

Video Article

Stereotactic Injection of MicroRNA-expressing Lentiviruses to the Mouse Hippocampus CA1 Region and Assessment of the Behavioral Outcome

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Abstract

MicroRNAs (miRNAs) are small regulatory single-stranded RNA molecules around 22 nucleotides long that may each target numerous mRNA transcripts and dim an entire gene expression pathway by inducing destruction and/or inhibiting translation of these targets. Several miRNAs play key roles in maintaining neuronal structure and function and in higher-level brain functions, and methods are sought for manipulating their levels for exploring these functions. Here, we present a direct *in vivo* method for examining the cognitive consequences of enforced miRNAs excess in mice by stereotactic injection of miRNA-encoding virus particles. Specifically, the current protocol involves injection into the hippocampal CA1 region, which contributes to mammalian memory consolidation, learning, and stress responses, and offers a convenient injection site. The coordinates are measured according to the mouse bregma and virus perfusion is digitally controlled and kept very slow. After injection, the surgery wound is sealed and the animals recover. Lentiviruses encoding silencers of the corresponding mRNA targets serve to implicate the specific miRNA/target interaction responsible for the observed effect, with naïve mice, mice injected with saline and mice injected with "empty" lentivirus vectors as controls. One month post-injection, the animals are examined in the Morris Water Maze (MWM) for assessing their navigation learning and memory abilities. The MWM is a round tank filled with colored water with a small platform submerged 1 cm below the water surface. Steady visual cues around the tank allow for spatial navigation (sound and the earth's magnetic field may also assist the animals in navigating). Video camera monitoring enables measuring the route of swim and the time to find and amount the platform. The mouse is first taught that mounting the hidden platform offers an escape from the enforced swimming; it is then tested for using this escape and finally, the platform is removed and probe tests examine if the mouse remembers its previous location. Repeated tests over several consecutive days highlight improved performance of tested mice at shorter latencies to find and mount the platform, and as more direct routes to reach the platform or its location. Failure to show such improvement represents impaired learning and memory and/or anxiety, which may then be tested specifically (e.g. in the elevated plus maze). This approach enables validation of specific miRNAs and target transcripts in the studied cognitive and/or stress-related processes.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50170/>

Introduction

The role of particular miRNAs in nervous system functioning has recently been challenged by lentiviral injection in several studies. MiRNAs have been found to be crucial for maintaining and re-shaping synapse structure¹, synaptogenesis² and synapse remodeling and maintenance³. These studies strongly suggest that miRNAs are engaged, via multileveled regulatory effects in both the shaping up and in maintaining the main output of the nervous system, cognitive function. Stereotactic injection of lentivirus particles into specific regions in the rodent brain enables searching for alterations in synapse morphology and neuronal activity, and was used for establishing the functional significance of over-expressed transcripts^{4,5}. Direct infection of neurons at well-defined brain areas with miRNA-expressing lentiviruses may be implicated in studies of aging, brain disorders and neurodegeneration; Studies of miRNAs in the realm of behavioral regulation⁶⁻⁸ are at a far less advanced state, and stereotactic injection of miRNA-expressing lentivirus particles followed by behavioral tests may be useful for such purposes. A considerably more laborious method for inducing over- or under-expression involves genetically engineered knock-in or knockout mice. Genetic systems can further allow for conditional and temporal control on expression (e.g. Cre-Lox, Tet systems), but these hardly offer the spatial specificity of the injection procedure and there is almost always a certain amount of leakiness. Also, the engineering procedures do not require surgery and can be used across laboratories with relatively good reproducibility; however, they are slower and require much more manpower and financial resources. In addition, the temporal control of the over- or under-expression in injected mice is far more accurate compared to genetically engineered mice.

