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Can transsynaptic viral strategies be used to reveal functional aspects of neural circuitry?

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ABSTRACT

Viruses have proved instrumental to elucidating neuronal connectivity relationships in a variety of organisms. Recent advances in genetic technologies have facilitated analysis of neurons directly connected to a defined starter population. These advances have also made viral transneuronal mapping available to the broader neuroscience community, where one-step rabies virus mapping has become routine. This method is commonly used to identify inputs onto defined cell populations, to demonstrate the quantitative proportion of inputs coming from specific brain regions, or to compare input patterns between two or more cell populations. Furthermore, the number of inputs labeled is often assumed to reflect the number of synaptic connections, and these viruses are commonly believed to label strong synapses more efficiently than weak synapses. While these maps are often interpreted to provide a quantitative estimate of the synaptic landscape onto starter cell populations, in fact very little is known about how transneuronal transmission takes place. We do not know how these viruses transmit between neurons, if they display biases in the cell types labeled, or even if transmission is synapse-specific. In this review, we discuss the experimental evidence against or in support of key concepts in viral tracing, focusing mostly on the use of one-step rabies input mapping and related methods. Does spread of these viruses occur specifically through synaptic connections, preferentially through synapses, or non-specifically? How efficient is viral transneuronal transmission, and is this efficiency equal in all cell types? And lastly, to what extent does viral labeling reflect functional connectivity?

1. Brief history of neural circuit mapping and development of one-step viral methods

The brain consists of a highly complex network of neurons which transmit information largely via synaptic connections. The understanding of how neurons are connected is thus critical to understanding both the basic and emergent properties of neural circuits. Though the advent of the microscope enabled an appreciation of the cellular structure of most tissues, the brain remained recalcitrant to study, in large part because thin sections yielded gray blobs of tissue that showed little about the organization of the brain. It was only through the work of Camillo Golgi and Ramón y Cajal that the Neuron Doctrine was established, which put forth the idea that the nervous system was made of discrete cells. This was facilitated by the advent of the Golgi staining method, which enabled Ramón y Cajal and others to detail the exquisite morphological details of individual neurons for the first time (Ramón y Cajal and Léon, 1909). Though this technique was groundbreaking, it requires staining whole blocks of tissue at once, stains neurons non-selectively, and requires thin sections of stained tissue to be imaged. It is thus not suitable for tracing long-distance connectivity between brain regions, nor for mapping connections in a targeted fashion.

These barriers were first overcome in 1948 by Paul Weiss and Helen Hiscoe (Weiss and Hiscoe, 1948), who demonstrated the transport of cellular components from the cell body into axonal processes. Later, axonal transport was used to label axon terminals with radioactive amino acids taken up by the cell body (Ochs et al., 1962; Taylor and Weiss, 1965; Lasek et al., 1968). For the first time, outputs of cells located in a defined brain region could be labeled without first

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significantly damaging the tissue. In an additional advance, Kristensson and colleagues demonstrated retrograde transport of the horseradish peroxidase (HRP) protein from the axon to the cell body (Graham and Karnovsky, 1966; Kristensson and Olsson, 1971; Kristensson et al., 1971). This method was more sensitive than previous approaches, as the catalytic properties of HRP enabled amplification of signals in labeled cells. Also, since HRP forms a visible reaction product with benzidine, several different histochemical methods can be used to label HRP-containing neurons, including those using benzidine hydrochloride or tetramethyl benzidine as a substrate (Mesulam and Rosene, 1979). Further improvements were made by conjugating HRP with plant lectins such as wheat germ agglutinin (WGA) which increased axonal uptake and retrograde transport (Gonatas et al., 1979; Gonatas and Avrameas, 1977). Though the lack of toxicity and increased sensitivity were both improvements on previous methods, HRP-WGA labeled both inputs (retrograde) and outputs (anterograde) of neurons near where the tracer was injected. In addition, spread could occasionally be observed to secondary neurons, which could either be a benefit or a drawback. Since this time, a number of retrograde tracing strategies have been developed, including several fluorescent molecules such as propidium iodide, Nuclear Yellow, and Fluoro-Gold, which are efficiently retrogradely transported from axons to cell bodies (Kuypers et al., 1979; Kuypers et al., 1977; Bentivoglio et al., 1980; Schmued and Heimer, 1990). Multiple strategies are available for anterograde tracing as well, such as the plant lectin Phaseolus vulgaris PHA-L (Gerfen and Sawchenko, 1984) and biotinylated dextran amine (BDA) (Veenman et al., 1992). Additionally, the lipophilic DiI (Honig and Hume, 1986; Honig and Hume, 1989; Sims et al., 1974) has been used widely to trace axonal tract projections. While these approaches enable identification of neuronal populations projecting to or from a targeted brain region, they all lack cell type-specificity, in some cases lack directional specificity, and those that can transmit transneuronally such as WGA do not amplify, leading to diminution of signal with distance. Though here we lump molecularly distinct methods into common categories for simplicity, each method has its unique pros and cons, which have been reviewed more extensively elsewhere (Lanciego and Wouterlood, 2011; Wouterlood et al., 2014).

