Video Article

A Simple Alternative to Stereotactic Injection for Brain Specific Knockdown of miRNA

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Abstract

MicroRNAs (miRNAs) are key regulators of gene expression. In the brain, vital processes like neurodevelopment and neuronal functions depend on the correct expression of microRNAs. Perturbation of microRNAs in the brain can be used to model neurodegenerative diseases by modulating neuronal cell death. Currently, stereotactic injection is used to deliver miRNA knockdown agents to specific location in the brain. Here, we discuss strategies to design antagomirs against miRNA with locked nucleotide modifications (LNA). Subsequently describe a method for brain specific delivery of antagomirs, uniformly across different regions of the brain. This method is simple and widely applicable since it overcomes the surgery, associated injury and limitation of local delivery in stereotactic injections. We prepared a complex of neurotropic, cell-penetrating peptide Rabies Virus Glycoprotein (RVG) with antagomir against miRNA-29 and injected through tail vein, to specifically deliver in the brain. The antagomir design incorporated features that allow specific targeting of the miRNA and formation of non-covalent complexes with the peptide. The knock-down of the miRNA in neuronal cells, resulted in apoptotic cell death and associated behavioural defects. Thus, the method can be used for acute models of neuro-degeneration through the perturbation of miRNAs.

Video Link

The video component of this article can be found at https://www.jove.com/video/53307/

Introduction

MicroRNAs have emerged as novel therapeutic targets due to their universal role in the regulation of gene expression and direct evidence for involvement in disease. MiRNAs are being actively explored for their potential as drug targets[1,2]. Further, alterations in miRNA expression are associated with several diseases[3] and simulation of these changes by artificial perturbation of miRNA expression can be used to study the cellular pathways involved in disease manifestation. Tissue specific delivery of miRNA targeting drugs is currently a major challenge for miRNA based drug development. Antagomirs and miRNA mimics are promising agents for perturbing miRNA levels[4–6]. However, special features that enhance their specificity and efficacy have to be incorporated into the design of antagomirs before they can be used for *in vivo* perturbation of miRNA expression.

MicroRNAs are especially relevant as targets in currently incurable neurodegenerative and neuro-developmental diseases. The blood-brain barrier poses a restriction to the delivery of antagomirs in the brain. Stereotactic injections are widely used in rodent models to deliver molecules to specific locations in the brain[7]. It requires skill, extensive investment in instrumentation and time. Stereotactic injections are invasive, involve surgery, cause at least minor injury and are restricted to local delivery. The use of cell penetrating peptides with a preference for targeting neurons can counter these limitations since they can be delivered through the trans-vascular route but breach the blood brain barrier. Such a peptide derived from the Rabies Virus Glycoprotein (RVG), was previously used to deliver siRNA against Japanese Encephalitis Virus in mice[8]. We found that using the peptide for antagomir delivery, miRNAs can be effectively knocked down in the mouse brain[9].

The second major challenge of miRNA knock-down arises from the small size of miRNAs and the presence of closely related sequence isoforms. We take the example of mmu-miR-29 family which consists of three closely related isoforms, miR-29a, b and c. Antagomirs are also generally modified along the backbone to increase their stability and render them resistant to attack by nucleases. Locked Nucleic Acids (LNAs) offer a further advantage that they enhance thermal stability and even lead to target degradation over and beyond steric hindrance[10]. Introducing modifications all along the backbone can be effective but expensive. We have earlier seen that modifications beyond an optimal number may not further enhance the efficacy. The design of the antagomir therefore involves the optimal modification of the antagomir.

To complex the antagomir non-covalently with the neurotropic peptide, a charged hepta- to nona-arginine extension is used. D-Arginine residues are used since they confer higher stability as they are not susceptible to cleavage by proteases. Hepta- to nona-arginine stretches act as efficient cell penetrating agents, although they do not confer cell type specificity. By covalently linking the RVG peptide to the nona-arginine linker, a

neurotropic, cell penetrating peptide was generated. The positively charged residues of the peptide interact with the negatively charged nucleic acid backbone, to form complexes. These complexes can be used to effectively transfect DNA or RNA into cultured cells and *in vivo* into tissues.

