

# **Modulating Sensory Systems Using RNAi**

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## Introduction

Since the synthetic technology for small DNA and RNA molecules was first developed over two decades ago, their use for transient disruption of gene expression has found extensive applications in biomedical research. So-called antisense technology uses short, synthetic, single-stranded oligodeoxynucleotides (ODN); ODN binds to specific mRNA sequences to interfere with the translation of the gene product or to promote the degradation of the mRNA by ribonuclease H. The more recent discovery of RNA interference has led to the development of short, synthetic, double-stranded small interfering RNA (siRNA), which direct the RNA-induced silencing complex (RISC) to degrade mRNA that contains sequences complementary to the siRNA (Hammond et al., 2001; Tuschl, 2003; Tijsterman and Plasterk, 2004; Liu et al., 2004). Both antisense ODN and siRNA reduce the level of the gene product that may lead to a significant functional downregulation of the targeted gene in a biological system. The loss of function by antisense ODN or siRNA allows the researcher to evaluate the function of specific gene products and is particularly valuable as a research tool for novel cDNA, or where selective antagonists or inhibitors for the gene product are unavailable. Both antisense ODN and siRNA are highly specific for their mRNA target by virtue of sequence complementation. Thus, target recognition is orders of magnitude higher in affinity and selectivity than are antagonists and inhibitors, and can serve as a very effective pharmacological tool for elucidating the physiological and/or pathophysiological function of structurally defined proteins in a variety of biological systems.

Extensive reviews and opinions in the literature have described the design of experiments using antisense ODN and siRNA for gene targeting. Certain criteria for using antisense ODN to target genes readily apply to the use of siRNA in gene targeting *in vivo*, but there are also important distinctions in the properties, and therefore the use and handling, of these two classes of molecules (Kurreck, 2003). This chapter will focus on the experimental design for synthetic siRNA-mediated gene silencing in order to target nervous system genes in rodents.

## The Choice of siRNA

Strategies for the design of siRNA are discussed in other chapters in this course and thus will not be dealt with here. Before the *in vivo* application of siRNA in experimental animal models, the siRNA's effect on the expression of its gene target was first established using a suitable *in vitro* system for selecting the most effective siRNA for experiment. As mentioned, siRNA mediate the downregulation of a

protein primarily through degradation of the mRNA for that protein. Thus, a quantitative reduction of the target mRNA, measured by reverse-transcriptase (RT)-coupled PCR (RT-PCR), is a criterion for validating the effectiveness of siRNA because it conforms to the defined mechanism of siRNA-mediated gene silencing (Elbashir et al., 2001; Tijsterman and Plasterk, 2004). This property of siRNA also provides a sensitive and reliable assay for validating the effects of siRNA *in vitro* and is quite valuable for validating the effects of siRNA in *in vivo* applications as well. Although a concomitant reduction in the level and activity of the protein *in vitro* would be predicted, the onset of downregulation of the protein also depends on its turnover rate, and the time course may lag the knockdown at the mRNA level.

In the *in vitro* testing of candidate siRNA, one typically transfects cultured cells with the siRNA and measures its effect on the level of targeted gene transcript and protein in the cells 48 or more hours later. The transfection's efficiency is ensured by the use of commercially available transfection reagent based on well-established protocols; otherwise, the uptake of "naked" siRNA by cells is very poor (Caplen and Mousset, 2003; Spagnou et al., 2004). The effective concentration of siRNA used in the *in vitro* testing for most targets published to date is 50 nM or lower. The effect of the siRNA should be dose-dependent, and its effective concentration depends on the efficiency of uptake and the level of the gene target's expression. Different cell types and cell lines in culture may differ in their propensity for taking up siRNA. It should be noted that the effective dose or duration of action of an siRNA *in vitro* likely bears little relationship to the dose or duration of action of that siRNA in an animal model.

