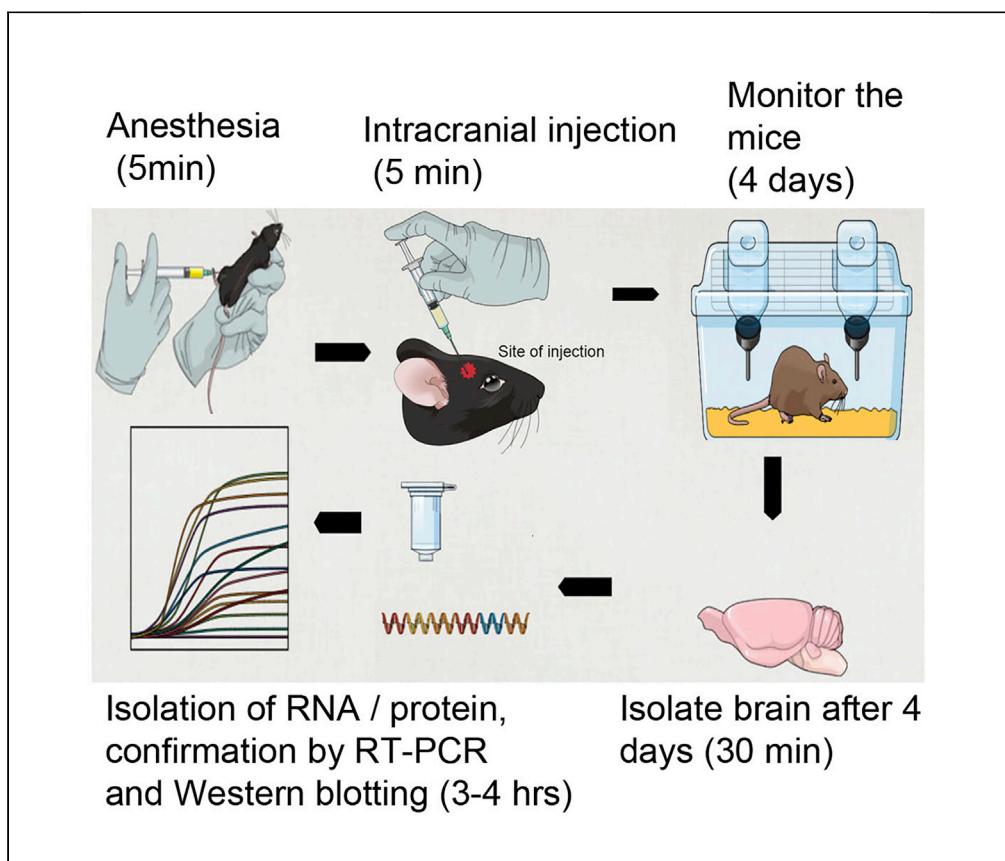


Protocol

Intracranial delivery of antisense locked nucleic acids for silencing microRNAs in the mouse brain



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Highlights
Intracranial delivery
of antisense LNAs

Noninvasive
procedure to silence
microRNAs
specifically in mouse
brain

Confirmation of
silencing effect of
LNAs on microRNAs

Antisense locked nucleic acid (LNA) technology has been widely used for silencing microRNAs with enhanced specificity and efficiency. In this protocol, we first describe the procedure for targeted intracranial delivery of LNAs to silence microRNAs specifically in the mouse brain. We then detail the steps to isolate RNA and protein from mouse brain, followed by using RT-PCR and Western blotting to confirm microRNA silencing. This noninvasive approach can only be applied to mouse brain to specifically target silencing of microRNAs.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Intracranial delivery of antisense locked nucleic acids for silencing microRNAs in the mouse brain

