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The hitchhiker's guide to the nervous system: axonal transport of neurotropic viruses and toxins

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Abstract

The central nervous system (CNS) offers a secluded hiding place from immune surveillance due to the existence of the blood brain barrier. However, the complexity of neuronal networks makes accessing the CNS a particularly difficult task for microbial pathogens. Some viruses and bacterial toxins can specifically interact with neuronal membrane receptors and trafficking components, allowing them to access the CNS and undergo long-range axonal transport from nerve termini to neuronal cell bodies. Even though these microbial agents cause a wide spectrum of neuronal pathologies, their mode of access may be similar in term of the molecular mechanisms involved. In this review, we highlight the mechanisms used by pathogens to exploit the axonal transport route during CNS invasion.

Introduction

Pathogens have long exploited the architecture and characteristics of neurons to invade the central nervous system (CNS), an immune-privileged organ due to its relative isolation from the blood stream by the blood brain barrier (BBB, **see Box 1**). Certain viruses and bacterial toxins possess unique properties that allow them to reach the CNS, replicate, spread and cause a range of severe pathologies. In many cases, disease progression following neuronal infection can be debilitating as neuronal homeostasis is impaired. For instance, tetanus caused by tetanus toxin (TeNT) and botulinism caused by intoxication with botulinum toxins (BoNT), are neurological disorders resulting from toxin-induced impairment of neurotransmission¹. Rabies virus-induced neurodegeneration is another classic example of the damage neurotropic viruses can cause². Theiler's virus can cause demyelination and is currently used as a model

for multiple sclerosis in rodents³, whereas Borna virus infections can lead to synaptogenesis impairment⁴ and behavioural changes in the host. Because inflammatory processes are frequent in patients with age-related neurological diseases, a link between some neuronal viral infections and neurodegenerative disorders has been proposed⁵. Indeed, Parkinson's disease may be linked to influenza virus or West Nile virus infection⁶⁻⁹.

The mode of access of pathogens to an healthy CNS can vary: access can occur through the BBB in the case of human immunodeficiency virus type-1 (HIV-1)¹⁰ (**Box 1**) or after axonal anterograde transport for measles virus¹¹ and retrograde transport for herpes simplex virus (HSV)¹², rabies virus, poliovirus¹³, borna virus¹⁴, and TeNT¹⁵ (**Table 1**). Interestingly, viruses such as West Nile virus¹⁶ or canine distemper virus¹¹, can use both routes of entry, giving rise to distinct

neuronal pathologies. The large distance that endogenous and exogenous ligands, including neurotropic pathogens, must overcome to reach the neuronal cell bodies illustrates the selective advantages that reaching the CNS may provide.

Recent advances in cell biology and imaging have allowed scientists to elucidate the molecular mechanisms underlying the entry and transport of several of these pathogens, revealing key features of the trafficking pathways involved. Long-range axonal transport is an essential process for neuronal differentiation and homeostasis and functionally connects peripheral organs with the soma of neurons located in the CNS. Its impairment is directly linked to several neurodegenerative disorders, such as Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis and hereditary spastic paraplegia¹⁷. Using viruses and toxins to study neuronal trafficking processes might therefore result in the characterization of factors involved in the regulation of axonal dynamics that are defective in neurodegenerative diseases. Neurotropic viral vectors are also promising platforms for gene delivery to the CNS to understand and treat neurological pathologies (see **Box 2**).

In this review, we focus on how viruses and bacterial toxins reach the CNS after long-range axonal transport and highlight the different strategies that are used to access the CNS via this essential route. Other mechanisms of entry such as passage through the BBB, or using haematopoietic cells to invade the CNS have been recently reviewed elsewhere¹⁸.

Neuronal architecture and axonal trafficking

Polarization is an essential aspect of neuron biology that allows the cell to receive, integrate and transmit vectorial signals within a neuronal network. Neuronal compartmentalization occurs rapidly during development when a specific neurite becomes an axon and the remaining projections become dendrites¹⁹. This unique architecture creates particular challenges when it comes to maintaining cell polarity and for the delivery of signals or cargoes over long distances, which for some human neurons are in the meter range (e.g. motor and sensory neurons)²⁰. To ensure proper spatial and temporal control of cargo progression along the secretory and endocytic pathways, cytoskeletal tracks and molecular motors must be finely regulated (**Figure 1**). Whereas microtubules have mixed polarity in proximal dendrites they are unipolar in axons

and in distal parts of dendrites, with their minus-end directed towards the cell body and the plus-end oriented distally²¹. Molecular motors move cargoes in ATP-dependent directional processes: cytoplasmic dynein is responsible for retrograde transport from the synapses to the cell body whereas members of the kinesin family mostly ensure anterograde traffic and delivery of cargoes to nerve endings (**Figure 1**). In spite of having opposite polarities, dynein and kinesin cooperate at the level of a single cargo to ensure precise localisation of key organelles (e.g. mitochondria), underlining the complex molecular interactions occurring during intracellular transport^{17,22}. Axonal transport is also regulated by a subset of specific microtubule-interacting proteins and neuronal subcompartments, such as the axon initial segment, which physically regulates the targeting of proteins and organelles and ensures proper protein segregation between the somato-dendritic and axonal compartments²³. Moreover, to ensure fast and restricted protein synthesis to rapidly expanding areas such as growth cones or during injury response, mRNA and microRNA are specifically transported and localized in axons, allowing neurons to synthesize proteins on demand^{24, 25, 26}.

