Methods for mechanical delivery of viral vectors into rhesus monkey brain

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ABSTRACT

Background: Modern molecular tools make it possible to manipulate neural activity in a reversible and cell-type specific manner. For rhesus monkey research, molecular tools are generally introduced via viral vectors. New instruments designed specifically for use in monkey research are needed to enhance the efficiency and reliability of vector delivery.

New method: A suite of multi-channel injection devices was developed to permit efficient and uniform vector delivery to cortical regions of the monkey brain. Manganese was co-infused with virus to allow rapid post-surgical confirmation of targeting accuracy using MRI. A needle guide was designed to increase the accuracy of sub-cortical targeting using stereotaxic co-ordinates.

Results: The multi-channel injection devices produced dense, uniform coverage of dorsal surface cortex, ventral surface cortex, and intra-sulcal cortex, respectively. Co-infusion of manganese with the viral vector allowed for immediate verification of injection accuracy. The needle guide improved accuracy of targeting sub-cortical structures by preventing needle deflection.

Comparison with existing method(s): The current methods, hand-held injections or single slow mechanical injection, for surface cortex transduction do not, in our hands, produce the density and uniformity of coverage provided by the injector arrays and associated infusion protocol.

Conclusions: The efficiency and reliability of vector delivery has been considerably improved by the development of new methods and instruments. This development should facilitate the translation of chemo- and optogenetic studies performed in smaller animals to larger animals such as rhesus monkeys.

1. Introduction

Modern molecular tools - chemogenetics, optogenetics, and calcium sensors – have introduced a new level of precision for examining neuronal circuits, both through manipulating and monitoring them (Boyden et al., 2005; Deisseroth et al., 2006; Armbruster et al., 2007; Pei et al., 2008; Alexander et al., 2009; Deisseroth, 2010; Stachniak et al., 2014; Oguchi et al., 2015; López et al., 2016; Stauffer et al., 2016; Pina and Cunningham, 2017; Ju et al., 2018; Choi et al., 2019). Application of these tools has been successful in rodents, especially mice, with a wide spectrum of applications examining the contributions of brain areas, individual pathways and specific cell types for behaviors such as memory, drug seeking and sleep. (Pologruto et al., 2004; Nawaratne et al., 2008; Ferguson and Neumaier, 2011; Sasaki et al., 2011; Bull et al., 2014; López et al., 2016).

Although there is ongoing success, application of optogenetics, chemogenetics, and calcium sensors in rhesus monkey research has proceeded slowly (Han et al., 2009; Heider et al., 2010; Diester et al., 2011; Eldridge et al., 2015; Nagai et al., 2016; Ju et al., 2018; see Galvan et al., 2018 for review of more recent progress in optogenetics). Contributing reasons for the slower adoption of molecular tools in larger mammals include, but are not limited to:

1. The larger size of the rhesus monkey brain presenting a challenge for delivering enough viral particles to discrete regions of cortex or subcortical structures for effective transduction.
2. Inter-species differences in viral tropism and transgene expression (Gray et al., 2011; Gerits et al., 2015; Scheyltjens et al., 2015; Watakabe et al., 2015; Galvan et al., 2019).
3. Off-target effects of chemogenetic ligands and/or their metabolites.
Achieva DStream), (Saunders et al., 1990). All scans were acquired
Mental Health. For subcortical injections, targets were derived from a
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brain that is comparable to expression levels previously achieved in
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4H2O (Mn2+) was co-infused with virus. To achieve
widespread and uniform viral expression in cortical areas, a set of
multi-channel injection devices was constructed that allow slow infu-
sion of large volumes of viral vectors across the targeted tissue(s). To
reduce needle deflections or bending during stereotactically guided
injections to subcortical structures, a needle guide that attaches to the
infusion apparatus was produced to improve mechanical stability. We
show that combining these three innovations produces a more uniform
level of expression of hM4Di across larger target areas within monkey
brain that is comparable to expression levels previously achieved in
rodent studies. All of these developments should be applicable to any
viral construct.

