Video Article

In Vivo SiRNA Transfection and Gene Knockdown in Spinal Cord *via* Rapid Noninvasive Lumbar Intrathecal Injections in Mice

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Abstract

This report describes a step-by-step guide to the technique of acute intrathecal needle injections in a noninvasive manner, *i.e.* independent of catheter implantation. The technical limitation of this surgical technique lies in the finesse of the hands. The injection is rapid, especially for a trained experimenter, and since tissue disruption with this technique is minimal, repeated injections are possible; moreover immune reaction to foreign tools (*e.g.* catheter) does not occur, thereby giving a better and more specific read out of spinal cord modulation. Since the application of the substance is largely limited to the target region of the spinal cord, drugs do not need to be applied in large dosages, and more importantly unwanted effects on other tissue, as observed with a systemic delivery, could be circumvented[1,2]. Moreover, we combine this technique with *in vivo* transfection of nucleic acid with the help of polyethylenimine (PEI) reagent[3], which provides tremendous versatility for studying spinal functions *via* delivery of pharmacological agents as well as gene, RNA, and protein modulators.

Video Link

The video component of this article can be found at http://www.jove.com/video/51229/

Introduction

The spinal cord is a very important center in a variety of key biological processes and physiological functions, including processing and transmission of painful (nociceptive) inputs[4-7]. Various experimental techniques have been developed to facilitate pharmacological modulation of the spinal cord, such as chronic implantation of intrathecal catheters[8], spinal cord microinjection, and intrathecal needle injection[9]. Each technique has its own advantages and drawbacks, and depending on the experiment paradigm one technique might be more suitable than the others. Whereas chronic implantation of intrathecal catheters is readily feasible in rat, this method is very difficult in the mouse, given size restrictions. Success rate is very low and motor deficits often occur due to the bulky presence of a catheter in the severely confined subdural space in the mouse. Moreover, long term delivery of drugs is rendered due to frequent clotting of chronically implanted catheters. Finally, immune reactions are common.

These problems can be circumvented using the method of acute intrathecal injection *via* a needle in the absence of a preimplanted catheter, which enables a fast and anatomically limited application of drugs and reagents to the spinal cords in mice. This method fully retains the benefits of intrathecal delivery over other systemic delivery routes (*e.g.* oral, intravenous, intraperitoneal, *etc.*) such as specificity of spinal modulation, which permits reduced dosages and limit side effects, as well as ability to deliver substances do not normally do not cross the blood brain barrier since during intrathecal injection, the needle is inserted between the dura mater and the spinal cord. Importantly however, in comparison to other methods of intrathecal delivery, the intrathecal needle injection method is the least invasive, allowing numerous applications in the same animal without causing any considerable tissue damage or evoking immune reaction due to implantation of foreign material. However, it requires technical skills for a very precise targeting of the needle to permit efficacy.

Here, we visually demonstrate the method for achieving an optimal rate of success for specifically targeting the lumbar spinal cord. The site of injection that is chosen in this experiment is the groove between L5 and L6 vertebrate column, near to where the spinal cord ends, to minimize the possibility of damaging the spine. Moreover, we demonstrate the use of this technique to knock down genes in the spinal cord using siRNAs.

Protocol

All animal usage procedures were in accordance with ethical guidelines laid down by the local governing body (Regierungspräsidium Karlsruhe, Karlsruhe, Germany).

1. Preparation of siRNA/PEI Complex

The siRNA/PEI complex solution is prepared using manufacturer's directions as follows:

1. Solution A: Dilute the desired amount of siRNA with sterile water (if necessary) up to a quarter of the end volume and dilute this further with 10% glucose solution up to half of end volume. Vortex gently or mix by pipetting up and down. The optimal amount of siRNA needs to be determined empirically but 1 µg siRNA in 10 µl complex solution per animal is a good starting point for optimization.
2. Solution B: Dilute the needed volume of PEI reagent with sterile water up to a quarter of the end volume and dilute this further with 10% glucose solution up to half of the end volume. Vortex gently or mix by pipetting up and down.

   Note: the amount of PEI reagent determines the ionic balance in the complex which influences the efficiency of the transfection. Likewise the optimal amount of PEI solution has to be determined empirically. In our hands, the optimal amount is 0.12 µl of PEI solution per 1 µg siRNA.
3. Mix solution A with solution B all at once, vortex gently.
4. Incubate the mixed solution for 15 min at RT before use. This complex is stable for 2 hr at RT and for 24 hr at 4 °C.

2. Intrathecal Injection

1. Anesthetize the mouse with 3% isoflurane, until it shows no signs of righting reflex. In addition, check for tail and/or paw pinch reflex to further ensure the state of anesthesia.
2. Shave around 2 cm^2 of fur at the posterior end of the animal near the base of the tail to facilitate a better visualization during needle insertion.
3. Place mouse in a nose cone for a continued isoflurane administration during the procedure, reduce the isoflurane to 1.5%, and cover the eyes of the mouse with eye lubricant.
4. Prepare the ready to use siRNA mixed solution using a 25 µl Hamilton syringe attached to a 30 G 0.5 in needle.
5. Locate the spinous process of the L6, which should be the most prominent one and fix the vertebrate column around this area by pressing it gently.
6. Carefully insert the needle between the groove of L5 and L6 vertebrae and observe for a tail flick as this sign indicates a successful entry of the needle in the intradural space.