Protocol

Note: All the procedure including animal subjects have been approved by Institutional Animals Ethics Committee (IAEC) at the Institute of Genomics and Integrative Biology, New Delhi (IGIB/AEC/10/2013). This protocol is specifically adjusted for targeted delivery of Antagomir-29 in the brain and knockdown of miR-29.

1. Antagomir Design Strategy

1. Retrieve the mature miRNA sequence from miRBase[11] (http://www.mirbase.org/).
2. Retrieve the sequences of related miRNA family members using the “Gene Family” link in the miRBase record (http://www.mirbase.org/cgi-bin/mirna_summary.pl?fam=MIPF0000009).
3. Reverse complement the sequence: http://www.bioinformatics.org/sms/rev_comp.html provides a convenient tool to reverse complement sequences.
4. Mark the positions to be modified by LNA using the following guidelines:
    1. Choose Thymine residues placed 3-4 bases apart for the LNA modification, since LNA pyrimidines are more stable than purines[12].
    2. Choose Cytosine residues separated by 3-4 residues if previous step produces less than five modifications.
    3. Prioritize the five modifications to the 5’ end of the antagomir, since this avoids the seed sequence.

2. Antagomir-neuropeptide Complex Preparation

Note: This is the most critical step in the protocol and it needs standardization. To standardize the protocol any fluorescently labeled oligonucleotide (FLO) can be used in place of the antagomir[9]. The peptides should be of high purity (HPLC grade, 98% purity).Sequences and charge of peptide and antagomir are given in Table 1.

1. Decide the number of moles of antagomir to be injected based on molar charge on antagomir, its toxicity and weight of the mouse. Calculate the number of moles of peptide to be injected based on molar charge ratio of antagomir : peptide.
   NOTE: Standardize molar charge ratio of antagomir : peptide for toxicity and effective delivery in the mouse brain. Use different molar charge ratios of fluorescently labeled oligonucleotide: peptide for standardization. We found that Antagomir-29 and control were both toxic at 4microgram/gram body weight of mouse. Antagomir-29 and control were used at 2microgram/gram body weight, but this effective concentration is likely to vary for each microRNA.
2. Dilute the peptide and antagomir in separate microfuge tubes from stock solutions with sterile 10% D-glucose to the desired final concentration as calculated in step 1.
3. Keep the microfuge tube with the peptide solution on a vortex at moderate vortexing speed.
4. Add 1/3rd of the antagomir solution to the peptide solution tube, slowly, drop wise, while it is mixed thoroughly on a vortex mixer.
5. Continue mixing of solutions on the vortex for next 1 min and then allow the mixture to stand for 1 min.
6. Repeat the step 4 and 5 two more times to mix the remaining antagomir solution with the peptide solution in the same microfuge tube. The slow addition is critical for the formation of mono-disperse complexes that are effective in transfection. Rapid addition can lead to aggregation of the complexes and their precipitation.
7. Incubate the complex for 30 min at RT without vortexing. During this time of incubation, acclimatize the mice to the lab where injections will be performed.

3. Tail Vein Injection of Antagomir-neuropeptide Complex

1. To restrain the animal, place the mouse in a restrainer or decapicone of proper size.
2. Clean the surface of the tail using cotton swab pre-soaked in warm water (about 40°C). This will promote vasodilation and increase the visibility of the vein.
3. Approach the tail with a small volume (0.5 – 1.0 cc) insulin syringe at a 15-20° angle. Be careful not to introduce any air into the syringe. Start at the distal portion of the tail. Inject the complex. Remove the needle and apply an antiseptic swab directly to the injection site (approximately 5-10 sec) to stop any bleeding.
4. Replace the mouse in an individually ventilated cage in the group of 3-5, with the corncob and tissue paper as enrichment. Do not keep uninjected and injected mice in same cage.

4. Behavioural Assays

Note: Keep the time interval of 3-4 hr in between injection and behavioural assays to allow recovery after injection. Bring the mouse to a quiet room. Do not disturb the animals during this period of acclimatization.