Viruses have in large part solved many of these shortcomings. Herpes simplex virus (HSV) and related family members were observed almost a century ago to transmit along nerve tracts (Goodpasture and Teague, 1923) and were the first viruses to be used to trace neural circuits. Unlike small molecular dyes, viruses replicate in second-order neurons and can therefore self-amplify. This enables robust labeling across multiple neuronal connections. Kristensson showed that HSV can spread along chains of neurons in both the retrograde and anterograde directions (Kristensson, 1970). Viral spread proceeds quickly from the initial inoculation site to higher order neurons. Despite its strengths, this approach has two key limitations: one, the virus can spread to inputs *and* outputs, and it can be difficult to discern if a given labeled cell is an input or output of the primary cell(s). Two, the virus can transmit along chains of connected neurons, and thus the presence of viral labeling cannot unambiguously delineate the order of connectivity. Early studies which traced the inputs to various muscles could mitigate this problem as the initial transfer could only be retrograde (muscle to motor neuron to motor input), and by quickly sacrificing the animal, uninterpretable multi-step viral transmission can be largely avoided. An example of this approach was illustrated by Rouiller and colleagues through combining HRP and pseudorabies (PRV) labeling of motor neurons and their inputs. In these experiments, HRP and PRV-labeled neurons were defined as first-order neurons, and neurons labeled by PRV but not HRP were defined as second-order neurons. To limit the extent of PRV spread, rats were sacrificed as soon as they started to show somatic signs of infection (Rouiller et al., 1989). In order to conduct circuit mapping using these polysynaptic viruses, careful titration of infection timing is essential (Kuypers and Ugolini, 1990).

HSV and similar viruses such as PRV were considered to spread via synaptic connections, largely based on the observation that the majority of viral spread is consistent with known anatomical connectivity. However, it is important to note that for these viruses, the synaptic specificity of transmission, relative to transmission to nearby non-connected neurons, has not been rigorously tested, or at least has not been publicly reported, and non-specific spread to surrounding non-neuronal cells such as glia is also observed (Fournier et al., 1977; Vahlne et al., 1978; Kristensson et al., 1979). In addition, some viral strains exhibit biases in their direction of transport. For example, the Bartha strain of PRV appears to exclusively transmit between neurons in the retrograde direction (Lomniczi et al., 1987; Strack and Loewy, 1990), while other viruses such as the HSV H129 prefer anterograde transneuronal transmission (Zemanick et al., 1991; Lo and Anderson, 2011). In the latter case, retrograde transmission has also been observed in multiple studies (Archin and Atherton, 2002a; Archin and Atherton, 2002b). In addition, most HSV/PRV recombinants are highly cytotoxic (with a few exceptions (Oyibo et al., 2014)), and their large genomes make genetic engineering relatively slow and laborious.

Due in part to these limitations, the rabies virus (RABV) has emerged as the most widely used vector for transneuronal labeling. Wild-type RABV was originally used to map circuits, using a careful titration of timing to construct circuit diagrams (Ugolini, 1995; Kelly and Strick, 2000). Although comparing cells labeled at different time points following viral injection can be used as an indicator of the degree of connectivity between labeled neurons in a circuit, it cannot unambiguously define connectivity relationships nor can it differentiate strong indirect inputs from weak direct inputs (Ugolini, 1995b). RABV spread appears to be retrograde-specific, with a few exceptions in the dorsal root ganglia (Tsiang et al., 1989; Zampieri et al., 2014; Bauer et al., 2014); however, its rapid transneuronal transmission and its ability to infect humans prevent the widespread use of wild-type rabies (Callaway, 2008). A major breakthrough occurred in 2007 when a genetically engineered version lacking the viral glycoprotein (Mebatsion et al., 1996) was used to enable single step-restricted viral spread (Wickersham et al., 2007). Rather than relying on a temporal analysis of the neurons labeled a given number of days after infection, by having an essential gene removed from the viral genome and having it provided only in select cell types, the virus could only label neurons projecting directly to the "starter" neurons expressing the glycoprotein, delivered via transfection or delivery via a different virus. This approach also allows for cell type-specificity, as RABV can be targeted to specific cell types by virtue of expression of an exogeneous viral receptor such as TVA on the cells of interest and the use of RABV pseudotyped with exogeneous glycoproteins, such as ASLV-A. Importantly, electrophysiological results in cortical slice cultures showed that 9 out of 11 virally-labeled cells were synaptically connected to labeled starter cells, suggesting that the virus transmits predominantly to synaptically-connected neurons (Wickersham et al., 2007). The fact that this virus cannot spread on its own has enabled its widespread use in neuroscience labs to map neuronal connectivity. In this review we will critically evaluate the use of transneuronal viral technologies for mapping neural circuits. We will focus principally on the use of one-step RABV input mapping, highlighting other viral technologies to emphasize critical or common features.