   Tip: Using fingernail, one should be able to locate the groove as well.
7. Once tail flick is observed, immediately, but carefully, secure the needle position with one hand and inject the desired volume of substance with the other hand slowly.

   Tip: a volume between 5-10 µl is optimal as volume less than 5 µl is unreliable and a volume bigger than 10 µl leads to too much pressure.
8. Once injection is performed, move the mouse back to the cage to recover from anesthesia.
9. Repeat this injection at least 2 more times every 24 hr to achieve optimal downregulation of the targeted gene.

Representative Results

In order to illustrate a successful injection, we performed this technique using Fast Green FCF dye in adult C57Bl6 mice (8-10 weeks of age). The animal was allowed to recover for a few minutes after the injection to provide enough time for the dye to spread and then killed with an overdose of CO_2. Subsequently, the vertebrate column was dissected and the spinal cord was exposed. The blue puncta corresponding to the diffused dye, marked the injection site. No sign of injury to the spinal cord could be seen, confirming the minimally invasive nature of this technique (**Figure 1A**). The injected dye diffused from the injection site rostrally, reaching up to the thoracic region of the spinal cord, only few minutes after injection (**Figure 1B**). Moreover the successful infusion of the dye in the epidural space could be proven by the stained surface of the spinal cord but not the interior (**Figure 1C**).

Furthermore, this technique, combined with PEI reagent kit, enabled a very effective siRNA *in vivo* transfection into the spinal cord. After siRNA intrathecal delivery (3x, once every 24 hr), the expression of the targeted protein (here WAVE1) on lysates derived from dorsal L3-L5 spinal tissue was reduced to around 70% in protein level (**Figure 2A**, n = 20) as well as in mRNA level (**Figure 2B**, n = 6). Moreover a downregulation of a similar magnitude could also be seen in the lysate of the ventral section (**Figure 2C**, n = 4). Interestingly, an even slightly stronger downregulation, was also seen in lysate of cervical spinal cord (**Figure 2D**, n = 4). But, despite the fact that a similar method has been used to downregulate gene in tissues outside the spinal cord[10], in our hands this downregulation was not observed in lysate of the brain (**Figure 2E**, n = 4) nor the DRGs (**Figure 2F**, n = 4).

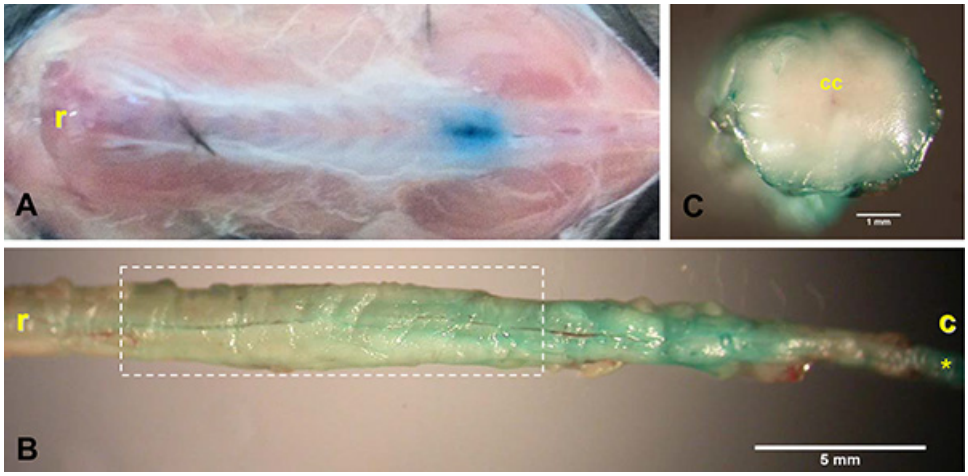

Figure 1. Spinal cord dissected under the microscope following non invasive, acute intrathecal injection with Fast Green dye. A, No visible sign of tissue injury can be seen on the site of injection, marked by the dye puncta. **B**, Excised spinal cord few minutes after injection showing the gradual diffusion of the dye in rostral direction. White box marks the lumbar region, and the star marks the injection site. **C**, Specific staining on the surface of the spinal cords (segment L3-L5), but not the interior of the spinal cord. r = rostral, c = caudal, cc = central canal. Click here to view larger image.