2. Materials & methods

2.1. Surgical procedure

All experimental procedures conformed to the Institute for
Laboratory Animal Research Guide for the Care and Use of Laboratory
Animals and were performed under an Animal Study Proposal approved
by the Animal Care and Use Committee of the National Institute of
Mental Health. For subcortical injections, targets were derived from a
pre-operative T1-weighted scan using a 3-Telsa MR scanner (Phillips,
Achieva DSStream), (Saunders et al., 1990). All scans were acquired
using the coronal orientation with a field of view of 100 × 100 × 60
mm³ and a matrix of 256 × 256 pixels in plane. The slice thickness for
each scan was 1 mm, the bandwidth was 260 Hz/pixel, the repetition
time was 2720 ms, and the echo time was 4.3 ms using a modified




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2.3.2. Lateral array assembly

To target the lateral surface of the cortex a square manifold of $4 \times 4 \times 3$ mm (see Supplemental Fig. 2 for additional detail) is used. Four surface holes with a diameter of 0.46 mm accommodate four 26-gauge hypodermic tubes of 8 mm length. Four 31-gauge needles of 5.5 mm length are inserted into the hypodermic tubing and glued in place. A central hole of diameter 0.65 mm accommodates a 23-gauge wire that attaches to the injection apparatus (Supplemental Fig. 1). The needles are arranged such that a single array placement will result in four separate injections at 2 mm square spacing (Fig. 2). All components are secured to the array using Krazy glue.

2.3.3. Linear-lateral array assembly

To target cortex in sulci on the lateral surface of the brain, a rectangular manifold of $6 \times 2 \times 3$ mm (see Supplemental Fig. 2 for additional detail) is used. Four holes with a diameter of 0.46 mm accommodate four 26-gauge hypodermic tubes of 8 mm length. Four 31-gauge needles of 5.5 mm length are inserted into the hypodermic tubing. A center surface hole with a diameter of 0.65 mm accommodates a 23-gauge wire that secures into the injection apparatus (Supplemental Fig. 1). The holes are arranged such that a single array placement will result in four separate injections at 2 mm linear spacing (Fig. 3). All components are secured to the array using Krazy glue.

2.4. Needle guide for subcortical targeting

A detachable needle guide made of anodized aluminum was built to mount onto the Nanomite pump. It is compatible with any system using $5/16''$ diameter stainless steel dowels. The arm can be sterilized with EtO prior to use. Once mounted, the aperture in the base of the needle guide is aligned to the trajectory of the needle. As the needle is lowered to target, the needle guide sits just touching the skull and the needle

![Fig. 1. Multi-channel array for surface injections into ventral cortex. (A) Components: four needles inserted into the dorsal surface holes, 3D printed base, hypodermic tubing is inserted into the lateral surface (see Supplemental Fig. 2 for additional detail). (B) Computer-Aided Design (CAD) model of the assembled ventral array. (C) Photo of assembled ventral array. The array is connected to the infusion apparatus (see Supplemental Fig. 1) by 10 cm silicone tubing for each channel, which is secured to the array using Krazy glue.](image)

![Fig. 2. Multi-channel array for surface injections of dorsal cortex. (A) Components: 4 hypodermic tubes inserted from above, 3D printed base, 4 needles inserted from below (see Supplemental Fig. 2 for additional detail). (B) CAD model of assembled array. (C) Photo of assembled array. The array is connected to the infusion apparatus (Supplemental Fig. 1) by 5 cm silicone tubing. The center hole (not shown) in the 3D printed base allows for the insertion of 23-gauge wire to stabilize the connection to the infusion apparatus.](image)
Fig. 3. Needle array for injections into sulci. (A) Components: hypodermic tubing inserted from above, 3D printed base, needles inserted from below (see Supplemental Fig. 2 for additional detail). (B) CAD model of assembled array. (C) Photo of assembled array. The array is connected to the infusion apparatus (Supplemental Fig. 1) by 5 cm silicone tubing. The center hole (not shown) in the 3D printed base allows for the insertion of 23-gauge wire to stabilize the connection to the infusion apparatus.

Fig. 4. Needle guide for precise sub-cortical targeting. (A) The pump is mounted on a stereotaxic arm. (B) The needle guide is attached to the arm with a ‘C’-bracket. (C & D) The needle guide aperture allows the needle to pass through. Component dimensions of the foot can be found in Supplemental Fig. 3.
passes through the aperture as it is lowered (Fig. 4C). The syringes were Hamilton (Cole-Parmer, IL) 81008 Gastight Syringes, 100 μL, cemented needle, 30-gauge, 45-degree bevel. Needles were sheathed with TSP polyimide coating (product TSP320450; Molex, IL) to create a reflux resistant step (see Supplemental Fig. 4 for details).