2. What is the evidence that viruses label synaptically-connected populations?

RABV and HSV/PRV are commonly assumed to spread through synaptic connections, and this assumption is rarely questioned. The original assertions about the transsynaptic preference of viral transmission can be traced back to an observational study by Ernest Goodpasture and Oscar Teague in 1923, where HSV was transferred from the lip of a human patient onto a rabbit's cornea, where it transmitted via the optic nerve to the brain and produced encephalitis (Goodpasture and Teague, 1923). RABV and HSV/PRV have been noted to label both

direct, weak connections and indirect, strong connections (Ugolini, 1995b). A commonly held assumption is that these viruses can label second-order neurons in a relatively unbiased fashion. However, it is worth revisiting and critically assessing the data in support of or against each of these assumptions.

Viral spread along known anatomical pathways forms the basis for the assumption that viral transneuronal transfer has a transsynaptic preference. This premise is inherently problematic; is viral spread to unexpected populations indicative of previously unknown synaptic connections, or of non-synaptic spread of virus? In some cases this has led to the discovery of new circuits, while in others the results have not been explained. In one example, the specificity of retrograde transmission was used to explain an enigmatic result whereby injection of a supposedly retrograde-specific PRV Bartha into one eye resulted in retinal ganglion cell labeling in the opposite eye (Pickard et al., 2002). This was first believed to have occurred through anterograde transmission from retinal ganglion cells along the optic nerve in the injected eye to retinorecipient regions, and subsequent retrograde transmission to the contralateral eye. However, this was inconsistent with the supposed retrograde specificity of this virus. A more thorough investigation found that the PRV Bartha traveled through autonomic circuits innervating the eye to retinorecipient regions, whereby it transmitted to the contralateral eye. These results are thus a classical example of the virus providing new information about unexpected connectivity. In a second example, using RABV in mice we identified unexpected inputs from lateral habenula (LHb) neurons onto ventral tegmental area dopamine neurons (VTA-DA) projecting to the nucleus accumbens lateral shell (NAcLat), a projection not hypothesized by previous work (Lammel et al., 2012; Beier et al., 2015). We will discuss the implications of this work in the following section. We also identified novel connections from starburst amacrine cells onto alpha ganglion cells in mice using the vesicular stomatitis virus (VSV), and Viney and colleagues identified novel inhibitory connections from monostratified amacrine cells onto intrinsically photosensitive retinal ganglion cells using PRV152 (Viney et al., 2007; Beier et al., 2013). In these cases, the presence of direct synaptic connections was confirmed using slice electrophysiology. However, other observations are more difficult to reconcile with synaptic transmission. For example, after injecting VSV into the nostril of young mice, while the pattern of transmission in the olfactory bulb was consistent with anterograde transsynaptic spread, we observed viral transmission into and along the rostral migratory stream (RMS) (Beier et al., 2011). The RMS is composed of neuronal progenitor cells migrating from the subventricular zone to the olfactory bulb. These cells are thought to make transient contacts, though whether synaptic-like contacts occur among these cells is not clear.

In general, the extent of spread to synaptically-connected neurons vs. nearby neurons based on proximity alone is not clear. The principal evidence for a synaptic mechanism of spread is that most viral labeling along defined neuroanatomical tracts is consistent with known connectivity. In support of this mechanism, simultaneous pre-synaptic uptake was observed of RABV virions budding from postsynaptic surfaces (Charlton and Casey, 1979). However, in vivo transmission of virions through a synapse-only route to mediate infection has not been observed. The relatively low level of glia labeling, especially at early time points post-viral injection, has been used as evidence in support of spread through synapses. Preferential viral spread to neurons could also be due to selective tropism or reduced infectivity of glial cells. However, transmission to glial cells has been noted to occur for all neurotropic viruses, including HSV (Fournier et al., 1977; Vahlne et al., 1978; Kristensson et al., 1979; Ugolini et al., 1987), PRV (Card et al., 1990; Rinaman et al., 1993), VSV (Plakhov et al., 1995; Chauhan et al., 2010; van den Pol et al., 2002), and RABV (Ray et al., 1997; Potratz et al., 2020; Pfefferkorn et al., 2016). This labeling undermines the absolute synaptic specificity of viral spread. The question then is whether the spread we observe is preferentially through synapses, or simply to cells with nearby processes.