Retrograde axonal transport allows signalling molecules activated in the periphery to be translated into specific nuclear responses. An example of this process is the retrograde transport of receptor signalling complexes triggered by neurotrophin binding. This long-range transport occurs during development when neurons reach their target organs that are the source of neurotrophins. "Signalling endosomes" contain neurotrophins and their receptors, as well as downstream activated molecules such as the extracellular-regulated kinase 5 (ERK5) and the transcription factor cAMP response element binding protein (CREB)²⁷. The delivery of these signalling organelles to the soma ensures the survival of neurons that have reached their physiological target, whereas cells that do not connect will undergo apoptosis²⁸. During adulthood this mechanism is maintained in specific neuronal populations, the survival of which is dependent on neurotrophin supply (e.g. basal forebrain cholinergic and striatal neurons). It is therefore not surprising that impairment of axonal transport, due to environmental factors or mutations in components of its molecular machinery, leads to neurodegeneration..

Neurotropic viruses and toxins

Given the essential role and robustness of axonal transport, it is not surprising that viruses and bacterial toxins take advantage of this pathway to access the CNS. Due to their impact on human health, poliovirus¹³, rabies virus³⁰, alphaherpesviruses¹² (*i.e.* HSV-1 and pseudorabies virus (PRV)), as well as TeNT¹⁵, are probably the best characterized microbial agents that undergo axonal transport. However, this intracellular route is also used by other viruses including such as West Nile virus^{16,31}, Theiler's virus³², measles virus³³, enterovirus 71³⁴ and influenza H5N1⁷. Moreover, botulinum neurotoxin serotype A (BoNT/A), which was thought to act mainly at the neuromuscular junction (NMJ) and other peripheral nerve terminals, can also be retrogradely transported in motor neurons³⁵ (see **Table 1**). Although common themes concerning a pathogen's *modus operandi* to invade the CNS have been found, distinct mechanisms underlying neuronal entry, long-range transport and somato-dendritic sorting are also emerging.

The preferential binding to nerve terminals is obviously a key step in the neuronal uptake of microbial agents. However, the mechanisms of neuronal entry can be quite different. Rabies virus³⁶ and alphaherpesviruses¹² must replicate and spread in non-neuronal cells before entering peripheral nerves, whilst other pathogens and virulence factors such as Borna virus³⁷ and clostridium neurotoxins can enter neurons directly (see below). Replication in non-neuronal cells not only boosts the chances of a viral particle accessing the CNS, but it could also favour the use of the endocytic and intracellular trafficking pathways that functionally couple neurons to their targets organs. Numerous studies on axonal transport of pathogens also involved direct intravitreal, intramuscular or intrasciatic injections (*e.g.* West Nile virus³¹, Theiler's virus³² or adenoviruses³⁸ (see **Box 3**); these sites of delivery may differ from the physiological route of entry and bypass some endogenous internalization mechanisms or force "unnatural" tropism. However, these approaches have helped to dissect the molecular mechanisms behind their transport and opened new avenues for their use in gene therapy or neuronal tracing. Indeed, vectors derived from neurotropic viruses or vectors pseudotyped with envelopes from neurotropic viruses can also use this pathway, making adenoviruses, adeno-associated viruses, pseudotyped lentivirus and HSV-derived vectors promising tools for gene delivery in the CNS³⁹ (**Box 2**). Because of their advantageous properties such as transsynaptic spreading after axonal

transport, some pathogens like herpesvirus and rabies virus have also been used intensively to map transneuronal circuits^{40, 41}.

Entering the Central Nervous System

The study of the intracellular trafficking of viruses, bacteria and bacterial virulence factors is a rapidly expanding field, which was initiated by the advent of "cellular microbiology" in the mid-1990's⁴². To date, most studies have focussed on epithelial-like cells⁴³, regardless in some cases of the natural cellular tropism of the infectious agent. While such studies have identified key cellular proteins and their functions in pathogen trafficking, some of these players also have specific roles in axonal trafficking.