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SUMMARY

Antisense locked nucleic acid (LNA) technology has been widely used for silencing microRNAs with enhanced specificity and efficiency. In this protocol, we first describe the procedure for targeted intracranial delivery of LNAs to silence microRNAs specifically in the mouse brain. We then detail the steps to isolate RNA and protein from mouse brain, followed by using RT-PCR and Western blotting to confirm microRNA silencing. This noninvasive approach can only be applied to mouse brain to specifically target silencing of microRNAs. For complete details on the use and execution of this protocol, please refer to Sharma et al. (2021).¹

BEFORE YOU BEGIN

Introduction

MicroRNAs are small noncoding RNAs that can regulate many cellular functions.² Exogenous manipulation of these cellular microRNA levels has been used as a tool to understand the role of various microRNAs in regulating cellular signaling mechanisms.³ MicroRNA inhibitors (or Anti-miRs) are antisense oligonucleotides which bind to complementary microRNAs and block their action. In recent years, different chemical modifications of these RNAs (e.g., cholesterol conjugation, introduction of Locked nucleic acid chain, 2' O-methyl nucleotides) have been designed for increasing their stability *in vivo*.⁴ To study the roles of specific microRNAs in different tissues of mice, miRNAs or Anti-miRs can be delivered to them by various routes e.g., intraperitoneal, intravenous, intranasal.⁵ Genetic knockout studies and use of antisense oligonucleotides are used for studying loss of function *in vivo*. Chemically modified antisense oligonucleotides targeting specific microRNA are widely utilized to sequester microRNAs causing de-repression of target mRNAs.⁴ *In vivo* use of antisense nucleotides needs to be optimized for their enhanced binding affinity and nuclease resistance in animal tissues. In our study¹ comprising the use of anti-miR-24 for silencing miR-24 in brain, customized antisense nucleotides (LNA) were injected into the cranium of mice. LNAs are bicyclic RNA analogs in which the furanose ring of the ribose sugar-phosphate backbone is chemically locked due to the introduction of a 2'-O,4'-C methylene bridge mimicking N-type (C3'-endo) conformation.^{6,7} This modification enhances nuclease resistance and binding affinity of antisense nucleotides to the complementary microRNA due to higher duplex melting temperature (T_m value). Apart from using LNA nucleotides, another common modification is the modification of phosphodiester linkages into phosphorothioate (PS) backbone in which one of the non-bridging oxygen group is replaced by sulfur atom in the phosphate group.⁸ The protocol below describes an easy noninvasive method for intracranial delivery of antisense nucleotides targeting miRNA-24 for achieving brain specific silencing of miRNA-24. This protocol will help the readers further to follow our study better and perform similar experiments in the future with precision.



Ethical statement

All animal experiments were performed in compliance with protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Note: Ethical permission from Institutional regulatory committee is mandatory before conducting this experiment.

Preparation of LNA solution

⌚ Timing: 10 min

1. Dissolve 15 pmol LNA oligonucleotide in 300 μ L nuclease-free water so that the final concentration is 1 pmol / 20 μ L. Customized oligonucleotides are sold by Qiagen in lyophilized form which are dissolved and stored at -20 degree.

Note: Store small aliquots at -20 degree to avoid freeze thaw cycles.

2. Procure thin needles (26 G 5/8 gauge) and 0.5 mL sterile syringes.

Preparation of anesthesia

⌚ Timing: 5 min

3. Prepare a mixture of ketamine and xylazine in sterile PBS accordingly (final volume 2 mL).

Reagent	Stock conc	Working conc	Volume to be added
Ketamine	100 mg/mL	25 mg/mL	500 μ L
Xylazine	100 mg/mL	2.5 mg/mL	50 μ L
Sterile PBS			1,450 μ L