3. Results

3.1. Injection visualization

The contrast reagent, Mn2+, was co-infused with the virus, lentivectorial hM4Di-CFP (titer of 1 × 10^9 i.u./mL), to provide post-operative confirmation that the virus was infused, and that it was targeted to the correct location. At each injection site, where 10 μL was injected at a rate of 0.5 μL/min, a MR signal was visible at the lowest concentration tested, 0.1 mM Mn2+ (Fig. 5B). Higher concentrations produced a higher contrast (as expected), but also made it more difficult to localize the focus of the injection (Fig. 5C). The addition of up to 10 mM Mn2+ did not interfere with the viability of the virus (Supplemental Fig. 6); the toxicity of 10 mM Mn2+ was not assessed in vivo.

3.2. Virus injection apparatus

Cortical injections were performed using a 2 × 2 multi-channel injector for targeting ventral cortex (Fig. 6AI), a 2 × 2 injector for targeting dorsal cortex (Fig. 6BI), and a 4 × 1 injector for targeting intrasulcal cortex (Fig. 6CI). 5 μL per channel was injected into the ventral surface, and 10 μL per channel was injected into the dorsal surface. Accuracy of injections and approximate coverage was assessed with post-operative MRI (Fig. 6, column II). Coverage was assessed at higher resolution (5X magnification) post-mortem with a Diaminobenzidine (DAB) stain; bright-field images reveal the somatic and dendritic expression profiles of each injection type (Fig. 6III). Somatic expression at high resolution (5X magnification) was examined with fluorescence immunohistochemistry under a confocal microscope (Fig. 6IV). A GFP antibody was used to detect CFP expression (Abcam, MA).

3.3. Needle guide

Sub-cortical injections were performed using a single sheathed syringe. The custom-built needle guide (Fig. 4) constrained the path of the needle to maximize accuracy of targeting. MR imaging was used to plan the location of the injection targets (Fig. 7A, B). Virus (lenti-hSyn-hM4Di-CFP) was co-infused with 0.1 mM Mn2+ to allow immediate post-op visualization of needle patency and accuracy of placement (Fig. 7C). Post-mortem visualization of hM4Di-CFP expression using bright field (Fig. 7D) and confocal (Fig. 7E) microscopy confirmed that injections accurately targeted the tail of the caudate nucleus. Contrasting this result with that of a surgery performed in the absence of the needle guide (Supplemental Fig. 5) illustrates the utility of the needle guide.

4. Discussion & conclusions

Achieving efficient and reliable viral vector delivery is critical for increasing the adoption of modern molecular tools in research with rhesus monkeys; we have described three new methods to this end. Co-infusion of the contrast reagent, Mn2+, permits a verification process that is advantageous in primate research. Being off target by even small distances can result in vastly different behavioral and physiological effects. Being able to verify injection accuracy immediately post-operatively provides the means to proceed with assurance that targeting of the injection was as intended. Thus, the likelihood of missing targets due to needle deflection, or slight differences in animal positioning can be minimized.

The use of Mn2+ as a reporter for virus injection and localization allows rapid verification of injection accuracy. Intraoperative MRI is superior, in the sense that it provides feedback during the virus injection, thus permitting adjustments of targeting in real-time (Szerlip et al., 2007). However, the use of a contrast reagent for post-operative imaging overcomes the difficulty of verifying injection accuracy when surgical procedures can only be completed with instruments that are not MR compatible (such as those involving the injector arrays described herein). The longevity of the contrast signal produced by Mn2+ allows injection accuracy to be verified several hours after the end of the procedure; an option that is not reliable when using contrast reagents with a shorter half-life, such as gadolinium. It is known that Mn2+ is toxic at high concentrations (120 mM) (Simmons et al., 2008). However, the concentrations of Mn2+ reported here (< = 5 mM) produced no detectable cell loss, in vivo. We have used 0.1 mM Mn2+ to
visualize injections into other brain regions not reported in this study, including ventral tegmental area, substantia nigra pars compacta, superior colliculus, amygdala, and head of caudate nucleus; we have never observed any reduction in transfection efficiency, cell loss, or other markers of tissue inflammation when using the Mn²⁺. Furthermore, concentrations up to 10 mM caused no loss of virus viability in vitro (Supplemental Fig. 6).