1. Acclimatize the mouse to the new room for 30 min and then start the following behavioural assays. Keep a gap of 10 min in between each behavioural assay.
2. Hindlimb clasping:
   NOTE: This test indicates motor dysfunction and neurological impairment.
    1. Lift the mouse gently by holding near base of the tail in the clear background.

2. Check the hindlimb position for 10-15 sec. Wild type mouse splays hindlimbs away from abdomen, while an ataxic mouse tends to retract one or both hindlimbs towards the abdomen more than 50% of time suspended[13].
3. Return the mouse to the cage

3. Ledge test:
   1. Lift the mouse gently by holding the tail and keep it on the ledge of a cage.
   2. Observe the mouse specifically at the corner of the cage. Wild type mouse can pass the corners easily without fear and losing the balance. Ataxic mouse lose its balance, freeze or shakes while walking along the cage ledge and at the corners.

4. Mouse foot print assay:
   NOTE: For this assay keep the absorbent sheet ready, on the floor of a narrow runway (~70-cm-long, ~5-cm-wide with ~5-cm-high walls.)
   1. Hold the mouse gently by one hand and apply ink on the hind limbs by a brush.
   2. Place the absorbent sheet on the floor of a narrow runway.
   3. Allow mouse to walk or run over an absorbent sheet in a straight line in a runway from one end to other.
   4. Repeat the process two more times with each mouse using fresh absorbent sheet.
   5. Measure the distance between two consecutive steps in the forward movement. Do not include first and last few footprints where the animal is just initiating and finishing its run, respectively.

Representative Results

Using the procedure presented here, complexes of 50microgram fluorescently labeled oligonucleotide (FLO) and ~850microgram RVG peptide of 1:15 molar charge ratio (FLO : peptide) were prepared and injected only once through tail vein. Complex of non-neurotropic Rabies Virus Matrix (RVM) peptide and FLO was used as a delivery control. Next day, mice brain and liver were isolated and single cell suspensions were prepared. Cells were observed under microscope for green fluorescence. FLO-RVG complex was successfully delivered with a shortest time of one day and detected as a green fluorescence in the brain cells but not in the liver **(Figure 2B,2D)** while RVM peptide delivered FLO to the liver but not in the brain cells **(Figure 2A,2C)**.

To knockdown miR-29 in the mouse brain antagomir against miR-29 were designed with five LNA modifications. The scrambled, non-miRNA targeting sequence used as an Antagomir-control. Complex of 50microgram Antagomir-control or Antagomir-29 with ~850microgram RVG peptide was prepared and injected through tail vein over a 3 day time course, followed by daily behaviour assays as shown in figure 1. In order to achieve maximum knockdown effect, complexes were injected once per day for 3 continuous days. On fourth day behaviour assays were performed without injection. Mouse footprint analysis showed significant reduction in the step length of Antagomir-29 RVG injected mice (**Figure 3A-3B**). Hindlimb clasping assay of Antagomir-29 RVG injected mice clearly showed ataxic behaviour with tendency to retract one or both hindlimbs towards the abdomen. In the ledge test Antagomir-29 RVG injected mice showed freezing or shaking behaviour with loss of balance at the corner of the cage. Mice were euthanized by injection of xylazine hydrochloride (16milligram/kg) and thiopentone sodium (80milligram/kg) on fourth day (24 hr after the last injection of complex). Brain samples were used to perform molecular and cellular analysis. Total RNA collected from cortex, cerebellum and hippocampus. Quantitative real time PCR assay showed clear reduction in the expression levels of both the isoforms of miR-29, miR-29a (**Figure 4A**) and miR-29b (**Figure 4B**), in the Antagomir-29 RVG injected mice brain parts.