Note: Make fresh anesthesia mixture. The anesthesia mixture can be stored at 4°C, for maximum 7–8 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-STING antibody	Cell signaling technology	13647S (1:1000 dilution)
Anti-actin antibody	Cell Signaling Technology	3700 (1:10000 dilution)
Anti-Myc protein antibody	Santa Cruz Biotechnology	Sc-40 (1:1000 dilution)
Chemicals, peptides, and recombinant proteins		
4% paraformaldehyde in PBS	Thermo Fisher Scientific	J61899.AP
Ketamine hydrochloride (ketaset)	Zoetis Inc.	100 mg/mL stock
Xylazine	Patterson Veterinary	100 mg/mL stock
Sterile PBS (endotoxin free)	MilliporeSigma	806552-1L
Critical commercial assays		
Taqman Universal RT-PCR master mix	Thermo Fisher Scientific	cat no-4304437
Taqman microRNA assay specific to miR-24	Thermo Fisher Scientific	catalog no.- A25576, assay i.d 481011
Taqman microRNA assay specific to miR-23b	Thermo Fisher Scientific	Catalog- 4427975, assay i.d- 000400
Taqman microRNA reverse transcriptase kit	Thermo Fisher Scientific	cat no.- 4366596
RNU6 control assay	Thermo Fisher Scientific	4427975, assay i.d 001006
miRVana PARIS kit for RNA/ protein isolation	Thermo Fisher Scientific	AM 1556

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
WT C57/BL-6 mice; male (6 weeks old)	The Jackson Laboratory	#000664 Strain- C57/ BL-6
Oligonucleotides		
miRCURY POWER LNA customized LNA inhibitor (15 pmol) (antimiR-24)	QIAGEN company	catalog no 339147, YCI0200961-FFA
POWER LNA negative control	QIAGEN	catalog no- 339147, YCI0201821-FFB
Other		
BD PRECISIONGLIDE™ 26 G x 5/8" HYPODERMIC NEEDLES	Becton Dickinson (BD)	305115
0.5 mL syringes	U.S. Plastic Corp.	82264
Diet gel	ClearH2O	72-07-5022
1.5 mL microcentrifuge tubes	Thermo Fisher Scientific	69715
Heating Pad for mice	Kent Scientific Corporation	RT-0515
Software and algorithms		
ImageJ	Schneider et al. ⁹	https://imagej.nih.gov/ij/
Microsoft Excel	Microsoft Corporation	https://www.microsoft.com/en-us/microsoft-365/p/excel/CFQ7TTC0HR4R?activetab=pivot:overviewtab&ef_id=EAlalQobChMlpKP3zNnO-glV0zizAB3hsAWcEAAAYASAAEgLIGPD_BwE:G:s&OCID=AIDcmm409lj8ne_SEM_EAlalQobChMlpKP3zNnO-glV0zizAB3hsAWcEAAAYASAAEgLIGPD_BwE:G:s&gclid=EAlalQobChMlpKP3zNnO-glV0zizAB3hsAWcEAAAYASAAEgLIGPD_BwE
GraphPad Prism 5.02	Dotmatics	https://www.graphpad.com/

STEP-BY-STEP METHOD DETAILS

Anesthesia

⌚ Timing: 5 min

This section describes the procedure to anesthetize the mice.

1. Weigh the mice before giving anesthesia and inject the anesthesia mixture into the peritoneum (I.P) of 6 weeks old C57/Bl6 male mice.
2. Inject 50 μ L of anesthesia into mice weighing 17–20 grams.

Note: This dose is used for mice ranging 5–6 weeks which weigh (16–20 grams) to temporarily anesthetize the mice. Mice with higher weight or age are not used for I.C injection as their cranium gets harder to inject. Confirm the effect of anesthesia by pinching the tail or foot pad of mice and do not proceed until they are completely unconscious.

Injection of the oligonucleotide into the cranium

⌚ Timing: 5 min

This section describes injection of oligonucleotide precisely into the cranium of mice and avoid any bleeding from site of injection.