Intracerebral virus injections in monkeys are often performed via visually guided hand-held injections for surface cortex (Eldridge et al., 2015; Upright et al., 2018) or stereotaxic injections of deeper cortical structures (Saunders et al., 1990). Hand-held injections of viral vectors can, in our experience, produce patchy expression in the target region (Eldridge et al., 2015). The use of the multi-channel injector array allowed the needles to remain stable within cortex, while virus was infused slowly. The needles remained situated for several minutes after the end of the injection, to allow residual pressure to dissipate. This approach appears to result in more uniform coverage of the target regions (Fig. 6), particularly for the ventral injector (Fig. 6A). The higher concentration of Mn²⁺ tested, 5 mM, produced an over-estimation of the area transfected (Fig. 6A-II cf. 6A-IV), although this higher concentration more closely approximated the region of dendritic + somatic expression (Fig. 6A-II cf. 6A-III). The lower concentration of Mn²⁺, 0.1 mM, more closely approximated the somatic expression profile of the virus (Fig. 6B-II and 6C-II cf. 6B-IV and 6C-IV). The difference in the density of staining between the DAB (Fig. 6III) and fluorescent images (Fig. 6IV) results from different staining protocols; we routinely visualize the fluorescent sections under high magnification to resolve individual somata, hence a lower density of staining is preferable for this application. As a result, dendritic expression tends to be less clearly visible in the fluorescent sections under lower magnifications (such as that used in Fig. 6).

Using stereotaxic co-ordinates to target subcortical structures is a method that has proven useful in past studies (Saunders et al., 1990). However, mistargeting can occur as a result of deflections, for example if the needle deviates following contact with a blood-vessel, or if the needle slides along the pial surface when inserted at a tangent. Needle deflections can be mitigated by using a guide tube. This approach is only convenient when the subject has a previously implanted chamber to provide access through a cranial window. The needle guide described herein seems to prevent the needle from deviating from the intended track during an acute surgery, providing the precision required to deliver virus to small subcortical structures.

In summary, co-infusion of manganese with the viral vector allowed for immediate post-operative verification of injection accuracy. The multi-channel injection devices increased the efficiency and uniformity of viral delivery to cortical regions of the monkey brain. The needle guide improved targeting accuracy for sub-cortical structures by preventing needle deflection. The combination of these three innovations allowed for the transduction of hM4Di in a reliable and reproducible manner across different regions of the monkey brain. These developments should facilitate the translation of molecular methods used in small animal research to studies with rhesus monkeys.

CRediT authorship contribution statement

J. Megan Fredericks: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing - original draft, Writing -
review & editing. **Kiana E. Dash:** Data curation, Formal analysis, Investigation, Visualization, Writing - review & editing. **Emilia M. Jaskot:** Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing - review & editing. **Thomas W. Bennett:** Data curation, Methodology, Resources, Software, Visualization, Writing - review & editing. **Walter Lerchner:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **George Dold:** Data curation, Methodology, Resources, Software, Supervision, Visualization, Writing - review & editing. **David Ide:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Alexander C. Cummins:** Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing - review & editing. **Violette H. Der Minassian:** Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing - review & editing. **Janita N. Turchi:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing - review & editing. **Barry J. Richmond:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Mark A.G. Eldridge:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

**Declaration of Competing Interest**

No competing interests declared

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**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jneumeth.2020.108730.

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**Fig. 7. In vivo and ex vivo visualization of subcortical viral injection/expression using a needle guide for enhanced accuracy.** Targeting tail of caudate: the pre-op scan of monkey C (A) used to calculate the co-ordinates (B) for targeting of injections. A post-op scan (C) was acquired 8 h after the injection of 10 μL of virus co-infused with 0.5 mM Mn2+. All MR images are skull stripped. (D) Bright field visualization of CFP expression in monkey D. (E) Confocal imaging of CFP expression in monkey D.
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