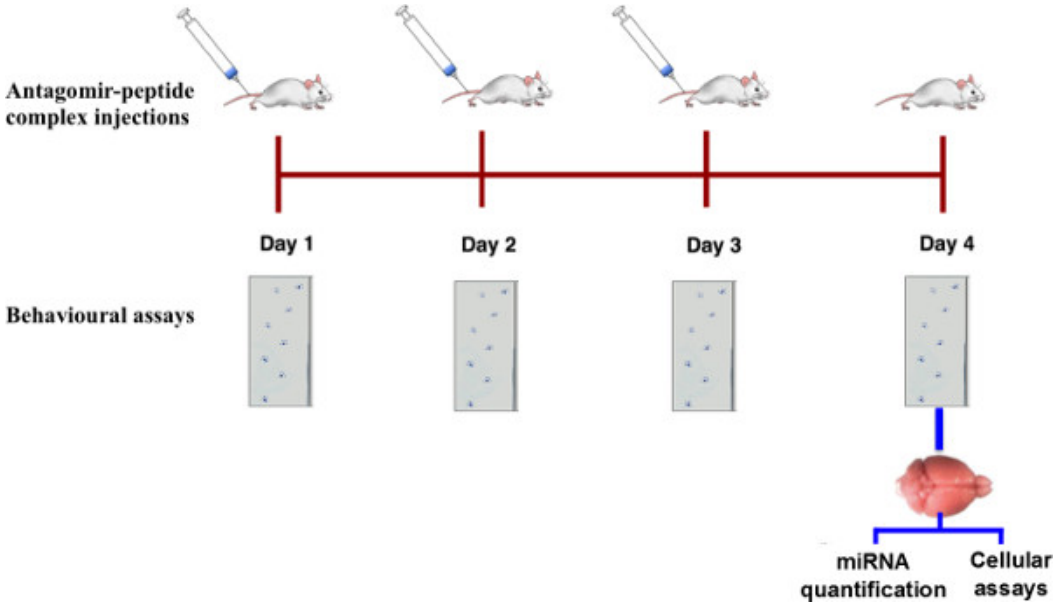

Figure 1. Schema for mouse tail vein injection and behaviour.
In order to target miR-29 in the brain, Antagomir-control or Antagomir-29 with RVG peptide was injected once a day for 3 consecutive days through tail vein. Behavioural assays were performed daily, after a 3 hr period, to allow the mouse to recover from injection. On fourth day only behavioural assays were performed and mice were euthanized and brain tissue was collected for real time PCR and cellular assays. Please click here to view a larger version of this figure.