3. How efficient is viral transmission? Do viruses label input cells equally?

An important consideration in any viral transneuronal experiment is the percentage of total input cells to the cell population of interest that get labeled by the virus. This number is difficult to discern when tracing inputs onto populations of starter neurons but is feasible when examining inputs onto single cells. The first study to provide this estimate was from Marshel and colleagues, who performed RABV one-step input tracing experiments from single layer 2/3 cortical neurons (Marshel et al., 2010). In this work, each starter cell gave rise to approximately 48 input neurons. As a layer 2/3 cortical pyramidal neuron receives input from ~1000 neurons at the age tested, the authors estimated that RABV was labeling ~5% of input neurons. Two newer studies performing a similar experiment in vivo reported a higher efficiency, with ~10 % and 40 % reported in the Rossi and Wertz studies, respectively (Wertz et al., 2015; Rossi et al., 2019). Given that the experiments are fundamentally similar, the reasons for this discrepancy are not clear, though it may reflect the longer incubation time used in the Wertz and Rossi studies (up to 11 days or two weeks post-RABV injection, versus up to 5 days in the Marshel study). Of note, we regularly terminate RABV input mapping experiments after 5 days post-RABV injection because while we indeed see more putative input neurons labeled with longer incubation times, the number of labeled glia cells increases rapidly with time (unpublished observations). In a third study sampling inputs from populations of layer 2/3 neurons instead of single cells, the ratio of total inputs/starter cells was approximately 50:1, also suggesting an input labeling efficiency near 5% (DeNardo et al., 2015). A fourth, unrelated study with a different virus in a different circuit used VSV mapping in the retina to estimate the efficiency of transneuronal labeling. We used a modified VSV tracing strategy to label starburst amacrine inputs onto direction-selective retinal ganglion cells (Beier et al., 2013). Given that each direction-selective ganglion cell is thought to receive input from ~200 starburst amacrine cells and we observed 4–5 labeled starburst amacrine cells labeled near each direction-selective ganglion cell after 2 days post-infection, we estimated a transmission efficiency of roughly 2–3 %. This number may be slightly lower than results obtained for RABV due to results being quantified after 2 days post-infection, rather than the 5 or more days used in the RABV studies. Therefore, while a range of values have been reported, most studies suggest that RABV and other transneuronal viruses label a minority of the total inputs to a given cell. However, it is important to note that there are many variables in any such experiment, including the expression of viral helper genes, number of cells expressing the helper genes, age of animal, incubation time, etc. Therefore, while the reported numbers provide a frame of reference, how various factors contribute to the efficiency of viral spread, individually or in combination, is not known, but is beginning to be explored (Lavin et al., 2020).

Another unanswered question is whether the virus can transmit via all synapses equally well. If only a small portion of the input population is being sampled in any given experiment, it is important to know whether labeling is an accurate representation of the actual synaptic connectivity or if it is biased towards particular cell types. Given that viruses use cellular receptors to gain access to cells and that these receptors are not all expressed equally in all cells, whether or not the viral receptor(s) is expressed on presynaptic cells may influence input labeling efficiency. For example, RABV has affinity for three known receptors: the nicotinic acetylcholine receptor, NCAM, and p75NTR (Lentz et al., 1982; Thoulouze et al., 1998; Tuffereau et al., 1998). However, each is sufficient but not necessary for viral infection. Thus which, if any, of these receptors are engaged in vivo is not known. Additionally, the topology of synapses may influence the efficiency of RABV transmission. For example, many GABAergic inputs are located proximally to the cell body, while excitatory inputs connect onto spines located more distally on the dendrites. However, identifying potential biases requires a detailed quantification of the numbers of synapses from excitatory and

inhibitory neurons onto defined starter cell populations in order to compare synapse numbers to inputs labeled by RABV. While a handful of such quantifications have been completed, they were not done on the same cell types that have been used as starter populations for RABV input mapping. Therefore, the possibility for biased transneuronal transmission remains an open question. Also, cells receive neuromodulatory inputs, many of which do not form classic synaptic contacts. Therefore, RABV may not transmit well to neuromodulatory connections such as from the dopaminergic nigrostriatal pathway. Indeed, one study compared RABV spread to midbrain DA inputs either from two different populations of medium spiny neurons in the dorsal striatum (when EnvA-pseudotyped RABV was injected into the striatum, infected medium spiny neurons, and spread to their inputs), or from G-deleted RABV containing its own envelope protein, RABV-G, injected directly injected into the striatum. The authors found that RABV one-step transmission to DA neurons from medium spiny neurons occurred at a much lower frequency than when the RABV-G-enveloped virus was injected into the striatum (Wall et al., 2013). These results suggest that transmission to neuromodulatory inputs may occur less frequently than to neurons making more conventional contacts, principally synapses. Note that reduced spread to neuromodulatory inputs does not distinguish synaptic vs. non-synaptic spread, it only suggests that spread to input cells that make non-canonical contacts onto starter cells may occur at a lower efficiency.

3.1. Limitations in interpreting inputs to cell populations

One-step RABV tracing is typically conducted from populations of cells defined by location in the brain, expression of a recombinase protein (e.g., Cre or Flp), and/or projection site. Therefore, the inputs labeled represent a composite of inputs to individual cells within the starter population. It is not known if the inputs labeled equally innervate all starter neurons, if they innervate only a subset of neurons, or somewhere in between. One study from Schwarz and colleagues attempted to use results from brains with sparse RABV input labeling to perform a simulation of the input distribution to each norepinephrine cell in the locus coeruleus (Schwarz et al., 2015). They estimated that each norepinephrine neuron receives a minimum of one input from at least 9 brain regions, but that the regions from which innervation is received are likely heterogenous. However, this estimation has two key limitations; one, RABV labels only a small fraction of the total inputs to starter cells, a caveat discussed above and acknowledged by the authors. Another limitation is that this simulation requires a fixed number of starter cells, which were quantified by visible expression of an mCherry fluorophore tagged to TVA, the receptor for EnvA-pseudotyped, G-deleted RABV. However, only a small amount of TVA protein is required for RABV entry (Gray et al., 2011). Therefore, it is possible that there may be starter cells that were not visibly expressing the mCherry protein. This limitation applies to most RABV studies where starter cells are quantified by expression of a fluorescent tag. The best way to ensure an accurate count of starter cells is to introduce TVA/RABV-G in a targeted fashion to each cell, for example through single cell electroporation (Marshel et al., 2010). Though this has been done for single cells, it is a highly laborious and low-throughput procedure not suited for rapid whole-brain quantification of inputs to targeted cells.