Protocol

1. Lentivirus Preparation

1. Grow HEK-293FT cells to 90% confluence.
2. On the day of transfection change cell medium to serum-free DMEM supplemented with 1 mM glutamine and 50 mg/ml penicillin-streptomycin.
3. Co-transfect the cells with a pLKO.1-Puro vector and with plasmids coding for the delta R8.2 and VSV-G moieties and the miRNA of interest, using 10 μ l of 1 mg/ml polyethylenimine as a carrier⁹.
4. Collect packaged lentiviruses at 24 hr and 48 hr post-transfection, filter through 0.45 μ m filter.
5. Concentrate the lentiviruses using ultracentrifugation in 70,000 x g, for 2 hr and 15 °C, aliquot and store at -70 °C.
6. Measure virus titer by infecting HEK-293T cells with serially diluted virus preparations (1 to 10⁻⁶ ml of vector per well). The resulting titer (a minimum of $\sim 1 \times 10^9$ infectious particles per ml is recommended) is evaluated for expression of the gene of interest, using puromycin selection and by quantifying GFP expressing viruses through counting fluorescent cells.

For more detailed protocol of lentivirus preparation see¹⁰.

2. Preparing Animals for Surgery

1. Surgeons garb should include surgical gown, sterile gloves, cap and mask.
2. Weigh the animal, then anesthetize it with an IP-injection of a Ketamine mixture, with the volume proportional to the animal weight as specified for each drug. For a Ketamine/Xylazine mixture, a dose range of 80-200 mg/kg Ketamine and 7-20 mg/kg Xylazine is usually used for mice.
3. Wait until the animal is fully anesthetized, then check for lack of withdrawal reflex in the hind limb of the animal by extending the limb and then pressing it with your finger.
4. Inject the animal subcutaneously with Rimadyl, as specified on the box, for pain relief. Typical dose range for Rymadil is 5-10 mg.
5. Place the animal on a heating pad to keep a steady body temperature and moisten the eyes with ointment.
6. Shave the area between the two ears with a trimming machine.
7. Sanitize the skin with betadine.

3. Exposure of the Skull and Drilling

1. Place the animal inside the stereotact by adjusting the rods into the crevices just anterior to the animal's ears.
2. Use a scalpel to make an anterior-posterior incision of about 1.5 cm between the ears and keep it open with surgery clamps (serfines).
3. Clean the surface of the skull with a cotton swab until the intersection between the coronal suture and sagittal sutures; *i.e.* the bregma and the intersection between the coronal and lambdoid sutures; *i.e.* lambda, are visible.
4. Point the tip of the syringe, held by the stereotact, to the bregma point, at all three axes. Write down the coordinates. This point would be considered as the zero point in all three axes.
5. Lift the syringe in the vertical axis so that a planar movement would not scratch the skull and move the syringe head to the correct location. Hippocampal CA1 injections require the following coordinates relative to the bregma in mm: -2 at the anterior/posterior axis, ± 1.8 at the lateral/medial axis and -1.5 at the dorsal/ventral axis.
6. Lower the tip of the syringe until it touches the skull and mark the spot with a marker. Remove the syringe back to avoid stabbing.
7. Drill a shallow hole, only in the skull bone using a fine driller. Hold the driller steady so it would not continue to drill into the soft tissue underneath. You can prevent this by steadying one hand with the other and by drilling in short pulses.

4. Injection of the Lentivirus

1. Withdraw 0.5 ml of the concentrated lentivirus solution.
2. Place the syringe above the hole and slowly lower it vertically until it reaches the surface of the skull. Continue to lower the syringe into the brain very slowly. At this step some bleeding might occur which does not necessarily indicate a failed penetration. If bleeding occurs mop it up with a cotton swab.
3. Set the digital pump to 0.02 ml/min (0.5 μ l would be injected in 25 min) and start infusion. Slow infusion allows for effective spreading of the virus into the tissue and prevents back flow. In some syringe types consider inserting the syringe for an extra depth of 0.5 mm before retreating to original coordinates and infusing.
4. After infusion is completed wait for additional 5 min to allow the material to spread into the brain instead of retreating back into the canal formed by the syringe.
5. Remove the syringe very slowly and watch for back flows. If a back flow is observed at this step, a fraction of the injected material was probably lost.