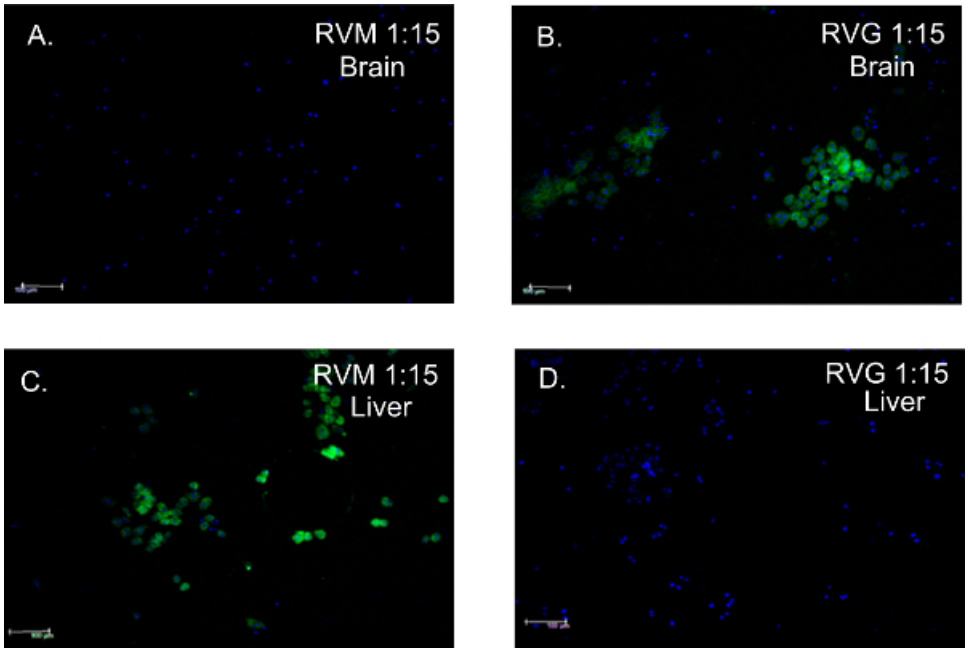

Figure 2. RVG peptide delivers fluorescently labeled oligonucleotide specifically to the mouse brain.
Using the method described in this article, complex of fluorescently labeled oligonucleotides (FLO) and peptide RVG/RVM were prepared and injected only once through tail vein. FLO-RVG complex was detected as a green fluorescence in the brain cells but not in the liver **(B,D)** while RVM peptide delivered FLO to the liver but not the brain **(A,C)**[9]. Please click here to view a larger version of this figure.

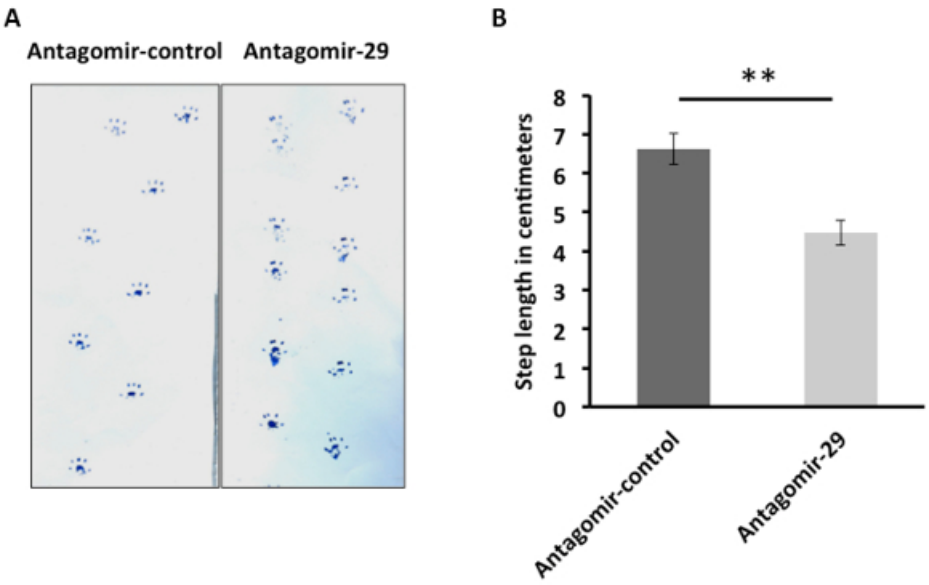

Figure 3. miR-29 knock down leads to the ataxic behaviour.
Mouse footprint assays (A) on fourth day showed clear reduction in distance between two consecutive steps for the mouse treated with Antagomir-29 (B) (**) *p*-value < 0.001; n=3 (Figure modified from[9].) Please click here to view a larger version of this figure.

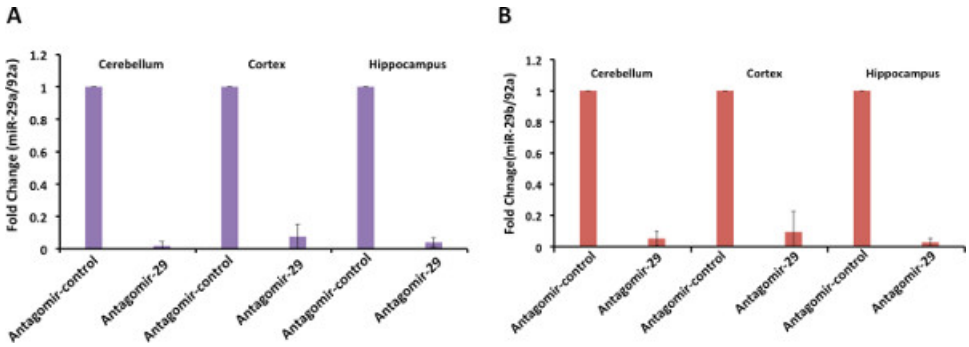

Figure 4. Antagomir-29 and RVG complex down-regulates miR-29 in mouse brain.
Total RNA collected from three brain parts. Quantitative real time PCR assay was performed to estimate expression levels of both the isoforms of miR-29, miR-29a **(A)** and miR-29b **(B)**, and normalized to miR-92a. (Figure modified from[9].) Please click here to view a larger version of this figure.