The fact that most experiments using one-step RABV are conducted on a population rather than single, defined cells makes it difficult to interpret the functional consequence of observed labeling patterns. For example, we and others demonstrated that RABV tracing shows that the LHb provides a quantitatively equal input to ventral tegmental area dopamine (VTA-DA) and midbrain GABA neurons (Beier et al., 2015; Watabe-Uchida et al., 2012; Faget et al., 2016). The LHb input comprised 5% of the total labeled inputs onto these cells. We also showed that the LHb provides ~5% of total inputs onto four different output-defined VTA-DA cell populations, including those that project to the nucleus accumbens lateral shell (NAcLat) that are involved in reward behaviors (Lammel et al., 2012). This connection was thought to be weak to non-existent based on a previous study, as tested through electrophysiological recording of 4 neurons (Lammel et al., 2012). Stimulation of the LHb is also highly aversive, likely through strong innervation of GABA neurons in the ventral midbrain (Matsumoto and Hikosaka, 2007; Ji and Shepard, 2007; Christoph et al., 1986). However, by conducting electrophysiological recordings from 27 neurons we were able to detect a weak but direct input from the LHb onto VTA-DA-NAcLat neurons (Beier et al., 2015). Thus, though RABV tracing data suggests that 5% of the inputs onto VTA-DA-NAcLat neurons arise from the LHb, electrophysiological and behavioral analyses suggest that this connection is functionally weak. In fact, RABV labeling in our study suggested that the LHb provides a ~3x larger input to VTA-DA-NAcLat neurons than the laterodorsal tegmentum (LDT), which is thought to provide a much stronger synaptic and functional input to these neurons (Lammel et al., 2012). Although the observation that LHb neurons are heterogeneous in their projections to the midbrain provides a potential explanation (Wallace et al., 2020; Maroteaux and Mameli, 2012; Li et al., 2011), population mapping using one-step RABV, even to defined subsets of neurons in midbrain, cannot alone be used to infer functional relationships between different circuit elements.

4. To what extent does viral mapping reflect functional connectivity?

The premise of neuroanatomy is that an understanding of neural connectivity is *necessary* for understanding brain function. However, it is clear that even a detailed understanding of connectivity is not *sufficient*. An important limitation of viral tracing is that the extent to which viral labeling reflects functional aspects of neuronal connectivity has not been thoroughly explored. One significant barrier is that for most circuits in the brain, we lack a rigorously validated connectivity map of the inputs and outputs of cell types. Without a "ground truth" of what the connectivity actually is, it is difficult to assess the performance of transneuronal viruses, and if the maps generated in this way can be used to infer circuit function. In fact, there are multiple pieces of evidence that RABV one-step input maps are insufficient to predict circuit function, several of which we will present here.

4.1. Using the somatosensory cortex as a model circuit

A precise and detailed understanding of the connectivity between neurons in the brain is required to assess factors that influence RABV spread. In terms of input-output connectivity, cortical microcircuits represent perhaps the best-studied circuit in the rodent brain. Though RABV spread is typically assumed to be proportional to the number of synaptic connections, this has never been shown. Karel Svoboda provided an analysis of this topic and the potential for non-synapse-specific RABV spread (Svoboda, 2019

). For example, DeNardo and colleagues examined inputs to excitatory neurons in different cortical layers in the somatosensory barrel cortex, and conducted electrophysiological recordings to validate several of these connections (DeNardo et al., 2015). However, the proportion of RABV-labeled inputs to these different cell types is not in quantitative agreement with measurements from a separate electrophysiological study, which performed paired recordings between excitatory neurons in different cortical layers (Lefort et al., 2009). For example, DeNardo and colleagues reported a relatively high proportion of layer 3 inputs to layer 6 starter cells, though that connection has been shown to be functionally weak (DeNardo et al., 2015; Lefort et al., 2009; Hooks et al., 2011). Additionally, RABV tracing in the DeNardo study identified few or no inputs from layer 1 inhibitory neurons to any deeper layer excitatory cells, despite the fact that layer 1 has been shown to provide direct inhibition onto layer 2/3 pyramidal cells (Cruikshank et al., 2012; Schuman et al., 2019). This same input from layer 1 inhibitory neurons onto layer 2/3 excitatory pyramidal cells was also

not captured in a more recent study (Yetman et al., 2019), suggesting this false negative may be a limitation of RABV one-step labeling.

In addition to the question of whether RABV labeling reflects functional connectivity, the extent of synaptic specificity vs. transmission to passing neurites is not clear. Electron microscopy studies have demonstrated that projections from nearby neurons are highly intertwined without regard to their functional connectivity, resulting in many more non-synaptic than synaptic contacts between neurons, and that there is not a significant difference in axon-synapse distance between axons that make connections with particular spines and ones that do not (Kasthuri et al., 2015). We previously provided evidence, discussed in the following section, against non-synapse-specific spread in the VTA, though it remains a possibility. It is also possible that synaptic specificity may occur in some cells/synapse types but not others. It is also important to note the limitation of electrophysiological analyses in assessing neuronal connectivity. Because slice electrophysiological recordings require thin sections of tissue, it is possible that the preparation may sever connections that exist in the brain. It is also possible that connections atrophy as a consequence of tissue slicing. Thus, the preparation used may significantly influence results, particularly in experiments that require paired recordings.

