

● INVITED REVIEW

Methods for the analysis of neuronal plasticity and brain connectivity during neurological recovery

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Abstract

The study of neuronal plasticity under pathological conditions is now a major point of focus on the field of neurological recovery. After the repeated failure of acute neuroprotection strategies for stroke treatment, the design of studies aimed at promoting the reconstruction of neuronal networks has become essential. Methods for the delivery of therapeutic agents on a steady dosage, thus preventing pharmacological peaks or excessive manipulation of experimental animals, are thus required. Additionally, methods that allow the visualization of neurological remodeling processes are fundamental to the understanding of how a therapeutic agent exerts its function. Here we describe how the use of miniosmotic pumps for the steady delivery of such agents, together with tract tracer injections, can be combined to unveil important information on how the brain changes after stroke and how therapeutic agents promote brain remodeling recovery.

Key Words: stroke; blood brain barrier; neuroplasticity; drug delivery

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Introduction

Modeling of neurological diseases or testing of possible treatments can be achieved by administration of chemical or biologically derived agents that can promote a certain response in the brain. For example, Parkinson's disease can be induced by peripheral administration of the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces specific degeneration of dopaminergic neurons projecting from the substantia nigra to the striatum (Hood et al., 2016). On the other hand, many potential therapeutic agents can be peripherally delivered by a variety of methods in order to promote neurological recovery after a lesion has been induced. However, the blood-brain barrier (BBB) often prevents the access of such experimental agents to the brain. One additional inconvenience for peripheral administration of drugs is the physical stress that may be induced to the experimental animal, which can have undesirable effects when performing behavioral testing.

On the other hand, the study of neuronal plasticity and mechanisms to promote it in experimental models with clinical relevance is clearly becoming the focus of intense research. Indeed, after the failure of a large amount of studies that have examined neuroprotective strategies after stroke, the promotion of neuronal plasticity seems mandatory in order to efficiently promote recovery of patients (Hermann and Chopp, 2012). Therefore the development of experimental methodologies that bring together reliable administration

of therapeutic compounds with well-established methods for the study of neuronal plasticity is of paramount importance.

Miniosmotic Pumps for Continuous Drug Delivery

Compounds of interest can be delivered by intravenous injection, which requires previous sedation of the animal, or by subcutaneous or intraperitoneal injection. Intravenous administration can only be applied once and involves severe risk for the animal. By comparison, for subcutaneous or peritoneal administration the animal has to be temporarily immobilized so that administration can be performed without damaging internal organs. However, the stress induced by such manipulation could have repercussions when behavioral testing is planned. One final, but not less important inconvenience of peripheral administration is the fact that injections create a peak concentration of the therapeutic agent that is reduced over time creating the necessity of continuous injections sometimes several times a day in order to maintain sufficiently high plasma concentrations of the agent being tested. Overall this adds to the problem of stress being caused to the animal. Agents can also be delivered by forced feeding or gavaging, or through drinking water. In the latter case an additional problem is created since the total amount of drug that the animal receives cannot be absolutely controlled.

One excellent and very efficient alternative method for

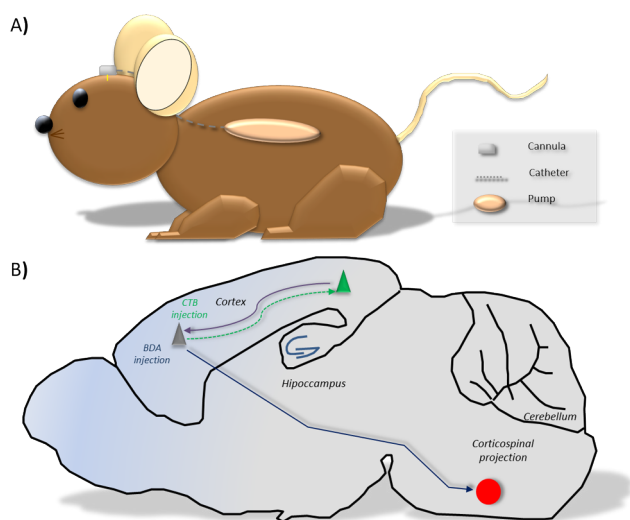


Figure 1 Methodology for the study of neurological remodeling using miniosmotic pumps and tract tracer injections.