5. Wound Sealing and Recovery

1. Seal the wound with histoacryl. Be sure to avoid leaking of the histoacryl into the eyes.
2. Inject the animal intraperitoneally (IP) with 1 ml pre-heated saline, to avoid dehydration.

3. Place the animal in a recovery cage that is positioned on a heated pad. Watch the animal while it recovers for an additional hour.

In case of weight loss, inappetance, weakness/inability to obtain feed or water, moribund state or infection, euthanasia of the animal should be performed. Acceptable methods for euthanasia of rodents are barbiturates, inhalant anesthetics, CO₂, CO, or potassium chloride in conjunction with general anesthesia.

After 4 to 6 weeks, assess the animal's navigation memory is assessed in the Morris Water Maze. The lentivirus will usually infect most cells within the injection sphere.

6. Behavioral Assessment in the Morris Water Maze

1. Train the animals in the Morris Water Maze. For exact training and testing protocol see ¹¹. After each trial, dry the animal with a dry towel, replace it in its cage and refresh the water in the tank.
2. Test the animals in the Morris Water Maze for 3 consecutive days, 4 trials each day. Each day insert the animal into the maze in each of the 4 directions of the maze (north, south, east and west) in a changing order, for example day one: east-west-north-south, day two: west-south-north-east. Wipe the animals after each trial.
3. During the test trials track the animal with a tracking system such as the Noldus tracking system.
4. After the 12th test trial, insert the animal into the water tank without the platform for one minute. This would be used to analyze the searching strategy the animal is taking.
5. Analyze the data as suggested in ¹².

Representative Results

Injection of 0.5 µl lentivirus into the CA1 region in the mouse hippocampus, with the flow rate indicated in the protocol section yields an infected sphere of about 1 mm in the rostral-caudal axis, and about 0.5 mm in the medial-lateral and the anterior-posterior axes (**Figure 3**).

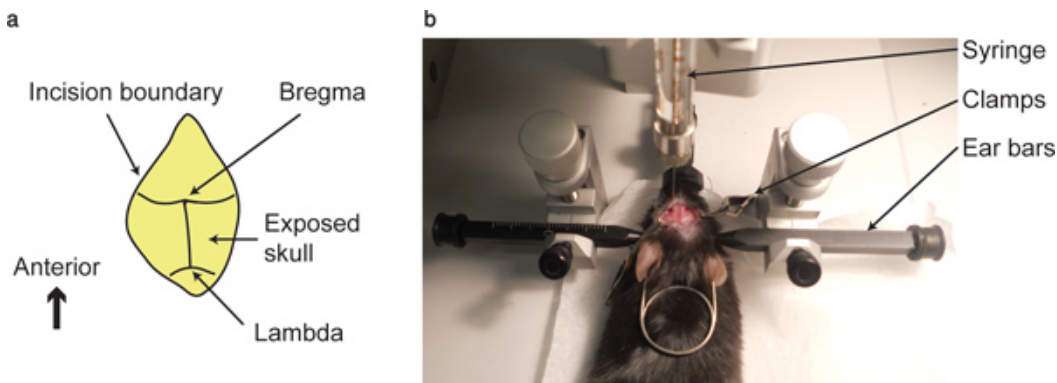


Figure 1. Bregma point and injection apparatus. (a) Shown is a schematic map of the Bregma (coronal and sagittal sutures intersection) and Lambda (Lambdoid and sagittal sutures intersection) as seen after performing the incision. (b) Picture depicting the main mechanical components of the stereotactic apparatus.