Another way to assess the functional interaction between RABV-labeled inputs and starter neurons is to measure correlated activity between these cells. Connected populations should either show correlated, or anti-correlated, activity, depending on if the connections are excitatory or inhibitory, respectively. One study using a combination of RABV tracing and two-photon GCaMP imaging in the mouse visual cortex found that a large fraction of RABV-labeled inputs to a defined pyramidal neuron did not show a common visually-tuned response. In the minority of labeled cells that did show a common tuning, the correlation was weak (Wertz et al., 2015). However, a more recent study separated the contributions from excitatory and inhibitory neurons in this microcircuit and determined that the combined excitatory and inhibitory activity in labeled presynaptic cells correlated strongly ($R = 0.67$) with the starter cell's activity (Rossi et al., 2019). These results together provide functional data to support the possibility that RABV spreads preferentially between synaptically-connected neurons, though it stops short of demonstrating that RABV spread occurs specifically through synaptic connections.

While the above analysis is focused on cortical microcircuits, similar discrepancies can be observed for long-distance inputs onto different cortical neuron types. For example, Wall and colleagues (Wall et al., 2016) used RABV tracing to characterize long-range inputs to three different cortical GABAergic interneuron types expressing either parvalbumin (PV), somatostatin (SST+), or vasoactive intestinal peptide (VIP). RABV labeling suggested that the ventral posteromedial nucleus (VPM) of the thalamus provides similar levels of input to cortical PV + and SST + interneurons. This observation is not consistent with previous results using optogenetic stimulation of thalamic inputs to the cortex, which suggested that PV + interneurons receive a strong thalamic input while the input to putative SST + interneurons is very weak (Cruikshank et al., 2010). Moreover, a more recent study (Yu et al., 2019) of touch-evoked responses in four different neuron types in the barrel cortex – excitatory pyramidal neurons, PV+, SST+, and VIP + interneurons – showed that while pyramidal and PV + interneurons were strongly driven by thalamic input (likely from the VPM), VIP + neurons did not respond, and SST + neurons were activated but only after a long (>10 ms) latency indicative of a multi-synaptic response. An optimistic interpretation of these data would be that post-hoc electrophysiological analysis of connections suggested by RABV labeling in most cases is able to detect functional connections, providing support for the hypothesis that at least on a population level, RABV labels synaptically-connected cells, an observation supported by results in other brain regions (Beier et al., 2015; Weissbourd et al., 2014). However, a more pessimistic outlook is that RABV one-step labeling neither provides an accurate picture of the synaptic weights between different cells nor labels all input populations onto starter neurons. RABV labeling appears to have a non-zero false negative rate (it does not label all inputs onto starter neurons, discussed above), a low to zero false positive rate (most labeled neurons are synaptically connected), and the percentage of inputs onto given cell type does not always scale with functional strength of connectivity.

4.2. Observations from other circuits

The general observation that RABV labeling does not comprehensively reflect functional connectivity extends to neural circuits in other brain regions. For example, in our study of the inputs onto different types of dopaminergic, GABAergic, and glutamatergic neurons in the VTA, biases in RABV labeling could be predicted by the density of input innervation onto each of these cell populations (Beier et al., 2019). Furthermore, the location of these cells in the midbrain was related to the number of RABV-labeled input neurons in different input regions, but the cell type, as defined by neurotransmitter, was not correlated with whole-brain input patterns. While this could reflect that connections in the VTA are roughly equally distributed onto each cell population, it has been shown that a number of input sites more robustly innervate either DA or GABA neurons in the ventral midbrain (Lammel et al., 2012; Beier et al., 2019; Jennings et al., 2013; Yang et al., 2018; de Jong et al., 2019; Edwards et al., 2017; Beier et al., 2017). Another potential explanation for our results is that the spread of RABV is not synapse-specific, and thus the virus is released and gets taken up by any neuronal process that passes by starter neurons. However, we performed a control experiment that argues against this possibility. We injected a G-deleted RABV directly into the VTA, where the virus could get taken up by passing inputs, and we observed a roughly ten-fold difference in the percentage of labeled inputs from the medial habenula (MHb) and the striatum. The presence of synaptic inputs from the MHb onto VTA neurons is controversial, since a dense fiber bundle, the fasciculus retroflexus, passes through the VTA en route to terminating in the interpeduncular nucleus (IPN). Though a direct connection from MHb neurons onto VTA-DA neurons has yet to be demonstrated, activation of the dorsal aspect of the MHb is reinforcing while inhibition is aversive, which is consistent with direct activation of VTA-DA neurons, not inactivation as would be expected if the projection was solely or preferentially to local GABAergic neurons (Hsu et al., 2014). Consistent with this observation, most studies conducting RABV tracing from VTA cells report a small input from the MHb (Beier et al., 2015; Watabe-Uchida et al., 2012; Faget et al., 2016), but the proportion is <3% of total inputs. The substantially larger proportion labeled when the G-deleted RABV was injected directly into the VTA was thus likely due to labeling of axons in passage.

