmaceuticals/Ribopharma) have announced the intention of progressing siRNAs into clinical trials in the near future; however, some researchers, including those from the commercial sector, warn that basic questions still need be answered before entering siRNAs into clinical development [65].

## Conclusion

Conventional AS strategies have contributed greatly to our understanding of the physiology and pathobiology of pain and analgesia. They offer a comparably straightforward approach for knockdown studies. Since target genes are specifically inhibited at the mRNA level, even closely related members of protein families can be investigated for which no specific small-molecule pharmacological tools are available. It should, however, be noted that AS approaches have had to struggle with problems due to unsatisfactory efficiency and delivery as well as toxic side effects. Some of these hurdles might be overcome by the recent development of novel, chemically modified nucleotides and new methods for delivery. Further progress will be achieved by using RNAi as a new technology for gene silencing. Numerous studies in cell culture and the first promising *in vivo* results have been published, and RNAi is expected to become a major tool for target validation in the field of pain research, as well as for other medically relevant targets in oncology and virology. However, all results have to be interpreted with caution and great care has to be taken before initiating therapeutic studies with humans. If researchers in the field of AS apply the knowledge gained over the past 20 years, great progress can be expected in the near future. RNAi might help to expedite drug development, since an siRNA has the potential to be a single molecule for target discovery, target validation and therapeutic application.

## Acknowledgment

The author wishes to thank Professor Dr Volker A Erdman for his support, all members of his research team for their excellent work, and Clemens Gillen, Erik Wade and Arnold Grünweller for critical reading of the manuscript and inspiring suggestions. Financial support of the author's research by the Deutsche Forschungsgemeinschaft (DFG Ku 1436/1-1) and the Fonds der Chemischen Industrie, as well as grants to Professor Dr Volker A Erdmann from the Bundesministerium für Bildung und Forschung (grant 01GG9818/0) and the National Foundation for Cancer Research (USA) are gratefully acknowledged.

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•• of outstanding interest
• of special interest

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