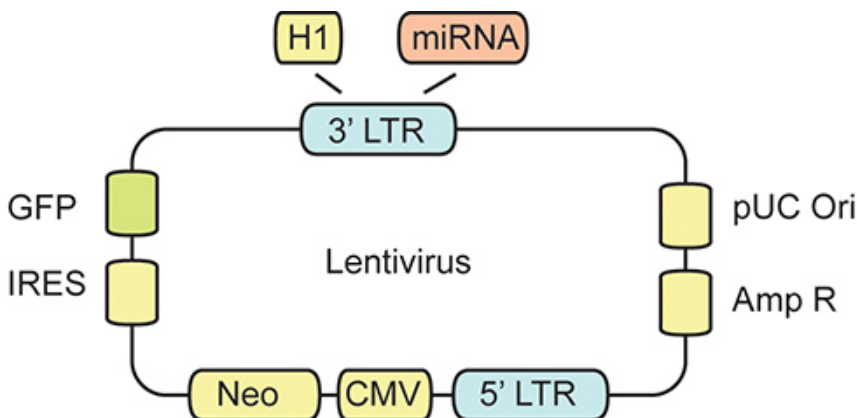


Figure 2. Lentivirus principal components. The lentivirus includes components that enable resistance to antibiotics (Ampiciline, Amp R), a reporter gene (GFP), 5' and 3' long terminal repeats (LTR) for effective integration and expression of the virus and the gene of interest (miRNA).

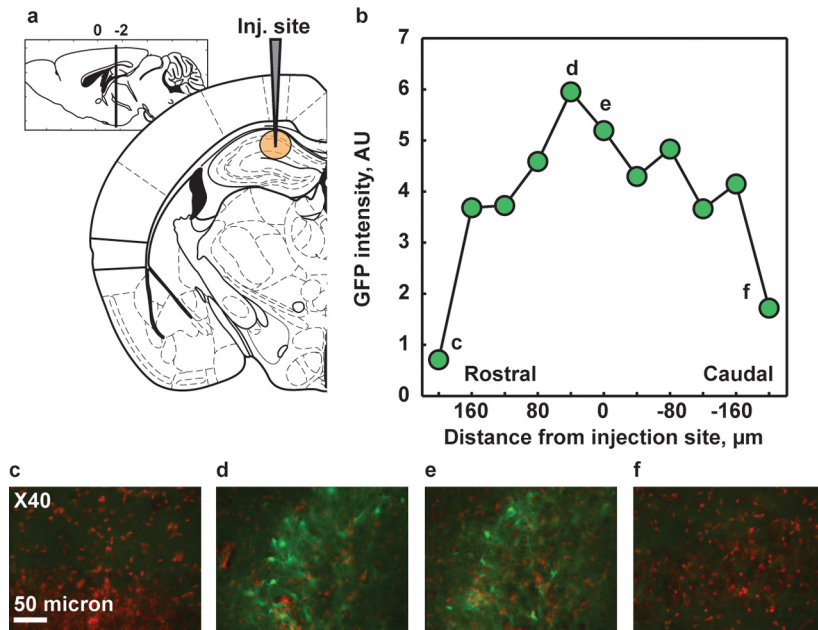


Figure 3. Injection site and *in vivo* Lentivirus transfection. (a) Injection site according to the mouse atlas. Injection is directed to the CA1 region of the Hippocampus. (b) GFP quantification in arbitrary units (normalized measure of the amount of 'green' seen in each field) along 11 slices, each one 40 μm thick, in the rostral-caudal axis. (c, d, e and f). Specific fields for the positions marked on the graph in b. DAPI staining in red and GFP in green. [Click here to view larger figure.](#)