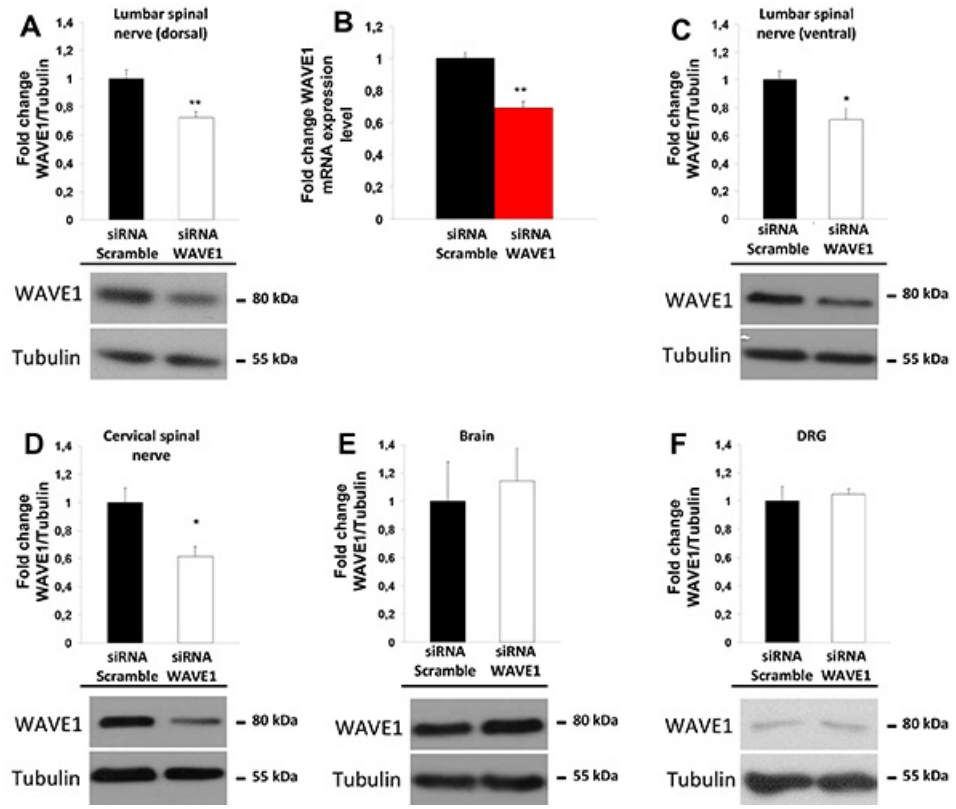

Figure 2. Successful downregulation of the targeted protein (here WAVE1) in the spinal cord after intrathecal siRNA delivery. Mice were intrathecally injected either with control siRNA or siRNA targeted against WAVE1 mixed with PEI reagent for 3 consecutive times every 24 hr; thereafter, the dorsal and ventral section of the lumbar spinal cord (segment 3-5), cervical spinal cord, brain and DRGs were excised, lysed and subjected to western blotting and qRT-PCR. **A-B**, Western blotting (n = 20) and qRT-PCR (n = 6) quantification from lysate of dorsal section of lumbar spinal cord, **C-F** Western blotting quantification from the lysate of ventral L3-L5 spinal cord, cervical spinal cord, brain and DRG respectively (n = 4). Analysis were by unpaired student's t-test (*P≤ 0.05, **P≤0,005). Click here to view larger image.

Discussion

Thus, the above-described technique of intrathecal needle injections is efficacious, fast, specifically-localized, and nondestructive. Technically, the most critical aspect of this procedure is the point of needle insertion into the groove. It is crucial that this procedure is done with very calm

hands and patience. Like many surgical procedures, training improves the rate of successful injection. This is also important because during an actual experiment, this technique does not provide an obvious indicator to directly confirm whether an injection is successful or not. The only visible indicator of correct needle insertion is the tail flick that is observed as a reflex.

This method fully retains the benefits of intrathecal delivery over other systemical delivery routes (*e.g.* oral, intravenous, intraperitoneal, *etc.*), such as specificity of spinal modulation, which permits reduced dosages and limits side effects; furthermore, intrathecal injections enable delivery of substances that are normally do not cross blood brain barrier since during intrathecal injection, the needle is inserted between the dura mater and the spinal cord. Importantly, however, in comparison to other methods of intrathecal delivery, the intrathecal needle injection method is the least invasive, allowing a numerous applications in the same animal without causing any considerable tissue damage or evoking immune reaction due to implantation of foreign material.

Altogether, in this report, we have shown not only the basic step-by-step guide to acute intrathecal needle injection, but also report an example of improvisation of this technique, where in an *in vivo* siRNA transfection and specific gene knockdown in the spinal cord can be achieved. Beside delivery of pharmacological reagents and PEI-facilitated siRNA delivery, the intrathecal needle injection method can also be used to facilitate other types of gene transfer, such as viral-mediated gene delivery[11]. Once sufficient expertise has been gained, this procedure can also be performed without anesthesia in awake, nonanesthetized mice[4,12]. This enables, for example, to study acute effects of pharmacological agents in behavioral paradigms provided mice are preacclimatized to prevent excessive stress.

Thus, acute intrathecal injections constitute a very useful tool for studies on the spinal dorsal horn and the versatility of the technique allows experimenters to customize and improvise to suit their objectives.

Disclosures

The authors declare no competing financial interests.

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