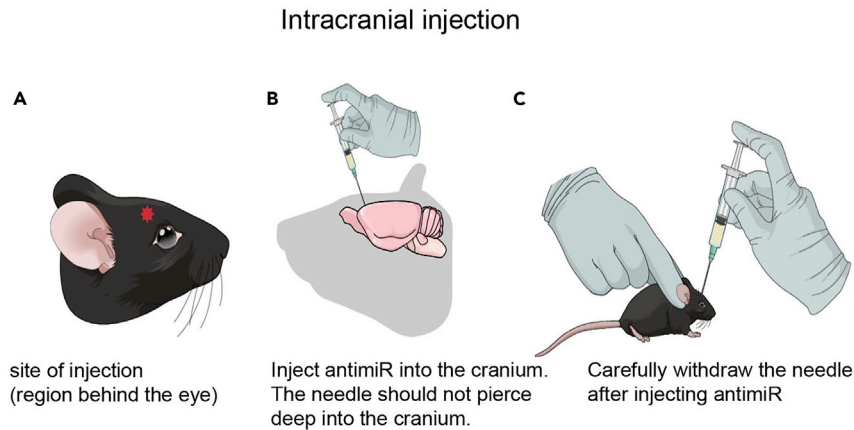


Figure 1. various steps of intracranial injection of antimicroRNA into mouse brain and position of needle in the cranium while injecting

3. Dissolve oligonucleotides and fill the solution into 0.5 mL syringe with thin needle (26 G 5/8 gauge).
4. Wipe the head region of mice (no shaving required) with 70% alcohol and hold the head of the mice from the side of its neck with left hand.
5. Very slowly start piercing the needle of syringe into the left side of the cranium at the frontal side of the cortex (left cerebral area as shown in [Figure 1](#)).

Note: Once the needle tip pierces into the cranium, no resistance to the needle is felt. Do not move the needle further into the brain as it may disrupt brain vessels and may cause bleeding.

6. As soon as the tip of needle penetrates the cranium, do not pierce the needle further deep into the brain and inject 20 μ L of antimicroRNA solution into cranium. The position of needle in the cranium is depicted in [Figure 1](#).
7. Slowly take the needle out of the brain and observe the site of injection carefully in order to check bleeding or popping out of the solution. Any leakage or bleeding will be clearly visible at site of injection.

Note: The solution should not leak out from the site of injection for efficient delivery of the oligonucleotide to the brain. [Figure 1](#) depicts injection of antimicroRNA efficiently into cranium without causing any bleeding from site of injection.

Recovery from anesthesia

⌚ Timing: 20 min

This section describes the recovery of mice after intracranial injection.

8. Keep the mice on a heating pad for some time until they start regaining consciousness.
9. Place the mice back into the cage and monitor them until they start moving.
10. Place a diet gel so that they have easy source of food in the cage.

Observation of injected mice

⌚ Timing: 4 days

This section describes observing injected mice for any symptoms and monitoring body weight.

11. Observe the mice daily and monitor its body weight.

Harvesting the injected mice

⌚ Timing: 30 min

This section describes perfusion of mouse brain with paraformaldehyde and isolation after 4 days post injection weight.

12. After 4 days, inject a terminal dose of anesthesia into the peritoneum of mice and allow the mice to lose consciousness.

Note: 80 μ L of anesthesia with mixture of ketamine and xylazine used at same final concentration as mentioned above is considered terminal dose for mice weighing 17–20 grams.

13. Place the mice on a surgical pad and make an incision to expose their hearts (on the ventral side of the body).
14. Inject 4% paraformaldehyde solution in PBS into the beating hearts of mice to drain out blood from the blood vessels.

Note: Perfusion is done only to remove blood from blood vessels.

15. Then make an incision at the snout area between eyes (dorsal side) and remove the skin to expose the cranium.
16. Cut the cranium by using scissors and isolate the brain. Snap chill brain tissue in liquid nitrogen and store them at -80 degree.

Confirmation of silencing of miRNA-24 in mouse brain

⌚ Timing: 3–4 h

This section describes isolation of RNA and protein from mouse brain and confirmation of miRNA-24 silencing by RT-PCR and Western blotting.