Binding the neuronal membrane

To reach the CNS viruses and toxins can enter different types of nerve endings such as sensory nerve terminals and NMJs, after for example dermal and muscle wounds. Receptors for certain pathogens appear to be concentrated at these sites, which would represent an ideal entry point to the CNS (**Figure 1**). Synapses are neuronal sub-compartments connecting neurons to each other or to their target organs. NMJs are specialized synapses that connect motor neurons to muscles, forming a functional motor unit (**Figure 1**). This highly organized structure has been intensively studied as a model for synaptic connection and for pathogen invasion of the CNS. "Classical" uptake of pathogens at the NMJs has been described in mice and non-human primates for poliovirus¹³, rabies virus⁴⁴ and clostridium toxins¹. By contrast, sensory nerve terminals are the main gateway for herpesvirus infection of the CNS¹². Intramuscular injection of poliovirus⁴⁵ and canine adenovirus serotype 2 (CAV-2)^{46,38} in leg muscles led to the uptake of the viruses at NMJs and subsequent axonal retrograde transport. Although rabies virus transport was reported in motor and sensory neurons, data from rodents and primates suggest that NMJs may be the exclusive point of entry for this virus⁴⁷. Targeting of motor neurons is likely due to the exclusive presence of the cellular receptors of rabies, the neuronal cell adhesion molecule (NCAM) and nicotinic acetylcholine receptor (nAChR) at NMJs⁴⁷. However, since NCAM is located at the presynaptic site and nAChR is post-synaptic, the mechanism of rabies binding and uptake probably involves complex interactions at the synapse.

Synapses are highly active structures characterized by distinct areas. Exocytosis of synaptic vesicles and granules occur at active

zones, which typically occupy the centre of synapses⁴⁸. In contrast, the endocytic machinery is selectively found outside active zones⁴⁸ and may regulate the internalization of the receptors that pathogens exploit to reach the CNS. Other subdomains at the synapse could also concentrate the receptors for a particular pathogen. For instance, lipid rafts contain several lipids and proteins that have been found to interact with bacterial toxins (e.g. polysialogangliosides for clostridium toxins⁴⁹) or viruses (e.g. cholesterol for pseudorabies virus⁵⁰). BoNT and TeNT can enter the axoplasm through the interaction with NMJ components such as polysialogangliosides and the synaptic vesicle proteins synaptotagmin I and II (BoNT/B and G) and SV2 (BoNT/A, E and F)¹ (see below). Membrane proteins associated with synaptic vesicles might therefore be preferential targets for neurotropic viruses and toxins due to the high rate of fusion and recycling of these synaptic organelles and their high concentration at active zones of nerve terminals.

Interestingly, certain classes of molecules found at nerve endings are preferentially recognized by infectious agents. For instance, cell adhesion proteins localized at NMJs often act as pathogen receptors (see **Figure 1**). Why have pathogens selected these molecules? Certainly their conservation through evolution plays a major role. This is illustrated by the broad host range of alphaherpesviruses⁵¹ and rabies viruses³⁰, and the fact that members of the same class of proteins can act as receptors for different viruses. Indeed, CD155, a member of the immunoglobulin superfamily, and the related members nectin-1 and nectin-2 serve as receptors for poliovirus and members of the *Herpesviridae* family, respectively⁵². The interaction of herpesvirus glycoprotein D with nectin-1 initiates viral uptake and is consistent with the preferential targeting to dorsal root ganglia neurons since nectin-1 is present at sensory endings but not NMJs^{53, 54}. Similarly, binding of poliovirus to NMJ-localized CD155 may be the first step in its neuronal spread^{55, 13}. The coxsackievirus and adenovirus receptor (CAR), also a member of the immunoglobulin superfamily, and NCAM, a key adhesion molecule in neurons, are found at NMJs and can act as receptors for adenoviruses^{38, 46} and rabies virus⁵⁶ respectively (**Figure 1**). Similarly, molecules involved in neuronal homeostasis may be ideal targets for pathogen recognition and uptake. The neurotrophin receptor p75^{NTR} is recognized by rabies virus glycoprotein G⁵⁷, although it is not essential for infection⁵⁸.

Entering neurons via endosomes

As mentioned previously, pathogen internalization and endocytic sorting have been predominantly characterized in epithelial-like cells⁵⁹, in which distinct endocytic mechanisms including clathrin-dependent and -independent entries, caveolae-dependent internalization, phagocytosis and macropinocytosis have been described⁶⁰. Receptor-mediated entry provides pathogens with an efficient way of exploiting the molecular motors and adaptors that control the endocytosis and trafficking. As a first step, the initial binding of viruses to surface molecules may be followed by β transfer to secondary receptors that induce internalization or direct the virus to membrane compartments where another receptor is found (e.g. DAF and CAR as primary and secondary receptors for coxsackievirus⁶¹). Following binding, conformational changes of the receptors can lead to the recruitment of endocytic components such as clathrin and its adaptors⁶⁰. In epithelial cells, the fate of endocytic vesicles varies: recycling allows internalized molecules to be redirected to the plasma membrane, “classical” retrograde transport occurs from endosome to the Trans-Golgi Network and degradation occurs after cargo progression into lysosomes⁶⁰. Although these pathways are conserved in neurons, some notable exceptions have been described. For instance, synaptic vesicles have distinct dynamic properties depending whether they belong to the readily releasable, recycling or reserve pools⁶². Yet, the contribution of these pools to the uptake of pathogens and their transfer to the synaptic cytosol is still unclear. Moreover, as synaptic vesicles mainly undergo cycle of exo-endocytosis locally, a mechanistic understanding of the link between these organelles, their trans-synaptic shuttling⁶³ and long-range axonal transport is still lacking. However, a recent study showed that BoNT/A, which interacts with the synaptic vesicle protein SV2 to enter NMJs, is transported back to the soma of motor neurons³⁵ (see below). Whether the transported BoNT/A is still in the lumen of a synaptic vesicle, sorted towards another type of endocytic organelle or transported directly in the axoplasm is not currently known¹.

