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BRAIN RESEARCH

Research Report

High-resolution neuroanatomical tract-tracing for the analysis of striatal microcircuits

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ABSTRACT

Although currently available retrograde tracers are useful tools for identifying striatal projection neurons, transported tracers often remained restricted within the neuronal somata and the thickest, main dendrites. Indeed, thin dendrites located far away from the cell soma as well as post-synaptic elements such as dendritic spines cannot be labeled unless performing intracellular injections. In this regard, the subsequent use of anterograde tracers for the labeling of striatal afferents often failed to unequivocally elucidate whether a given afferent makes true contacts with striatal projections neurons. Here we show that such a technical constraint can now be circumvented by retrograde tracing using rabies virus (RV). Immunofluorescence detection with a monoclonal antibody directed against the viral phosphoprotein resulted in a consistent Golgi-like labeling of striatal projection neurons, allowing clear visualization of small-size elements such as thin dendrites as well as dendritic spines. The combination of this retrograde tracing together with dual anterograde tracing of cortical and thalamic afferents has proven to be a useful tool for ascertaining striatal microcircuits. Indeed, by taking advantage of the trans-synaptic spread of RV, different subpopulations of local-circuit neurons modulating striatal efferent neurons can also be identified. At the striatal level, structures displaying labeling were visualized under the confocal laser-scanning microscope at high resolution. Once acquired, confocal stacks of images were firstly deconvoluted and then processed through 3D-volume rendering in order to unequivocally identify true contacts between pre-synaptic elements (axon terminals from cortical or thalamic sources) and post-synaptic elements (projection neurons and/or interneurons labeled with RV).

1. Introduction

Charting the complex connections of the central nervous system is fundamental to understand how brain networks functions. Remarkable advances have been made in this field in recent decades due to the expansion of neural tracing paradigms. Anterograde tracers such as *Phaseolus vulgaris*-leucoagglutinin (PHA-L; Gerfen and Sawchenko, 1984) and

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biotinylated dextran amine (BDA; Veenman et al., 1992) provide a proper elucidation of the pre-synaptic element. However, the accurate visualization of the post-synaptic element remained elusive by means of retrograde tract-tracing methods. When injected in a given brain area, retrograde tracers are taken up by axon terminals innervating this area and retrogradely transported to the parent cell body (for a review, see Lanciego and Wouterlood, 2006). When relying on "classical" retrograde tracers, the main caveat is that the transported tracer remains restricted to the cell body and to the thickest, main dendrites, without significant labeling of neuronal processes located far away from the parent cell body such as thin dendrites and dendritic spines. This is also a common drawback when using retrograde tracers with a different nature such as for example several strains of trans-synaptically transported viruses (Geerling et al., 2006), and as such, it represents a major limitation for the detailed analysis of anatomical interactions.

In summary, there is a clear need for a tracer that meets several demands, including: (i) to allow complete visualization of the labeled neurons, including the full dendritic tree as well as small structures such as dendritic spines, (ii) to be transported exclusively in the retrograde direction, therefore allowing the accurate identification of the brain areas targeted by labeled neurons and (iii) to be compatible with other existing tools for tract-tracing. When searching the technical arsenal currently available, it becomes evident that rabies virus (RV; Challenge Virus Standard strain) does approach the ideal. When compared to other commonly used trans-synaptically transported viruses such as the herpes viruses, rabies virus exhibit higher efficiency for retrograde tracing and low cytopathicity within infected cells (Ugolini, 1995). Furthermore, the lack of viral transport through fibers of passage also represents an additional advantage (Ugolini, 1995; Kelly and Strick, 2000; Nassi and Callaway, 2007). Indeed, the trans-synaptic spread of the virus is a useful strategy for the analysis of neuronal microcircuits, and this specially holds true in brain areas such as the striatum, in which interneurons play key roles in modulating the activity of projection neurons (Kawaguchi et al., 1995). More recently, a deletion-mutant version of RV lacking the glycoprotein responsible for viral neuronal tropism became available to study the morphology and physiology of projection neurons (Wickersham et al., 2007a). The virus incorporates the glycoprotein in its envelope using a transcomplementation strategy, so that it can infect neurons projecting to the injection site but it cannot spread beyond the infected cells. Furthermore, a trans-synaptic tracer based on RV able to cross only one synapse beyond the primary infected cells has been designed for *in vitro* studies in brain slices. This provides an elegant technique to label neurons that project to a single, genetically targeted synapse (Wickersham et al., 2007b). To date, RV has been essentially used to trace multi-synaptic circuits and it is generally detected with antibodies against the RV nucleoprotein, which provides labeling restricted to the cell body and the proximal dendrites.