The control for the nonspecific, sequence-independent effects of the siRNA is a mismatch RNA (mmRNA) whose sequence is derived from the siRNA by scrambling 20–30% of the siRNA sequence, resulting in a loss of affinity and specificity for complementation with the targeted mRNA (Fig. 1). There is no strict and fast rule for scrambling

### Design of siRNA for the Rat $\delta$ -Opioid Receptor

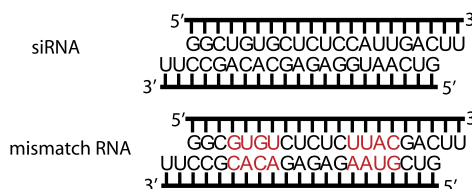


Figure 1. The sequence of an siRNA and a corresponding mismatch RNA for the rat  $\delta$ -opioid receptor. The sequence mismatch introduced into the mismatch sequence is highlighted in red (Luo et al., 2005).

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a sequence, except that mismatch in the center 6 nucleotides of the siRNA sequence may be tolerated; that is, despite the sequence mismatch in this region, the mmRNA may still interact with the targeted mRNA sufficiently to induce knockdown. Several candidate mmRNAs can be derived from an siRNA, and can be screened against the genome database to exclude sequences that have significant homology with known cDNA; this procedure is followed by *in vitro* screening (as for the selection of the siRNA), with the exception that mmRNAs are selected for lack of effect on the expression of the gene of interest.

### siRNA Delivery to the Nervous System

A major challenge in the use of RNAi for studying the function of specific peptides and proteins in the nervous system is the delivery of these molecules to their target. Compared with antisense ODN, for systemic delivery, the double-stranded siRNA are significantly more stable in the blood (Soutschek et al., 2004). Chemical modification (such as limited phosphorothioate substitution of phosphodiester linkages) further enhances the resistance of siRNA to nucleases in the circulatory system, and a number of strategies have been applied successfully to facilitate the uptake of siRNA by target tissue (Soutschek et al., 2004; Kim et al., 2006; Nawrot and Sipa, 2006; Tagami et al., 2007; Cardoso et al., 2007). These advances are pivotal to the therapeutic potential of siRNA. On the other hand, siRNA cannot penetrate the blood–brain barrier. Thus, to use siRNA as a research tool to probe nervous system function, direct delivery methods that bypass the blood–brain barrier are necessary.

The success of gene targeting by siRNA depends on efficiently delivering the molecules to the appropriate region of the nervous system, as well as on conditions that would maximize their penetration into cells once they reach the region of interest. The various procedures described below are well established for delivering drugs to the nervous system of experimental animals, including the rat (Waynforth and Flecknell, 1992). However, they differ as to the proximity from which a drug is deposited into the target region of the brain or spinal cord. Certainly, close proximity enhances the precision of targeting cells to a region of interest and maximizes the concentration of the siRNA available to that region (the latter is a major determinant of cellular uptake of these molecules, as shown from our experience and the extensive evidence in the literature on delivering antisense ODN and siRNA *in vivo*). Other strategies, including the use of transfection reagents and chemical modifications that facilitate the cellular uptake of siRNA, would likely enhance the success of RNAi.

### siRNA/Cationic Lipid Complexes

A number of commercial reagents for transfection comprise cationic lipids which, when mixed with DNA or RNA, form micelle complexes incorporating the polynucleotides. Fusion of the micelle complexes with cell membranes enhances the probability of the DNA or RNA being taken up by the cells. Some of these reagents have been optimized for siRNA delivery *in vitro* (Spagnou et al., 2004). However, neither *in vitro* nor *in vivo* delivery of antisense ODN requires the use of such reagents; rather, uptake of ODN by target cells or tissues is evident if the ODN is administered at a sufficiently high concentration (Lai et al., 1996; Lai et al., 1997; Lai et al., 2002). Uptake of the ODN has also been correlated with a knockdown of the protein target *in vitro* (Lai et al., 1997).

The initial impetus for exploring the use of siRNA/cationic lipid complexes for delivering siRNA *in vivo* was the prohibitive cost of synthetic siRNA, if a dose equivalent to antisense ODN were to be used for the experiment. Furthermore, from a mechanistic point of view, it has been established that siRNA is highly efficient in activating RISC, suggesting that the effective concentration of an siRNA inside a cell is low. Based on the *in vitro* evidence of poor uptake by cells, the large dose of siRNA required to produce an effect *in vivo* is likely to be primarily the result of poor uptake. Strategies that can lower the required dose of siRNA not only enable the experiment to be cost-effective but also reduce the off-target, or non-specific, effects associated with a high concentration of siRNA. The siRNA against the  $\delta$ -opioid receptor produced a significant knockdown of the receptor in the spinal cord at a dose that was 40 times less than the required dose of an antisense ODN to knock down the same receptor (Luo et al., 2005).