The “classic” endocytic pathways at nerve terminals can also provide endogenous molecules and pathogens with a direct link to the axonal retrograde transport machinery. Using co-localisation and overexpression of dominant negative constructs, we found that the TeNT heavy chain binding fragment (TeNT

Hc)⁶⁴, CAV-2 and CAR⁴⁶ were co-internalized by clathrin-coated vesicles and progressed from an Rab5- to Rab7-positive endosomes that eventually underwent axonal transport (see below). Similarly, poliovirus and CD155 enter together endosomal structures, which eventually will be coupled to the axonal machinery^{45, 65}, while p75^{NTR}, which binds rabies virus can be endocytosed through a similar pathway involving clathrin-coated pits and directed towards trafficking endosomes⁶⁶. Interestingly, the onset of axonal transport of TeNT, CAV-2 and p75 occurs 30-45 minutes post-internalization, suggesting a complex series of sorting events from ligand binding at the neuronal membrane to the engagement of motors and/or their attachment to the microtubule tracks^{17,64}.

Some membrane receptors are involved in neuronal transcytosis, a mechanism that allows the transfer or targeting of ligands from axon terminals to the somato-dendritic compartment and then to other cells in direct contact with the neuron. This axo-dendritic pathway is paralleled by a somatodendritic-axonal targeting mechanism, which is used by some newly synthesized proteins such as the adhesion molecule NgCAM and the neurotrophin receptor TrkB. Both proteins are targeted to axons following a prior step of ligand-independent internalization/recycling at the level of the somatodendritic membrane^{67,68}. It is tempting to speculate that similar mechanisms exist to target axonal receptors during endocytosis back to the cell body. Pathogens using these molecules may exploit this process to be transported along the axon back to the soma. For example, CAR, which is transported in the sciatic nerve apparently without ligand⁴⁶, may be an ideal choice for coxsackieviruses B and some adenoviruses to reach the CNS.

Direct membrane fusion and endosomal escape

The internalization of pathogens in a cell does not always involve progression through the endocytic pathway. Some enveloped viruses can directly fuse with the plasma membrane and find themselves “naked” in the cytoplasm. For example, enveloped alphaherpesviruses undergoes pH-independent fusion with the plasma membrane to enter neurons^{12, 69}. Once in the axoplasm, the exposed tegument proteins may recruit adaptor proteins and molecular motors to be transported along microtubule tracks (see below). Similarly, rabies virus can undergo pH-dependent fusion with the cellular membrane that would lead to axoplasmic localisation of the capsid⁷⁰.

However, Klingen *et al* described neuronal transport of fully enveloped virus occurring in endosomes⁷¹, suggesting that entry mechanisms can be cell-specific. Non-enveloped viruses can also trigger endosomal lysis. This is well described in epithelial cells for some adenoviruses: adenovirus serotype 5 escapes Rab5 early endosomes after acidification and is then transported to the nuclear pore by direct recruitment of dynein onto the capsids⁷². A recent study highlighted the difference existing between epithelial cells and neurons since the majority of the axonal transport of adenoviruses occurred in intact endosomes⁴⁶. However, these findings do not exclude the possibility that some virions may escape axonal endosomes and traffic directly by interacting directly with molecular motors as it was reported in epithelial cells.

Long-range Axonal Transport of Pathogens

Why is motor-based axonal transport a key mechanism for CNS invasion by pathogens? The distances that can separate cell bodies from nerve terminals mean that viruses, as well as endogenous molecules, cannot rely on passive diffusion. Indeed, Sodeik calculated that viral transport through diffusion would take hundreds of years per centimetre of cytoplasm⁷³. By contrast, molecular motors ensure the transport of cargoes at speeds exceeding several $\mu\text{m/s}$ (equivalent to 1-3 cm/h)¹⁷. Neurotropic viruses and toxins were therefore selected, evolutionary speaking, owing to their ability to take advantage of the axonal transport machinery. Advances in live-cell imaging techniques, the use of compartmentalized chambers and classical biochemical approaches are leading to better characterization of the axonal dynamics of several viral pathogens.