(A) Miniosmotic pump implantation for delivery of experimental or therapeutic agents. The miniosmotic pump is implanted in the back of the animal and compounds may be delivered peripherally or directly to a tissue of interest such as the brain by using a catheter (dashed line) and an infusion cannula. (B) Tract tracer injection. Neurons of the motor cortex or other brain nuclei can receive stereotactic injections of tracers. Here the efferent projections from the motor cortex to the spinal cord are indicated by the blue line (BDA injection). Corticocortical projections to the motor cortex are exemplified by an injection of CTB. CTB is retrogradely transported to a cortical area connected to the injection site (green dashed line) thus revealing an afferent connection from the distal site to the injection point (purple line). BDA: Biotin dextran amine; CTB: cholera toxin B.

drug delivery is the use of miniosmotic pumps. These devices (**Figure 1A**) can contain a wide variety of DMSO and water soluble compounds that can be locally or peripherally delivered to the animal at a constant known rate for periods ranging from one to several weeks, and also using substantially less amount of the substance of interest than when systemically delivered (compare Reitmeir et al. (2011) with Pellegrini et al. (2013)). Once filled and implanted, the substance is continuously delivered to the body by absorption of water from the animal, which swells the interior of the pump thus expelling the solution at a fixed known rate. When necessary, the pump is connected through a catheter and delivery cannula which is positioned on the skull thus allowing delivery into the brain and avoiding the BBB (**Figure 1A**). By using stereotactic coordinates it is then possible to deliver the substance of study into a specific area of the brain that might be of interest (Sanchez-Mendoza et al., 2016). Mice quickly recover from the implantation procedure and can perform a wide variety of motor tests without any inconvenience. These pumps have been successfully applied to the study of brain responses to inflammatory stimuli in models of psychiatric diseases (Hoyo-Becerra et al., 2014), experimental studies to unveil new pathways involved in the pathogenesis of Alzheimer's disease (Herring et al., 2016), and to study brain plasticity after stroke (Reitmeir et al., 2011; Wang et al., 2016).

The Study of Neuronal Plasticity Using Anterograde and Retrograde Tract Tracing Techniques

Brain plasticity is a very complex feature of neuronal biology that involves a wide variety of responses from the neurons ranging from brief synaptic alterations to large structural modifications. Therapeutic agents, toxic stimuli or trauma can promote profound tissue alterations that may lead to increase or reduction of dendritic spines, formation of new primary dendrites or sprouting of long distance connections (Reitmeir et al., 2011, 2012; Hoyo-Becerra et al., 2014; Wahl et al., 2014; Herring et al., 2016; Wang et al., 2016). There are a wide variety of methods to study brain connectivity. A remarkable example is the recently developed method of brain clearing, which makes the brain transparent and has been improved to the point that it is now possible to study the fine morphology of neurons in a similar way to the long known Golgi-Cox staining but preventing the preparation of tissue slices (Aoyagi et al., 2015; Costantini et al., 2015), whilst in humans diffusion tensor imaging and functional connectivity MRI are used to finely determine the borders of areas and connections of the brain and to understand how neuronal networks distant to the stroke core modify their interaction during recovery (Carter et al., 2012; Sporns et al., 2005). These are strong methods that together with animal studies similar to ours, might lead to stronger therapeutic design (Silasi and Murphy, 2014). However, to study long distance connections in the mouse, methods should be selected according to the age of the animal. For the developing brain it is possible to use the lipophilic indocarbocyanine dye DiI on brain slices, which are then uptaken by growing axons (Mire et al., 2012). However, these tracers tend to be uptaken by the myelin sheet, thus being unsuitable for the study of adult brains.