Name	Sequence	Charge	Length
RVG-peptide	YTIWMPENPRPGTPCDIFTNSRGK RASN-GGGG-RRRRRRRRR*	11 +	41
RVM-peptide	MNLLRKIVKNRRDEDTQKSSPASA PLD-GGGG-RRRRRRRRR*	11 +	40
Antagomir-control (Scrambled)	5' ACCAATCGACCAAC 3'	14 -	14
Antagomir-29	5' CACTGATTTCAAATGG 3'	16 -	16
	*Arginine residues at the C-terminal end of RVG and RVM peptides are in D-form.		
	LNA modified bases in the Antagomir sequences are underlined.		

Table 1. Sequences of peptide and Antagomirs

Discussion

Here we demonstrate a widely accessible methodology to study the effects of miRNA modulation. Currently, most attempts at *in vivo* characterization of miRNA functions involve the creation of knockout mice or a transgenic that expresses a miRNA sponge. Most miRNAs, even the cell type specific ones are expressed in more than one organ. For instance, miRNAs initially thought to be specific to the hematopoietic system are also expressed in the brain, due to the presence of microglia. Thus even a cell type specific knock-down strategy, expressing anti-sense agents against miRNAs under the control of cell type specific promoters is likely to produce pleotropic effects. miR-29 family of miRNAs for instance are expressed in the brain, blood cells and several other sites in the body. Thus genetic tools for modulation of miRNA are not widely applicable. It is also not amenable to drug development or testing of potential therapeutic strategies.

The ability to deliver the cargo through simple tail vein injections makes the technique widely accessible. It also circumvents the use of lentiviruses for neurotropic delivery. This method, originally developed for knockdown of a neurotropic virus, is amenable to modular changes in the three domains that produced cell type specific knockdown of miRNA, namely, the cell penetrating peptide, the neurotropic peptide and the anti-sense molecule. For instance, we found that using a different miR-29 specific antagomir leads to knockdown but fails to produce the behavioural effects[9]. Besides cell penetrating peptides, non-viral methods of gene delivery to neuronal cells include nano-particles, lipids and cationic polymers. However, many viral vectors and cationic lipids still need invasive methods of administration due to the blood brain barrier. One approach to this problem has been to use mimics of endogenous proteins that can cross the blood brain barrier.

Monoclonal antibodies against the insulin receptor and transferrin receptor have been used effectively as *in vivo* brain delivery vectors since the blood brain barrier expresses these receptors[14]. The Rabies virus glycoprotein derived, RVG peptide is known to bind to the nicotinic acetylcholine receptor and has been used in combination with liposomes and nano-particles. The unique ability of this peptide to cross the blood brain barrier with minimal toxicity and carry siRNA, small molecules, anti-sense molecules or plasmid for delivery to the brain can be very useful in drug delivery to the CNS[8,15,16]. More specifically, a simple conjugate of the peptide with a modified anti-sense molecule, for CNS delivery of microRNA inhibitors by-passing the blood brain barrier can accelerate *in vivo* functional studies on neuronal miRNAs.

The most critical step in this protocol is antagomir-neuropeptide complex preparation. Rapid addition of antagomir to the peptide solution may cause precipitation. This reduces the targeting efficiency. Also dose of peptide and antagomir has to be standardized because low dose of peptide or antagomir may not be efficient for the effective knockdown of target molecule while higher dose may be toxic to the mouse due to off-targets.

A minor limitation of this technique is that repeated injection of the complex has to be given for at-least 3 days to achieve phenotypic effect. The treatment regimen is likely to vary from microRNA to microRNA. Also the effect of knockdown of the molecule is transient, lasting for a few

days.We found that a treatment regimen of single daily dose of antagomir:peptide for 3 continuous days resulted in knockdown for five days after which expression of miR-29 was restored (unpublished observation).