Direct recruitment of motors The recruitment of molecular motors by HSV-1 (human) and PRV (pig) has been intensely studied, and several viral proteins have been shown to interact with dynein or dynactin¹². Early studies showed the crucial role of microtubules in herpes virus transport⁷⁴ and live-cell imaging in the giant squid axon allowed one of the first visualizations of viral axonal transport (see Box3)⁷⁵. Incoming capsids were associated with the dynein/dynactin complex and their transport blocked by inhibiting dynein-dependent transport with dynamitin overexpression⁷⁶. Several alphaherpesvirus proteins associate with members of the dynein complex: pUL34 interacts with the dynein intermediate chain 1⁷⁷ whereas the viral helicase pUL9 contains the dynein light chain LC8 binding site “KSTQT” and associates with

LC8 *in vitro*⁷⁸. However, the relevance of these interactions for the retrograde transport of herpesvirus is unclear because these viral proteins are not found in mature virions¹². More relevant in the context of transported virions, the HSV-1 capsid protein pUL35 (VP26) can interact with the dynein light chains RP3 and Tctex1⁷⁹. However, capsids lacking pUL35 or most of the tegument proteins retained retrograde transport capacity^{80,81}. Other tegument proteins associated with entering capsids have been proposed to mediate retrograde transport such as pUL25, pUL36 and pUL37^{12,82}. Moreover, dynein is not the only motor that alphaherpesviruses can recruit: the HSV-1 tegument protein US11 can bind conventional kinesin heavy chain *in vitro*⁸³. Whereas retrograde transport is the key process in CNS invasion by herpesviruses, it is interesting to note that bi-directional transport was observed during both entry and egress in sensory neurons^{84,85}. Not surprisingly though, the average speed of retrograde traffic is higher during entry, whereas the anterograde transport becomes dominant during egress, suggesting an efficient regulation of motor coordination and/or recruitment. Although the mechanism underlying this phenomenon is still unclear, it may be due to a difference in the capsid composition during these two phases^{85,12}. Interestingly, LC8 binding domains are also present in other viral proteins⁷⁸ such as the rabies virus protein P^{86,78} (RV P). However, depending on the immune status of mice, mutations in this domain had contrasting results on the pathogenic effect of rabies virus after peripheral infection^{87,30}. Moreover, rabies virus protein G, which binds to p75^{NTR}⁵⁷, conferred the axonal retrograde transport of pseudotyped lentivirus⁸⁸, proving unlikely the unique role of RV P interaction with LC8 in the neuronal spread of the virus. This mechanism is however complex because rabies virus is still able to enter p75 knock-out neurons⁵⁸, suggesting some redundancy in viral entry and/or transport (see below).

Linking receptors to molecular motors

One of the simplest ways for a pathogen to access the CNS would be to bind a receptor that can be internalized and retrogradely transported. A good example of this is the poliovirus receptor CD155, which has a Tctex-1-binding site that allows the recruitment of the dynein/dynactin complex and the transport of endosomes containing the virus^{89,55}. In PC12 cells, direct interaction between the cytoplasmic domain of CD155 and Tctex-1, as well as transport studies with mutants of CD155 unable to bind Tctex-1 suggested that

this interaction is required for the retrograde transport of poliovirus and its receptor⁵⁵. However, a recent study showed that some CD155-independent transport of poliovirus may occur in mice expressing human CD155, again indicating that multiple trafficking routes may be used by pathogens *in vivo*⁶⁵.

Pathogens hijacking long-range vesicular transport pathways

Endocytic and exocytic vesicles are constantly trafficked in axons and therefore provide a continuous source of membranes and signals. Some pathogens can access some of these organelles to disseminate in the CNS. For instance, TeNT H_C exploits the vesicular pathway used by neurotrophins such as NGF and BDNF, and their receptors Trk and p75^{NTR} to be transported in a retrograde manner^{90,64} (**Figure 2**). These endocytic structures are also responsible for the receptor-dependent axonal transport of poliovirus⁶⁵ and an adenovirus⁴⁶ (**Figure 2**). Importantly, these axonal endosomes display a pH close to neutral^{91,46}, which allow the transport of cargo over long distances in a protective environment precluding pH-induced conformational changes or avoiding degradation. This environment could also explain why adenoviruses are mainly found in the lumen of endosomes in axons and not in the cytoplasm as shown in epithelial cells, where the pH drop that occurs in early endosomes causes viral escape^{92,72}. Together, these observations led to the suggestion that these endosomes could represent a multifunctional transport pathway for ligands with distinct somato-dendritic fates.

Pathogenic proteins and axonal transport

Viral proteins can also be transported in axons independently of fully-assembled viruses. Although HIV is often found in the brain of AIDS patients, the virus itself does not appear to directly infect neurons⁵. However, dementia is commonly associated with AIDS. This pathological effect is due, at least in part, to two viral proteins, gp120 and Tat, which trigger neuronal apoptosis⁹⁶ (Table 1). Axonal transport is important in the spread of gp120 and Tat and their associated neurotoxic effects. The envelope protein gp120 undergoes microtubule-dependent axonal retrograde transport in the rat CNS and is responsible for the neurotoxic activity^{97,98}. Similarly, Tat-induced apoptosis was detected at distal sites of injection of Tat-producing astrocytes⁹⁹. To date, the molecular mechanisms underlying the transport of these

two HIV proteins remain largely uncharacterized.