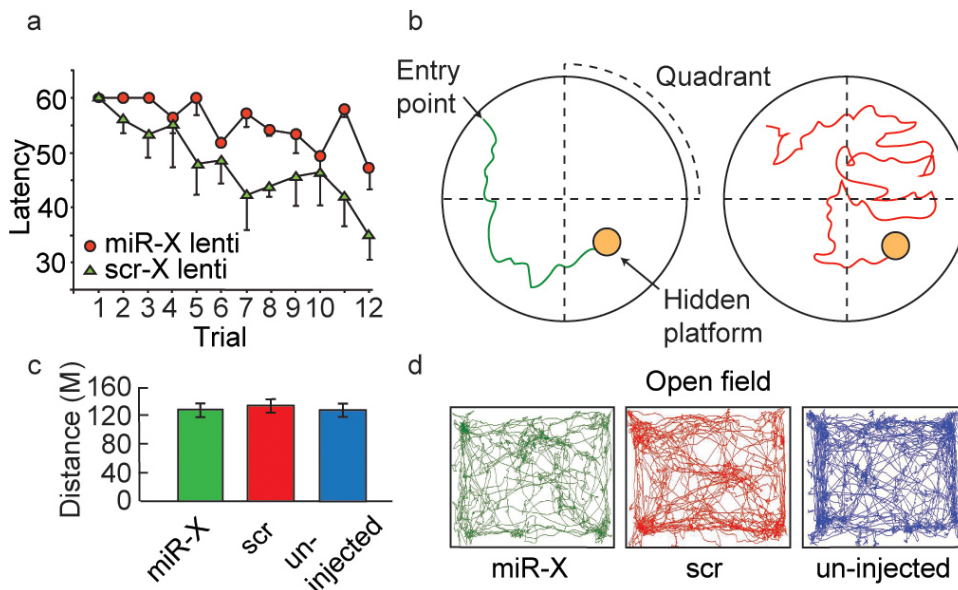


Figure 4. Behavioral assessment of mice in the MWM. (a) Mean latency to find the hidden platform for mice injected with lentivirus expressing a specific miRNA (designated miR-X in the figure, red circles, $n=10$) and mice injected with the scramble expressing lentivirus (green triangles, $n=10$). Error bars represent SEM. ANOVA P -value <0.05 . (b) Representative trajectories of the last trial for the two groups. (c) Mean distance in 30 min in the open field for mice injected with the miR expressing tested lentivirus (green), scramble expressing lentivirus (red) and un-injected animals (blue). No difference in the global locomotion activity was identified (ANOVA $P>0.1$). (d) Representative trajectories of mice from each group in (c).

Discussion

Stereotactic injection of a lentivirus is a relatively rapid method for *in vivo* assessment for both up- or down- regulation of different genes and miRNAs. The main alternative is a genetically engineered mouse which is a much more laborious and time consuming technique than direct lentivirus injection. In addition, the up regulation, in lentivirus injection, occurs at a specific time in the adult mouse and does not include any possibility of leakiness during development, as is most often the case in the genetically engineered mice that include temporal control. Complete

temporal control is essential when assessing the relevance of different genes in neurodegenerative diseases in which the altered regulation occurs late in the life of the animal. Additionally, lentivirus injection offers precise spatial control which, again, is subject to a certain amount of leakiness in the genetically engineered systems. The mouse engineering systems utilize the endogenous specificity of different transcription factors by adding an insert of a specific promoter that would be activated only in a certain region or cell type. In comparison, lentivirus injections can be limited in space without changing the vector. The disadvantages of lentivirus injection are the requirement for a laborious surgical procedure and questionable reproducibility between laboratories. After the injection surgery, a period of time, between a week and 3 weeks must pass for the virus to induce its effect and for the animal to recover, before additional protocols can be performed such as behavioral and biochemical assessments. For studies in which the time of induction should be in close proximity to the different assessments the injected lentivirus can include a temporal control system, such as the Cre-Lox system, and antibiotics can be used in such a case as the inducer long after the animal completely recovered. This technique was presented in ¹³. Regarding reproducibility between injections, groups that perform this method routinely have showed a fairly reproducible targeting and delivery of injected material between animals ¹³. Reproducibility between laboratories is a matter that requires great care, since different laboratories use different syringes, different types of syringe edge (blunt or sharp), and different flow rates, which affect the resulting injection sphere. In both methods a positional effect due to genomic integration site might occur. In the transgenic line the consequence would be gene silencing in the whole line. In the lentivirus injected population, on the other hand, the effect would be limited to a small subset of the population. Combining miR experiments together with shRNA experiments *in vivo* can shed light on the specificity of the effect of a certain miR on its target ^{6,8}. Lastly, lentivirus injection can be used in a transgenic knockdown mouse for testing a knock-in effect in a specific brain region and serve for a loss of function / rescue of function experiments.

Disclosures

The authors declare that they have no competing financial interests.

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