Viruses or tract tracer injections are excellent tools to study long distance connections in the adult mouse brain (Lo and Anderson, 2011; Oh et al., 2014; Wahl et al., 2014; Wang et al., 2016). They can enter neurons and are distributed along their structure, thus allowing the study of short and long distance connections. With the use of anterograde or retrograde tract tracers it is possible to study efferent or afferent connections to a particular nucleus of interest, respectively (**Figure 1B**). We and others have successfully used the anterograde tract tracers cascade blue and biotin dextran amine (BDA) to evaluate plasticity of the corticospinal tract after stroke (Li et al., 2010; Reitmeir et al., 2011; Wahl et al., 2014; Wang et al., 2016). Viruses can however expand through the brain and induce toxicity, whereas tract tracers are inert and well tolerated by the brain, thus they can be used in experimentation without specific biosafety measures for the laboratory or adverse effects on the experimental animal. They can be injected by iontophoresis or pressure injections in very little amounts at the surface or deep nuclei of the brain and are stable for long periods of time. The tissular distribution of the tract tracers can be then studied by immunodetection using specific antibodies.

However, there also exist auto-fluorescent retrograde tract tracers, like Fluorogold (FG), which can be excited at 414 nm to deliver a white-blue light that can then be imaged on a confocal microscope (Smith and Alloway, 2010; Sanchez-Mendoza et al., 2016). There have also been generated fluorochrome coupled derivatives of the retrograde tract tracer cholera toxin B (CTB), so that it can be directly visualized by confocal microscopy. The greatest advantage of such coupled CTBs is that different parts of the brain can be injected with the same tract tracer in order to study interconnectivity without having conflict in the analysis of distribution as would be expected from comparing two different tracers (*i.e.*, FG vs. CTB) (Li et al., 2010; Smith and Alloway, 2010). The combined use of retrograde and anterograde tracers allows for the characterization of neuronal networks during neurological recovery.

In our experimental approach for the study of brain remodeling on stroke, an animal is initially subjected to transient middle cerebral artery occlusion and is given three days to recover from the acute phase of stroke. At three days post injury, animals are placed on a stereotactic device and miniosmotic pumps are implanted for either local brain delivery (Reitmeir et al., 2011) or peripheral delivery (Wang et al., 2016) of a therapeutic agent (**Figure 1A**). Several weeks later the pumps are removed once they have surpassed the recommended period of use. At 4-6 weeks after stroke animals receive stereotactic injections of anterograde tract tracers to then analyze sprouting along the corticospinal tract (**Figure 1B**) (Reitmeir et al., 2011; Sanchez-Mendoza et al., 2016; Wang et al., 2016). Both the implantation of pumps and injection of tract tracers require very little time of anesthesia and the technique can be mastered in a relatively short amount of time by an unexperienced experimenter.

Experimental models with clinical relevance require that animals receive sustained administration of therapeutic agents for long periods in a way that does not affect their normal behavior. Additionally, methods of tract tracing allow us to easily uncover pathways of brain connections that could be modified during the recovery process after an injury. Therefore the combination of miniosmotic pumps and tract tracing studies have allowed us to study in depth the reorganization of the brain in models of stroke. Thus, we have been able to extensively characterize the response of the corticospinal tract in response to growth factors and chemical agents that are being used in the regular clinical practice or that are undergoing clinical trials. The delivery of recombinant human erythropoietin or the NMDA receptor inhibitor memantine with the use of miniosmotic pumps for periods of approximately 30 days, was shown to promote neurological recovery and sprouting of the corticospinal tract at the level of the red and facial nucleus as evaluated by tract tracer analysis (Reitmeir et al., 2011; Wang et al., 2016). Therefore, this is a combined methodology with great application possibilities for the study of neurological recovery after stroke that could possibly be applied to traumatic brain injury or neuronal degeneration paradigms in addition to stroke.

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Conflicts of interest: None declared.

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