Transport efficiency and redundancy

As this point it is worth speculating on why some pathogens use multiple determinants for their axonal transport, and why mutations in viral proteins involved in motor binding do not always abrogate neuronal trafficking. From an evolutionary perspective, natural selection is likely to favour microbial agents that can use several pathways to reach their final destination. Viruses that engage the trafficking machinery in different ways (e.g. poliovirus can be transported in a CD155-dependent and independent manner⁶⁵) will have an advantage in CNS invasion. Along this line, HSV and PRV contain several proteins directly binding to the dynein complex but mutations in these viral components have only mild effect on its retrograde spread^{30,80}. This redundancy in viral proteins and motor binding is probably the mechanism that allows the virus to be efficiently transported in the CNS¹². However, it is worth considering that some of these reported interactions might correspond to egressing viruses or viruses trapped in an egress route.

Different pathogens also recruit molecular motors with distinct polarity to ensure their efficient axonal transport. Using live-cell imaging, alphaherpesviruses and CAV-2 were found to undergo bi-directional transport with a bias for the retrograde direction during their first phase of infection^{85,46}. Inhibition of either dynein or kinesin-mediated trafficking led to a strong reduction of the overall transport of CAV-2, suggesting a possible regulation or co-ordination between these two motors⁴⁶. Interestingly, motor coordination was also observed for mitochondria, which also display bi-directional trafficking and for TeNT Hc, which only moves retrogradely¹⁰⁰ (reviewed in¹⁷). In this light, viruses and toxins may be ideal tools to understand the regulation between molecular motors.

Reaching the neuronal cell body

The last step in translocation of a cargo from an axon to the cell body is still poorly understood. In epithelial-like cells, numerous viruses en-route to the nucleus transit at the microtubule-organizing centre (MTOC)⁴³. Even though in neurons the MTOC may be a potential sorting platform for axonally-transported material, no clear role of this structure has been reported. Pathogens follow different intracellular fates once they have arrived in the soma (see **Figure 3**). In the case of DNA viruses, targeting to the nucleus is the

final destination for incoming particles. Herpesvirus is a classic example of how some viruses can take advantage of the immune-privileged status of the nervous system to establish latency, in part by “silencing” the expression of its episomal DNA. Periodic reactivation of latent HSV leads to DNA replication and *de novo* viral protein synthesis, resulting in new viruses travelling along axons back to the primary infection site. Studies on the mechanisms responsible for herpesviruses anterograde transport and viral egress remain controversial¹². Live-cell imaging with fluorescently labelled pseudorabies viruses showed anterograde transport of fully formed (teguments and envelop) viruses^{102,103} whereas others studies mainly found unenveloped capsids in axons^{104,105}. In the “unenveloped” or “subassembly” transport model, assembly of virus with membrane would only occur at distal axonal sites prior release¹². Interestingly, the glycoprotein E (gE) that is needed for replication in epithelial cells and subsequent retrograde transport of HSV-1 in neurons¹⁰⁶ is also crucial in the anterograde spread of HSV-1¹⁰⁷. Other viruses do not need access to the nucleus and can replicate in the cytoplasm. The RNA virus rabies virus uses protein aggregates called Negri bodies, which contain the innate immune response receptor TLR3, as a viral factory to enhance its replication in neuronal cells¹⁰⁸. Whether axoplasmic replication can also occur remains to be documented.

Transcytosis

Some toxins (e.g. TeNT) and viruses (e.g. HSV and rabies virus) undergo transcytosis and continue their journey into a new connecting neuron. Again, the mechanisms used by infectious agents seem to differ: neuron to neuron spread can occur across synapses, as has been shown for measles³³, West Nile¹⁶ and rabies² viruses, or by direct cell-to-cell transfer as has been shown for PRV¹⁰⁹. The molecular mechanisms involved in trans-neuronal transfer remain however mostly uncharacterized. It is tempting to speculate that viruses engaging receptors naturally involved in transcytosis, as was discussed above, may cross neurons efficiently. Data regarding the trans-synaptic spread of measles virus shed light on how viruses can force this cellular crossing. Interestingly, this synaptic transfer was not dependent on CD46 and SLAM, the receptors involved in the entry of measles in neurons, but instead on the interaction between viral proteins and neurokinin-1 at synapses, leading to microfusion events³³ (**Figure 3**). In another

model system, Roussarie and collaborators showed that Theiler's virus was transferred to myelin sheets of oligodendrocytes after axonal transport in the optic nerve to allow persistent infection³². In mice expressing mutants of myelin components, Theiler's virus was unable to provoke persistent infection after intravitreally injections. Finally, clostridial neurotoxins can also reach secondary neurons. Once in the cell body of motoneurons, TeNT is transcytosed to inhibitory neurons synapses where it cleaves locally components of synaptic vesicles and inhibit neurotransmission release¹. Similarly, after intramuscular injections of high dose of BoNT/A, this neurotoxin can be found in adjacent neurons of the spinal cord, resulting from transcytosis after axonal retrograde transport in motor neurons³⁵.