In a second study, investigators examined inputs onto different cell types in the dorsal striatum (Choi et al., 2019). This question had been investigated previously, and both studies found that, by and large, direct and indirect pathway medium spiny neurons share a common set of inputs and receive a quantitatively similar proportion of inputs from each brain region (Wall et al., 2013; Guo et al., 2015). Note that in these studies the location of starter neurons was not carefully controlled, and thus may have played a role in the differences that were observed. In addition, statistical corrections for multiple comparisons were not conducted, which has historically been the case for nearly all papers employing the one-step RABV input mapping method or its variants. Choi and colleagues examined inputs onto direct pathway medium spiny neurons as well as two types of interneurons intermingled in the same region, PV + and SST + cells (Choi et al., 2019). They noted a significant discrepancy between anatomical and physiological connectivity. Specifically, despite the fact that RABV tracing data suggested that SST + interneurons and direct pathway D1-positive neurons received similar fractions of inputs from the anterior cingulate cortex and parafascicular thalamic nucleus, the SST + neurons showed significantly reduced synaptic strength due to multiple factors including reduced probability of release, fewer release sites, and reduced postsynaptic sensitivity.

Therefore, while RABV labeling suggested an equal number of inputs from two excitatory inputs onto these cells, the functional aspects of those connections were quite different.

5. Conclusion

In consideration of over a decade of RABV one-step tracing experiments from multiple cell types over many different circuits, there is no clear evidence that the extent of viral labeling can be used on its own to reproduce all aspects of functional connectivity. However, the majority of the data suggest that RABV input mapping can be used to detect synaptically-connected neurons, as where RABV labeling has been reported and tested, synaptic connections have also been observed, at least on a population level. Therefore, strictly from an anatomical standpoint, RABV labeling can be used to detect the presence of synaptic connections. In addition, it may be used as a predictive identifier for behaviorally relevant cell populations by detecting differences in connectivity induced by experience (Beier et al., 2017; Wall et al., 2019).

The lack of clarity regarding synaptic transfer of virus gives rise to a significant chicken and egg confound. When the virus labels unexpected connections, is this because spread of the virus is non-synaptic or non-specific, or is it because this connection has been missed using other methods? This question could be important for precise anatomical mapping of neural connectivity and perhaps development/pruning of synaptic connections, though if the connections are all weak or functionally not relevant for behavioral outputs, the impact of discovering these connections may be minimal. On the other hand, elucidation of previously unknown direct connections may be relevant to explain unexpected behavioral results. We discussed earlier that RABV tracing detects a small but reliable input from the MHb to VTA-DA neurons. Whether this connection could explain the reinforcing properties of dorsal MHb stimulation remains to be tested.

If RABV labeling does not reflect the strength of connectivity, it must be influenced by factors that do not strictly relate to functional connectivity. However, the nature of these factors has largely been unexplored. Part of this is due to an incomplete understanding of the anatomical architecture and functional properties of circuits throughout the brain. We recently showed that RABV transmission can be influenced by changes in neuronal activity (Beier et al., 2017). In animals that had received a single dose of cocaine, we observed increased RABV labeling of inputs from the globus pallidus external segment (GPe) onto VTA-DA neurons. We then tested if this increase in inputs was due to a change in the number of synapses, strength of synapses, or activity of connections. We found no evidence of a change in the numbers or strength of synaptic connections from the GPe onto midbrain neurons but did find that activity in PV-positive GPe neurons was elevated 24 hours following the cocaine exposure (Beier et al., 2017). Thus, RABV labeling can be influenced by other factors besides the presence or absence of synaptic connections. Interestingly, changes in neuronal activity were not observed to alter spread of RABV in primary neuronal cultures (Bergami et al., 2015). We also failed to observe differences in RABV or VSV transmission in organotypic hippocampal cultures when tetrodotoxin was applied (data not shown), suggesting that activity dependence of viral transmission may not be universal, and may not occur in all circuits and cell types. However, evidence from our ongoing studies suggests that RABV labeling can detect changes in multiple cell types/circuits in vivo, and that differences can be detected with stimuli other than drugs of abuse. However, the potential contribution of changes in the numbers or strength of connections remains to be tested. Furthermore, just because we did not detect a change in synapse number or strength does not mean one did not occur. We prefer the use of RABV as a comparative tool to assess input changes in response to an experience (Beier et al., 2017), or to generate hypotheses to be tested using functional methods (Beier et al., 2015), rather than to provide a quantitative map of the input landscape to targeted cell types. This is because so little is known about the mechanisms of RABV transmission, and therefore we should interpret as little as possible about the meaning of differences in viral labeling. We favor the more conservative interpretation that identification of differences in viral labeling should be interpreted as a change in *something*, which then should be interrogated using more widely validated approaches. Questions about viral transneuronal labeling could only be answered through an increase in funding and research effort geared towards elucidating the basic cellular mechanisms of viral transmission in neuronal systems. Such efforts would likely give rise to improved viral tracers as well as a more sophisticated understanding of how data from these tracers should be interpreted.