17. Confirm the silencing of miR-24 in mouse brains by RT-PCR using a miR-24 specific Taqman probe. For extraction of miRNA and protein from same tissue, use the miRVana PARIS kit using manufacturer protocol available online.

https://www.thermofisher.com/documentconnect/documentconnect.html?url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanuals%2Ffm_1556.pdf.

Note: To ensure that the injected oligonucleotide has been well absorbed in the brain tissue, use the right hemisphere for RNA and protein isolation (as the oligonucleotide is injected into the left side) for confirmation of miR-24 silencing.

18. Reverse-transcribe the extracted RNA into cDNA using Taqman reverse transcriptase kit following the manufacturer protocol:

<https://genome.med.harvard.edu/documents/qpcr/microRNATaqManAssayProtocol.pdf>.

19. Perform RT-PCR using the Taqman probes specific to miR-24 using manufacturer protocol.

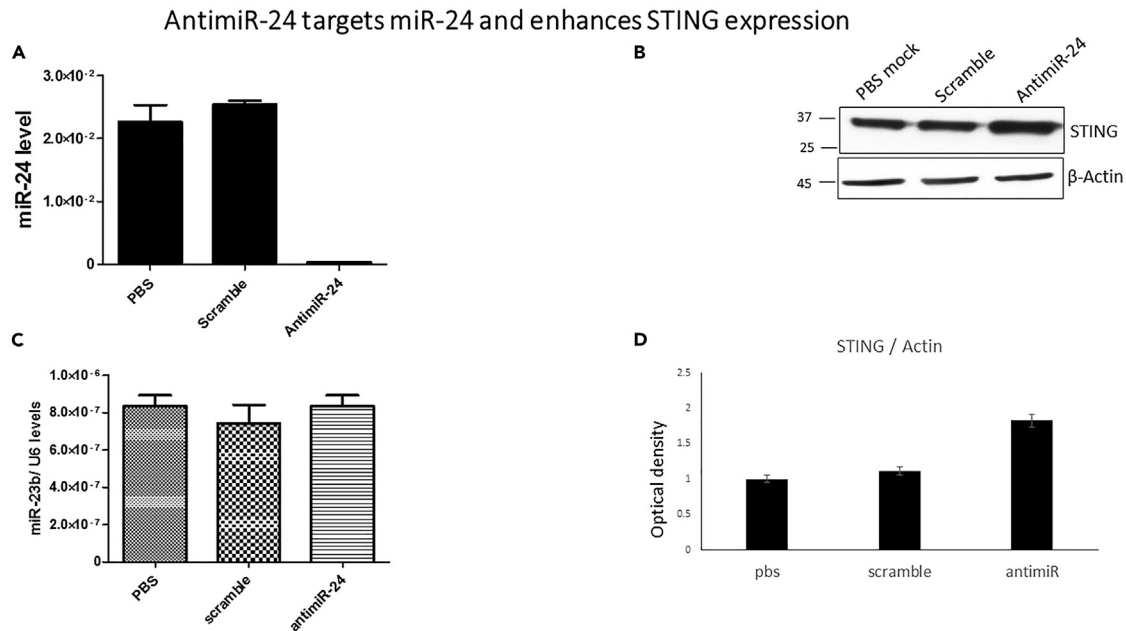


Figure 2. Confirmation of silencing of miR-24 by RT-PCR and western blot of STING protein

(A) Taqman RT-PCR results confirming silencing of miR-24. PBS, Negative control scramble or AntimiR-24 oligonucleotide is injected into mouse brains and brains are harvested after 4 days. RNA is isolated from brain tissues and miR-24 specific Taqman probes are used for RT-PCR. RNU6 specific Taqman probe is used for normalization. (Mean ± SEM, N = 3).

(B) Western blot image depicting enhanced STING protein expression in AntimiR-24 injected brain. Protein is isolated from brain tissues of these mice and expression of STING is analyzed by western blotting. AntimiR-24 injection in brain enhances STING expression.