Conclusions and Perspectives

Understanding the function of neuronal networks remains one of the exciting challenges of modern biology. Many pathogens undergo axonal transport to reach and damage the CNS, but we are only just starting to understand the molecular mechanisms which provide the basis for their neuronal targeting. Novel therapeutic approaches may be found through a more thorough characterization of the cellular steps involved in the entry and transport of viruses and toxins in the CNS.

Many still unexplored key areas and open questions regarding the axonal transport of viruses and pathogenic proteins remain. For example, there is increasing evidence that local translation is a key process to ensure fast and robust response to acute stimuli in axons such as injury or local growth. Local translation of importin α is required for the retrograde transport of signalling complexes containing nuclear localization signals (NLS)²⁵. Because numerous viruses or endosomal proteins involved in the axonal transport of pathogens bear these targeting motifs (e.g. pUL36 of alphaherpesviruses), signals activated during entry of infectious agents at nerve terminals might stimulate local translation leading to enhanced axonal transport. Along these lines,

little is yet known about the signalling molecules activated during entry that may regulate pathogen axonal transport. Whether these signals are similar to those used by neurotrophin during neuronal differentiation and survival, or closer to injury and stress-related signals is still unknown. Similarly, the recruitment of molecular motors mediated by some viruses is only beginning to be characterized. There are still important questions that remain on how viruses and toxins can regulate an efficient retrograde transport when multiple motors of different polarity seem to be engaged. In this light, dynactin is a potential regulator of bi-directional transport by binding both dynein and kinesin. Interestingly, dynactin is involved in HSV transport⁷⁶ and could be engaged by other neurotropic viruses and toxins.

Open questions also remain concerning the molecular mechanisms of post-axonal transport, when viruses and toxins reach the somato-dendritic compartment. How are vesicles or non-membranous cargoes directed to their target sub-compartments such as the perinuclear space, nuclear membrane or synapses? What is the role of the MTOC in this process? The Golgi network is well described in epithelial cells to act as a sorting platform both during endocytosis and exocytosis. Interestingly, in neurons, Golgi outposts exist also in dendrites and are involved in the targeting of membrane receptors in this cellular compartment¹¹⁰. Non-conventional Golgi was also reported in axons¹¹¹. Is this pathway involved in the transcytosis of some viruses and toxins? Disruption of the Golgi was involved in the anterograde transport of HSV-1¹¹², but no clear role of this organelle in direct transcytosis after axonal transport of a pathogen has been described. Even though *in vivo* models represent the best system to study neuronal invasion after axonal transport of pathogens, the use of compartmentalized systems, combined with cell and molecular biology approaches will be essential to further dissect the molecular machinery use by pathogens to efficiently access and spread in the CNS.