Here, we designed a protocol in which RV is used as a tool to study morphology and connectivity of neurons identified from their projection site (e.g., first-order neurons). Viral detection is accomplished by relying on a mouse monoclonal antibody against a viral phosphoprotein present in the whole cytoplasm of the RV-infected neuron (31G10 isolated during the fusion experiment described by Raux et al., 1997), therefore enabling the unambiguous characterization of the post-synaptic element. The conducted procedure for viral detection is fully compatible with existing tools for anterograde tract-tracing neurons, which represents its main added value. It is combined in the present study with anterograde axonal tracing with PHA-L an BDA followed by multiple fluorescence labeling. By this combination, the inputs to a specific retrogradely-labeled population of efferent neurons and hence connected interneurons (first-order and second-order neurons, respectively) can be analyzing qualitatively and quantitatively under the confocal microscope. Structures displaying labeling were visualized under the confocal laser-scanning microscope. The subsequent use of powerful post-acquisition image software resulted in the unequivocal visualization of the pre- and the post-synaptic elements with an unprecedented level of detail.

Fig. 1 – Injection sites and Golgi-like labeling obtained with retrograde tracing using rabies virus. (A) Low-magnification picture showing the deposit of rabies virus (RV) in GP. Epifluorescent illumination. Scale bar = 1 mm. (B) Iontophoretic delivery of the anterograde tracer *Phaseolus vulgaris-leucoagglutinin* (PHA-L) at the level of the parafascicular nucleus of the thalamus (PF). Epifluorescent illumination. For reference purposes, the position of the *fasciculus retroflexus* (fr) and the boundaries of the subthalamic nucleus (STN) are delineated. Scale bar = 1 mm. (C) Delivery of the anterograde tracer biotinylated dextran amine (BDA) in the cortical area M1. Injected BDA was visualized by an HRP-coupled streptavidin followed by a DAB-Ni precipitate. Scale bar = 1 mm. (D and E) Comparison between two different ways to visualize striatopallidal medium-spiny neurons using the retrograde tracer Fluoro-Gold (D) or Rabies Virus (E). Fluoro-Gold is currently the first choice retrograde tracer to identify projection neurons that innervate a given brain area to which the tracer is stereotaxically delivered. The main caveat of using Fluoro-Gold (as well as any other of the retrograde tracers available so far) is that the transported tracer is restricted to the parental cell body and the thickest dendrites. By contrast, rabies virus offers better performance. Instead of using antibodies directed against viral genome-associated proteins, our protocol involves the use of a primary monoclonal antibody that binds to a form of the viral phosphoprotein present in the whole cytoplasm of the infected neuron. With this strategy, the entire dendritic tree can be visualized by immunofluorescence and even the thinnest dendrites and dendritic spines are clearly labeled (as shown for striato-pallidal neurons in panels F and G). To the best of our knowledge, this is the first time in which the post-synaptic elements of a given population of projection neurons are revealed at this unprecedented level of detail. Scale bar is 60 μm for D and E and 120 μm for F and G. (H–I) Any other type of projection neuron innervating the area of RV deposit also exhibited Golgi-like labeling, such as STN neurons projecting to GP (H), thalamo-pallidal projecting neurons (I; a projection initially described by Kincaid et al., 1991), as well as neurons within the raphe nuclei and the pedunculopontine nucleus innervating the SNr (panels J and K, respectively). Scale bar for panels H–K = 40 μm.