The choice of transfection reagent is based on several criteria. Foremost, it should have no apparent adverse effects on the animal. Bolus dosing, given in small volumes with long intervals between doses and low overall dose, is more amenable to using such reagents as drug vehicle than is continuous infusion, which requires a large volume of vehicle over a long period of time (see below). The maximum amount of siRNA that can be packaged into stable siRNA/lipid complexes within the limited injection volume is also critical because it is the major limiting factor of the bolus dose of siRNA (the maximum concentration possible to achieve is 0.2  $\mu\text{g}/\mu\text{L}$ , or about 15  $\mu\text{M}$ ). The stability of the siRNA/lipid complexes is essential for presenting the siRNA when it reaches the target tissue. Premature breakdown of the complexes voids the utility of the vehicle. (Another reason that

transfection reagent is not suitable for infusion is the prolonged storage of the drug in the infusion pump.)

### Delivery of siRNA via CSF

siRNA can be delivered to the nervous system via the CSF that bathes the cells. Drugs can be delivered to the brain by injecting them into the ventricular space (intracerebroventricularly, or i.c.v.). In the rat, this is accomplished by delivering the drug with a fine injection cannula that is in turn guided into the lateral ventricle by a guide cannula. The guide cannula is positioned stereotaxically and implanted according to the coordinates of the ventricle (Paxinos and Watson, 1997). The siRNA can be delivered in a volume of up to 10  $\mu\text{L}$ . The flow of the CSF will likely carry the siRNA to the other ventricles; however, because the effect of siRNA is concentration dependent (Luo et al., 2005), when its concentration becomes progressively diluted as it diffuses from the injection site, the corresponding effect should diminish exponentially. Thus, the region of the brain most likely to be affected by the siRNA is the paraventricular region close to the guide cannula. Increasing the concentration of the siRNA may allow uptake at more distal sites.

To deliver siRNA to the spinal cord of the rat, animals can be injected intrathecally (i.th.) via an implanted catheter (Yaksh and Rudy, 1976; Malkmus and Yaksh, 2004). Drugs are typically delivered in a volume of 5  $\mu\text{L}$ . Intrathecal delivery also allows siRNA to access the dorsal root ganglia, which lie within the dura mater of the spinal cord and are protected by the blood–brain barrier. Because dorsal root ganglia contain the cell bodies of peripheral sensory afferent fibers, intrathecal administration of siRNA also effectively targets the peripheral sensory nervous system (Fig. 2).

### Microinjection of siRNA

Administering siRNA to specific brain regions by stereotaxic microinjection has certain advantages: It pinpoints the region of siRNA's uptake and correlates the physiological consequences of the siRNA treatment directly with a knockdown of the targeted gene in that region. The disadvantage of microinjection is its invasiveness and the potential for injury-related effects — particularly if multiple injections are necessary and the siRNA is complexed with transfection reagent (see next section). Microinjection is guided typically by a stereotaxically implanted guide cannula directed to the target region according to the coordinates of the target region. Injection is made with a stylus that penetrates 1 mm deeper than the guide

cannula to avoid backflow into the guide cannula, at a volume of 0.5  $\mu\text{L}$  over 1 minute (Fig. 3).