Svoboda (2019) distinguishes four possibilities for synaptic specificity of RABV spread, summarized below:

1 Strong synapse-specificity: Viral spread strongly reflects functional connectivity - spread is dictated by the number or strength of synapses. Viral transmission is not biased to and from specific cell types. Virus transmits primarily through synaptic contacts.
2 Medium synapse-specificity: Viral spread reflects functional connectivity - spread is largely dictated by the number or strength of synapses, but some biases may influence proportions of inputs labeled. Virus transmits primarily through synaptic contacts.
3 Weak synapse-specificity: Viral spread no longer reflects functional connectivity. Biases appear in transneuronal spread. Virus transmits primarily through synaptic contacts.
4 No synapse-specificity: Viral spread no longer reflects functional connectivity. Biases appear in transneuronal spread. A significant amount of viral transmission occurs via local contacts, not synapse-specific.

He concludes that, at best, RABV transmission exhibits weak synaptic specificity and suggests that there is no compelling evidence that RABV spreads predominantly through synapses and does not simply spread to nearby processes. Our conclusions are largely in agreement with this assessment, though we are more optimistic that RABV transneuronal transmission occurs predominantly through synaptic connections, as direct injection of RABV containing its own envelope glycoprotein (RABV-G) on the viral surface into the brain labels inputs with a different quantitative distribution than one-step RABV labeling from neurons located at the same brain site (Wall et al., 2013; Beier et al., 2019), and simple proximity labeling would be likely to label more glial cells when viral spread occurs from starter neurons. However, the distribution of neurites in the extracellular space that could be infected by injected RABV may be different than neurites in close proximity to starter cells. Also, RABV may have a preferential tropism for neurons. It appears that, even though spread of RABV between neurons is not entirely synapse-specific, at least on a population level the inputs that are detected reflect the presence of synaptic connections. One key consideration is that the inputs quantified in RABV labeling experiments are those that were successfully infected by virions that completed the entire replication/infection cycle. This includes: replication in starter cells, assembly in starter cells, release from starter cells, entry into secondary cells, translocation of the intact virion to the cell body, uncoating of the virion, and successful replication/expression of fluorophore. If any of these steps fails, it would result in aborted infection events, which would not be scored. More likely than not, one of these steps is limiting and dictates specificity of transmission. However, without a clearer understanding of the basic mechanisms of viral transmission between neurons, we do not know which if any of these steps may disproportionately influence which cells become infected. One hypothesis is that one or more of these steps successfully occurs more frequently in synaptically-connected neurons. It is also possible that the mechanisms that dictate specificity for short-distance and long-distance connections are different. Successful infection of long-distance connections requires efficient long-distance retrograde transport from the axon terminal to the cell body, whereas infection of

neurons whose soma is nearby to the starter neuron would not require efficient retrograde transport in secondary neurons, as the virion would already be close to the soma upon entry. Thus, it is possible that experiments using RABV to examine microcircuits near starter cells may be more susceptible to non-synaptic transmission events that occur through proximity labeling. However, without rigorous studies of the mechanisms of transneuronal viral transmission, this is strictly speculation. All these conclusions would be greatly strengthened by a more rigorous assessment of the synaptic specificity of RABV transneuronal labeling, as the electrophysiological evidence to date has fallen short of providing a clear answer to these questions.

Transneuronal viral tracing has emerged as a useful anatomical tool to label connected cells, and improved versions have the potential to interrogate functional properties of these connections. These advantages ensure that viral labeling methods, in particular RABV one-step input labeling, will remain a staple technique to investigate neural circuit structure/function. However, if one-step RABV truly has only weak synaptic specificity, this substantially limits our interpretations of RABV tracing data, and significant questions remain. These include, but are not limited to: Do viruses have cell type biases? If so, what are they? Do biases differ between viruses? Do viruses transmit through conventional synapses/neuromodulatory synapses/other contacts, or all of the above? To what extent is spread synapse-specific? To what extent is viral transmission reflective of synapse number/strength/activity? Do the properties of viral spread change with the time of incubation or the multiplicity of infection (MOI) of the initial starter population? What fraction of inputs per cell does RABV label? Are these values consistent across cell types/brain regions or is there substantial variation?

To date, we have accumulated a large amount of indirect evidence that is consistent with synaptic specificity of viral transneuronal transmission. However, we still lack direct evidence of specificity (e.g., through electron microscopy). Though the majority of studies using RABV report results consistent with retrograde transsynaptic spread, in cases where different results are observed (glial transmission, anterograde spread, etc.), it is not clear why. Clearly, there are many questions that remain to be answered, which can only occur through more detailed research. Until we understand more about the basic mechanisms underlying viral transneuronal transmission, a cautious interpretation of viral mapping results is warranted. The more we know about how the virus works, the more we can take advantage of its unique properties to interrogate and decode the complex information carried by neural circuits.

CRediT authorship contribution statement

Alexandra Rogers: Writing - original draft, Writing - review & editing. **Kevin T. Beier:** Conceptualization, Visualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

We have no competing interests to declare.

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