The applicability of this method to knockdown of larger molecules like long non-coding RNAs need to be tested since it is not clear if effective complexes can be formed with larger RNA. A potential limitation could be the high cumulative negative charge on long RNAs, and consequently, the large amount of peptide required to achieve the required charge ratio.

Using a fluorescent, non-targeting oligonucleotide we found that the peptide effectively delivers the cargo to the cortex, cerebellum and the hippocampus. Thus pan-brain delivery was achieved. The knockdown of the miRNA was also comparable in all three parts of the brain. The modular nature of the method allows it to be adopted in future for delivery of miRNA mimics to restore pathologically down-regulated miRNAs.

Disclosures

The authors have nothing to disclose.

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References

1. Roshan, R., Ghosh, T., Scaria, V., & Pillai, B. MicroRNAs: novel therapeutic targets in neurodegenerative diseases. *Drug discovery today.* **14**, 1123-1129 (2009).
2. Maes, O. C., Chertkow, H. M., Wang, E., & Schipper, H. M. MicroRNA: Implications for Alzheimer Disease and other Human CNS Disorders. *Current Genomics.* **10**, 154-168 (2009).
3. Soifer, H. S., Rossi, J. J., & Saetrom, P. MicroRNAs in Disease and Potential Therapeutic Applications. *Mol Ther.* **15**, 2070-2079 (2007).
4. Bader, A. G., Brown, D., & Winkler, M. The Promise of MicroRNA Replacement Therapy. *Cancer research.* **70**, 7027-7030 (2010).
5. Stenvang, J., Petri, A., Lindow, M., Obad, S., & Kauppinen, S. Inhibition of microRNA function by antimiR oligonucleotides. *Silence.* **3**, 1-17 (2012).
6. Trang, P. *et al.* Systemic Delivery of Tumor Suppressor microRNA Mimics Using a Neutral Lipid Emulsion Inhibits Lung Tumors in Mice. *Molecular Therapy.* **19**, 1116-1122 (2011).
7. Barbash, S., Hanin, G., & Soreq, H. Stereotactic Injection of MicroRNA-expressing Lentiviruses to the Mouse Hippocampus CA1 Region and Assessment of the Behavioral Outcome. *J Vis Exp.* (76) e50170 (2013).
8. Kumar, P. *et al.* Transvascular delivery of small interfering RNA to the central nervous system. *Nature.* **448**, 39-43 (2007).
9. Roshan, R. *et al.* Brain-specific knockdown of miR-29 results in neuronal cell death and ataxia in mice. *RNA.* **20**, 1287-1297 (2014).
10. Kaur, H., Wengel, J., & Maiti, S. Thermodynamics of DNA−RNA Heteroduplex Formation: Effects of Locked Nucleic Acid Nucleotides Incorporated into the DNA Strand. *Biochemistry.* **47**, 1218-1227 (2008).
11. Griffiths-Jones, S., Grocock, R. J., Van Dongen, S., Bateman, A., & Enright, A. J. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Research* . **34** , D140-D144 (2006).
12. Kaur, H., Babu, B. R., & Maiti, S. Perspectives on Chemistry and Therapeutic Applications of Locked Nucleic Acid (LNA). *Chemical Reviews.* **107**, 4672-4697 (2007).
13. Guyenet, S. J. *et al.* A Simple Composite Phenotype Scoring System for Evaluating Mouse Models of Cerebellar Ataxia. *J. Vis. Exp.* (39) (2010).
14. Bergen, J. M., Park, I.-K., Horner, P. J., & Pun, S. H. Nonviral Approaches for Neuronal Delivery of Nucleic Acids. *Pharmaceutical Research.* **25**, 983-998 (2008).
15. Zou, L.-L., Ma, J.-L., Wang, T., Yang, T.-B., & Liu, C.-B. Cell-Penetrating Peptide-Mediated Therapeutic Molecule Delivery into the Central Nervous System. *Current Neuropharmacology.* **11**, 197-208 (2013).
16. Hwang, D. W. *et al.* A brain-targeted rabies virus glycoprotein-disulfide linked PEI nanocarrier for delivery of neurogenic microRNA. *Biomaterials.* **32**, 4968-4975 (2011).