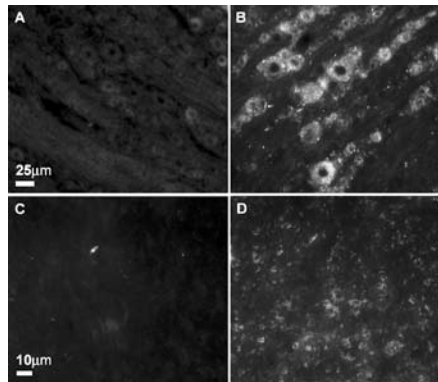


Figure 2. Fluorescence microscopy of the distribution of a rhodamine-tagged siRNA in the lumbar dorsal root ganglion (A, B) and the lumbar dorsal horn of the spinal cord (C, D). The siRNA (2  $\mu\text{g}$ ) was complexed with i-Fect (1:4 w/v) and delivered as a bolus dose of 10  $\mu\text{L}$  via an intrathecal catheter directed to the lumbar spinal cord. The tissues were fixed and harvested 24 hours after the injection, and frozen sections (20  $\mu\text{m}$ ) were then prepared for microscopy. Fluorescence was clearly visible in the cytoplasm of dorsal root ganglion neurons (B) and spinal cord neurons (D). Little autofluorescence is seen in sections of dorsal root ganglion (A) and spinal cord from untreated rat (Luo et al., 2005).

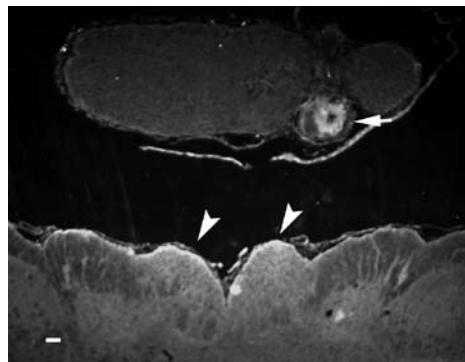


Figure 3. Delivery of a Texas-Red tagged siRNA to the caudal medulla of a rat via an implanted cannula directed to the nucleus gracilis (arrowheads). The siRNA (0.1  $\mu\text{g}$ ) was complexed with i-Fect (1:4 w/v) and delivered in a volume of 0.5  $\mu\text{L}$ . The tissue was fixed and harvested 4 hours after the injection, and frozen sections (20  $\mu\text{m}$ ) were prepared for microscopy. The fluorescent signal was further amplified by a rabbit anti-Texas Red primary antibody (1:500) and a Texas-Red tagged goat anti-rabbit IgG secondary antibody. Fluorescence is seen in the nucleus gracilis immediately adjacent to the fourth ventricle. The paraventricular region is highly labeled. The injection site is visible as a bright circular area at the base of the cerebellum (arrow), which lies immediately dorsal to the medulla in the intact brain.

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**Infusion of siRNA**

Mini-osmotic pumps (Alzet) are widely used to infuse drugs continuously. siRNA can be infused into the spinal cord by intrathecal catheter connected to an infusion pump that is implanted subdermally; siRNA can also be infused into the ventricle, or parenchymally into a specific brain region via an implanted cannula similarly connected to an infusion pump. A major advantage of infusion is the need for minimal handling of the animal subjects, but the total cost of infusing siRNA is high because of the large quantities that are delivered over many days. Typically, infusion delivery uses “naked” siRNA to avoid the potential unwanted side effects of sustained exposure to vehicle, such as transfection reagent. The solubility of the siRNA in aqueous solution allows the siRNA to be prepared at millimolar concentration (Dorn et al., 2004).

**Optimal Effective Dose of siRNA**

The optimal effective dose of siRNA for a particular target is empirically determined as the dose-to-effect ratio for a particular physiological or behavioral end point. Whether the siRNA is delivered by infusion or bolus injection, implantation of cannulae or catheters is necessary because repeated bolus injections of siRNA may be required before the onset of effect and to maintain the knockdown of the nervous system targets evaluated to date (discussed next). The effect of the siRNA tends to dissipate within 24–48 hours upon cessation of the siRNA treatment (our unpublished observations). By comparison, the action of some siRNAs for non-neuronal targets lasts considerably longer. Whether this is the result of differences in the properties of RISC in various tissues, or the stability of siRNA in different tissues, or is attributable to the chemical structure of the siRNA or the gene target, remains to be evaluated. Nevertheless, the need for repeated administration of siRNA limits its use to targeting the nervous system of smaller rodent models such as mice because they are not amenable to catheterization or cannulation.