(C) Taqman RT-PCR results showing no effect of antimiR-24 on other non-targeting miRNA (miR-23b). PBS, Negative control scramble or AntimiR-24 oligonucleotide is injected into mouse brains and brains are harvested after 4 days. RNA is isolated from brain tissues and miR-23b specific Taqman probes are used for RT-PCR. RNU6 specific Taqman probe is used for normalization. (Mean ± SEM, N = 3).

(D) Graph showing densitometric analysis of western blot image (B). The western blot image of blot (B) is quantified by using ImageJ software to determine fold change of enhanced STING expression upon antimiR-24 injection. (Mean ± SEM, N = 3).

<https://genome.med.harvard.edu/documents/qpcr/microRNATaqManAssayProtocol.pdf>

- Silencing of microRNA causes de-repression of the target mRNA. Confirm the silencing of miR-24 by assessing the expression of STING and Myc protein (which are direct targets of miR-24) through Western blotting.

EXPECTED OUTCOMES

Brain tissues are homogenized and miRNA / protein is isolated by using the miRvana PARIS kit (Thermo Fisher Scientific). Successful *in vivo* delivery of the oligonucleotide blocks miR-24 action and de-repress its target mRNAs expression. In mice injected with a miR-24 targeting oligonucleotide, miR-24 is not detected by RT-PCR analysis of brain RNA (Figure 2A). Also the enhanced expression of STING protein is confirmed by Western blotting (Figure 2B). AntimiR-24 injection into the brain specifically targets miR-24; not other microRNAs. The expression of other non-targeting microRNA (miR-23b) which gets expressed from same gene cluster along with miR-24, is also examined to confirm specific targeting by antimiR-24 (Figure 2C). AntimiR-24 does not affect the expression of miR-23b and specifically silence miR-24 only. As miR-24 also targets expression of Myc protein,¹⁰ antimiR-24 inoculation enhances the expression of Myc protein in mouse brain (Figure 3A). As a negative control, a non-targeting LNA oligonucleotide is injected into the mouse brains. These results confirm the specific silencing of miR-24 by antimiR-24 in mouse brain. Because the oligonucleotide is injected to the left side of brains, isolate the RNA and protein from the right side of the brain cortex. This ensures the oligonucleotides are well absorbed throughout the brain and effectively silence miR-24 in the whole brain. Also, RNA and protein is isolated from the same brain tissue