**Validation of the Knockdown of Gene Targets by siRNA**

To show that these effects result from down-regulation of the targeted protein or peptide, the target tissue is harvested when maximal effect is seen and then analyzed for both its content of mRNA for the target gene (using quantitative RT-PCR [qRT-PCR]) and that of the gene product. Appropriate controls for these assays comprise separate groups of experimental subjects that are treated with saline, vehicle, or mmRNA under the same conditions as for siRNA; the tissues from these control groups are

then analyzed in parallel with the siRNA-treated group.

Additional controls or strategies further strengthen the specificity of the siRNA against the intended gene target and should be considered for the experimental design whenever possible. These include (1) testing more than one siRNA against the same target to show that the effect against the same target is consistent; (2) confirming the delivery and/or uptake of siRNA in the region of interest using a tagged siRNA; (3) demonstrating that the effect of the siRNA is reversible, i.e., that it takes place only during siRNA treatment; and (4) showing that the siRNA is selective for the defined gene target by evaluating its effect, or lack thereof, on highly homologous genes (e.g., enzyme isoforms, receptor subtypes).

**Targeting Peripheral Neurons**

The peripheral nervous system, which consists of the sympathetic and parasympathetic nerves as well as primary sensory afferent fibers, is amenable to RNAi. The sympathetic and parasympathetic ganglia lie outside the blood–brain barrier, and thus may be targeted by systemic delivery of siRNA. In contrast, the cell bodies of most primary afferent sensory neurons reside within the dorsal root ganglia; thus, targeting these cells with antisense ODN or siRNA has relied on intrathecal delivery. The region of delivery can be localized by adjusting the placement of the catheter tip and limiting the drug volume to within 10  $\mu$ L. It should be noted that the intrathecal route delivers drugs to the dorsal root ganglia and the spinal cord equally efficiently; therefore, this route does not differentiate between presynaptic (dorsal root ganglia) and postsynaptic (spinal cord neuron) effects of the gene knockdown unless the gene target is differentially expressed in either tissue. The trigeminal neurons are the primary afferent sensory neurons that innervate the face; their cell bodies reside in the trigeminal ganglia (spinal 5 nuclei) in the caudal medulla (Paxinos and Watson, 1997). Delivery of antisense ODN or siRNA to these ganglia requires stereotaxically implanted cannulae.

The primary afferents are the first-order neurons for nociceptive transmission. Therefore, they have been a major focus in understanding the physiological and molecular mechanisms of pain transmission as well as the potential pathophysiology of these neurons under chronic, abnormal pain conditions due to inflammation of, or injury to, these peripheral nerves. The antisense ODN strategy set the precedent for using gene targeting as proof of principle of the role of the voltage-sensing sodium-channel subtype  $Na_v1.8$  (Porreca et al., 1999; Lai et al., 2002; Gold et al.,

2003; Lai et al., 2004) and the purinergic channel subtype P2X3 (Honore et al., 2002; Dorn et al., 2004; North, 2004) in inflammatory and neuropathic pain states but not in normal nociception. The use of antisense ODN was justified because of the absence of selective antagonists for these channels. The outcome of these findings supports the potential of these channels as therapeutic targets for abnormal pain. In particular,  $\text{Na}_v1.8$  is expressed predominantly in peripheral sensory neurons; drugs that are selective for this channel subtype should have substantially fewer adverse side effects compared with current sodium-channel blockers. siRNAs to  $\text{Na}_v1.8$  are highly effective when delivered intrathecally in a rodent model of neuropathic pain (D. Sah, J. Lai, and F. Porreca, unpublished data), consistent with previous observations of the use of antisense ODN.

Viral delivery of antisense ODN or siRNA is an alternative approach in which the molecules can be delivered specifically to peripheral sensory neurons by inoculating the innervated skin. To date, viral delivery has focused mainly on the overexpression of genes as pain therapy rather than on the disruption of gene expression. More recently, an article described a lentivirus-mediated expression of an siRNA for  $\text{Na}_v1.8$  *in vitro* (Mikami and Yang, 2005).

### Targeting Highly Expressed Genes

siRNA rapidly supplants the use of antisense ODN for gene targeting *in vivo* because the former has a clearly defined mechanism of action, is more stable, and thus more amenable to systemic delivery, and may have a longer duration of action. These advantages over antisense ODN make siRNAs particularly promising as therapeutic agents and as superior basic research tools for applying functional genomics. Because current technology has not circumvented the blood–brain barrier in order to deliver siRNA, the therapeutic potential of these molecules in nervous system diseases remains limited. However, as a research tool, if siRNAs are more efficient and longer acting than antisense ODN, they may overcome some limitations of using antisense ODN for gene targeting. One such limitation is the inability of antisense ODN to significantly knock down targets that are either highly expressed or have slow turnover rates. The former trait is exemplified by the expression of many neuropeptides.

In our initial optimization of the conditions for RNAi by intrathecal delivery, we chose a well-characterized protein that we previously targeted successfully with antisense ODN — the  $\delta$ -opioid receptor, one of the G-protein coupled receptors (GPCRs), which as a family of proteins are quite amenable to knockdown by antisense ODN. Intrathecal once-daily injection

of a bolus dose of 2  $\mu\text{g}$  (complexed with i-Fect siRNA Transfection Reagent; Neuromics Antibodies, Northfield, MN) blocked the anti-nociceptive action of a  $\delta$ -receptor–selective agonist, deltorphin II, by day 3 compared with vehicle or mmRNA-treated control (Luo et al., 2005). The onset of effect was similar to that seen previously using antisense ODN (Bilsky et al., 1996). The functional inhibition of the  $\delta$ -opioid receptor was concomitant with a significant knockdown of the  $\delta$ -opioid receptor but not of the  $\mu$ -opioid receptor in the same tissue. Uptake of the siRNA was observed in both the spinal cord and the dorsal root ganglia (Fig. 2). The inhibitory effect of the siRNA resolved upon cessation of the siRNA treatment.

The intrathecal administration of siRNA was then used to deliver an siRNA against neuropeptide Y (NPY). NPY has been shown to be significantly upregulated in the large-diameter dorsal root ganglion neurons upon ligation injury of the L5 and L6 spinal nerves and appears to underlie the development of hypersensitivity to innocuous touch stimuli (tactile hypersensitivity or allodynia) (Ossipov et al., 2002). The once-daily intrathecal administration of the siRNA for NPY was initiated 1 day before the L5/L6 spinal nerve injury and continued once daily for up to 7 days. Sensitivity to touch in the injured paw was monitored daily. A significant attenuation of tactile hypersensitivity was established by day 3 of the siRNA administration, but not of the mmRNA (Fig. 4).

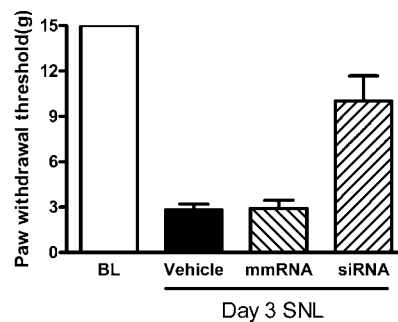


Figure 4. Intrathecal administration of an siRNA for NPY attenuates the development of tactile hypersensitivity in spinal nerve ligation (SNL)-injured rats. Rats were implanted with an intrathecal catheter directed to the lumbar spinal cord. The baseline paw withdrawal threshold in response to Von Frey filament probing was recorded for all the experimental subjects (BL). Administration of siRNA or a mismatch RNA was given once daily at 2  $\mu\text{g}$  in 1:4 (w/v) complexes with i-Fect, or i-Fect alone (vehicle). One day after initiating treatment, rats were subject to L5/L6 spinal nerve injury (Ossipov et al., 2002). The paw withdrawal threshold was recorded daily while administration continued. Both the vehicle-treated and the mmRNA-treated groups developed tactile hypersensitivity after the ligation injury, as reflected by the significant lower thresholds for paw withdrawal. The siRNA-treated group showed a significantly higher threshold of paw withdrawal, suggesting that the siRNA treatment was anti-allodynic. The data were collected on day 3 after the ligation injury (4 injections were given by this test day). Each group consisted of 6 rats. The baseline threshold was pooled from all 3 groups (N= 18).

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The significant anti-allodynic effect of the siRNA to NPY suggests that siRNAs are likely more effective against highly expressed targets.

### Acknowledgments

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