AntimiR-24 enhances Myc protein expression

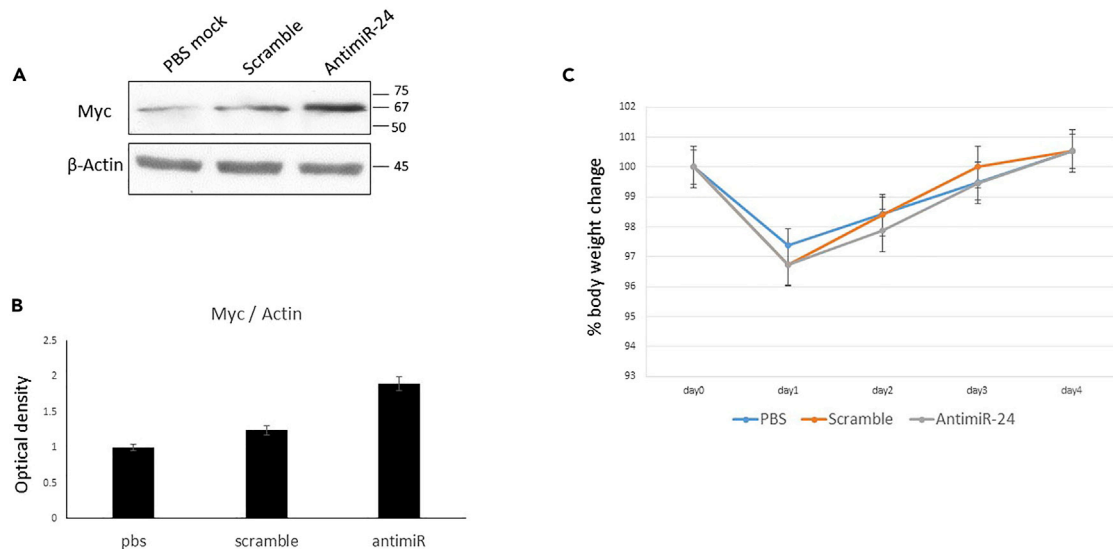


Figure 3. AntimiR-24 de-represses expression of Myc protein and does not affect body weight of the mice

(A) Western blot image depicting enhanced Myc protein expression in AntimiR-24 injected brain. Protein is isolated from brain tissues of these mice and expression of Myc is analyzed by western blotting. AntimiR-24 injection in brain enhances Myc expression.

(B) Graph showing densitometric analysis of western blot image (A). The western blot image of blot 3A is quantified by using ImageJ software to determine fold change of enhanced Myc expression upon antimiR-24 injection. (Mean \pm SEM, N = 3).

(C) Graph showing percentage of body weight changes in mice. Mice injected with PBS or Scramble / AntimiR-24 oligonucleotide are monitored daily after procedure. Due to anesthesia, the mice display slight weight loss post injection but completely recover later. (Mean \pm SEM, N = 4).

(by using miRvana PARIS Kit) to avoid any discrepancy in results. Injection of oligonucleotides into the brain does not cause any significant body weight loss (Figure 3C) in mice.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Graph Pad Prism 5.02 software. The mean \pm SEM of all biological replicates was used to make all graphs. For analyzing body weight changes, percentage of body weight was plotted using Microsoft excel sheet. ImageJ software was used for quantification of Western blots.

LIMITATIONS

This protocol is used for localized silencing microRNAs only in mouse brain by intracranial injection. This I.C injection of antimiR will not silence miRNA in other parts or organs of mice. It does not guarantee long term systemic silencing of miRNAs. This protocol cannot be used for intracranial injection of mice more than 6 weeks of age, as the cranium of mice gets harder with age. Moreover volume more 20 μ L will cause leakage from site of injection.

TROUBLESHOOTING

Problem 1

While injecting into the cranium, excessive penetration of needle into brain may cause bleeding and oligonucleotides may flow out of the cranium (step 5).

Potential solution

The needle should be used with thickness mentioned above only (26 G 5/8 gauge). Once tip of the needle enters into the cranium, stop inserting it further into the brain and inject the oligonucleotide very gently.

Problem 2

Mice may appear sick and display neurological symptoms like paralyzed limbs, impeded movements or severe weight loss 1–2 days post injection due to internal bleeding or any other damage to the brain tissue (step 11).

Potential solution

Such mice should not be used for further studies.

Problem 3

The oligonucleotide solution may ooze out from site of injection (step 6).

Potential solution

Do not inject more than 20 μ L of solution as more volume will ooze out from the site of injection and cause leakage of the oligonucleotide.

Problem 4

Excessive I.P injection of anesthesia may lead to death of mice (step 2).

Potential solution

Carefully weigh all the mice and inject anesthesia accordingly. For 16–20 gm mice, 50 μ L anesthesia should be given (final concentration of ketamine 25 mg/mL and xylazine is 2.5 mg/mL).

Problem 5

Clogging of spin columns while isolating RNA from brain tissue (step 17).

Potential solution

Do not use excess tissue to isolate miRNA / protein as it can clog the columns and reduce the yield. Use 100 gm of brain tissue for miRNA / protein isolation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ganes C. Sen (seng@ccf.org).

Materials availability

The study did not generate any new unique reagents.

Data and code availability

This study did not generate unique codes. The data generated during this study have been published by the authors.¹

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

N.S. performed the experiments, analyzed the data, and wrote parts of the paper, and G.C.S. designed experiments, interpreted the results, acquired funding, and wrote parts of the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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