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Figures & Legends –

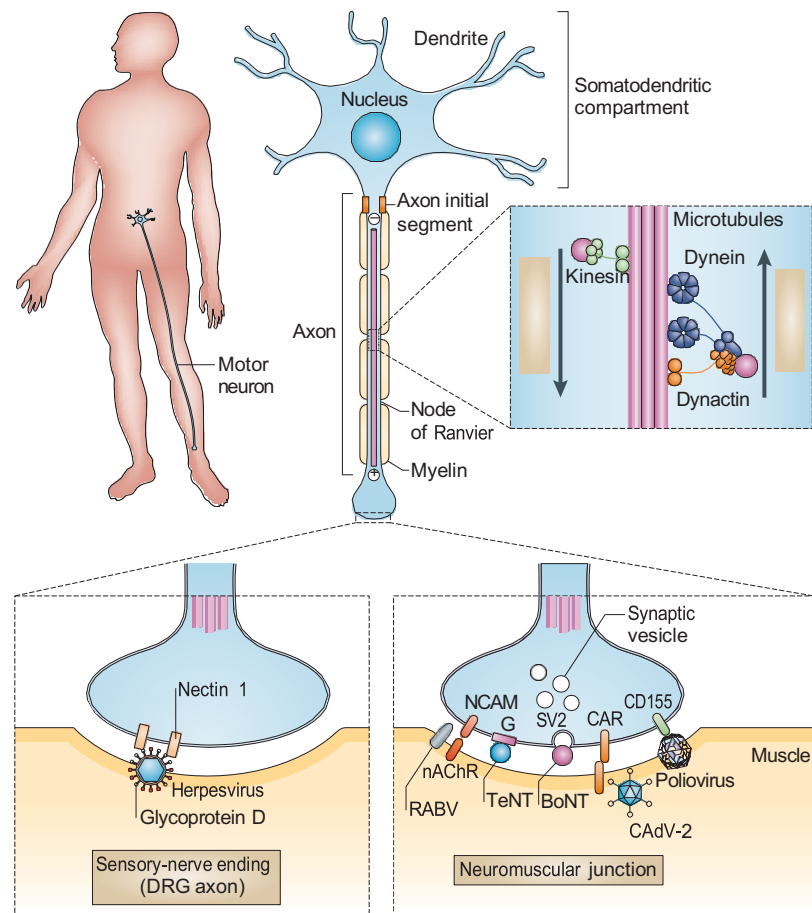


Figure 1- *Neuronal architecture and axonal transport.*

Neurons are highly polarized cells with extensions (axons) that exceed a meter in large mammals and represent the functional cellular unit of the nervous system. Axons can be enclosed in myelin sheaths that consist of several layers of cellular membranes coming from oligodendrocytes or schwann cells that provide enhanced propagation of electrical signals. Nerve terminals such as neuromuscular junctions (NMJs) contain numerous membrane molecules that can serve as receptors for virus and toxins. For instance, Tetanus toxin binds to polysialogangliosides and unidentified protein receptor at NMJs and strongly labels this structure when injected intramuscularly. To ensure communication between nerve termini and soma, intracellular transport plays an essential role in the distribution of neuronal proteins and cargos. Microtubules display mixed polarity in proximal dendrites and are unipolar in axons and distal dendrites. In this configuration, cytoplasmic dynein is the motor responsible of retrograde transport of cargos whereas members of the kinesin family move in the opposite direction from the cell body to the nerve terminals.

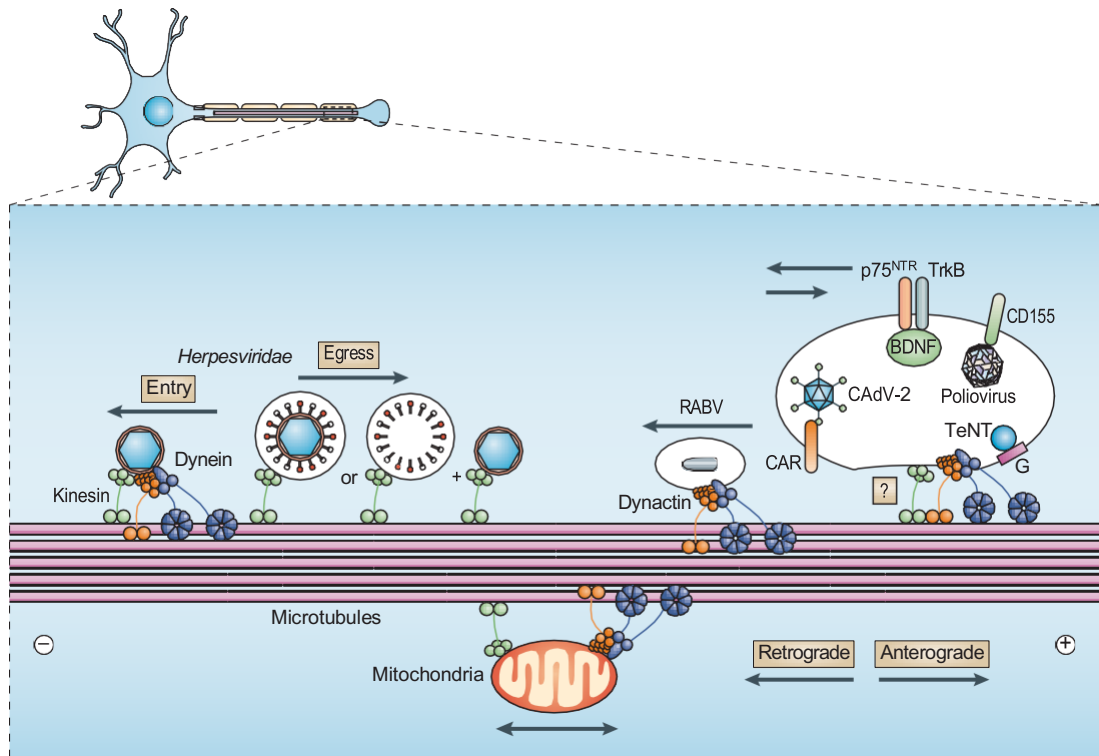


Figure 2- Herpes virus and TeNT transport in neurons.

Despite many still unresolved controversies, studies addressing herpesvirus transport are shedding light on how the virus can access the CNS after entry at peripheral nerve endings. Numerous viral proteins, in particular tegument components, can bind molecular motors and play a role during axonal transport or virus assembly. Egress was reported to involve fully formed (enveloped) virions or separate capsids, tegument proteins and viral membranes, with the assembly occurring in growth cones. TeNT is entering an endogenous vesicular pathway that neurotrophins and their receptors are using to be transported over long-distances in a protective environment. Poliovirus, CAV-2 and their respective receptors CD155 and CAR, are also found transported in this multitasking endosome.

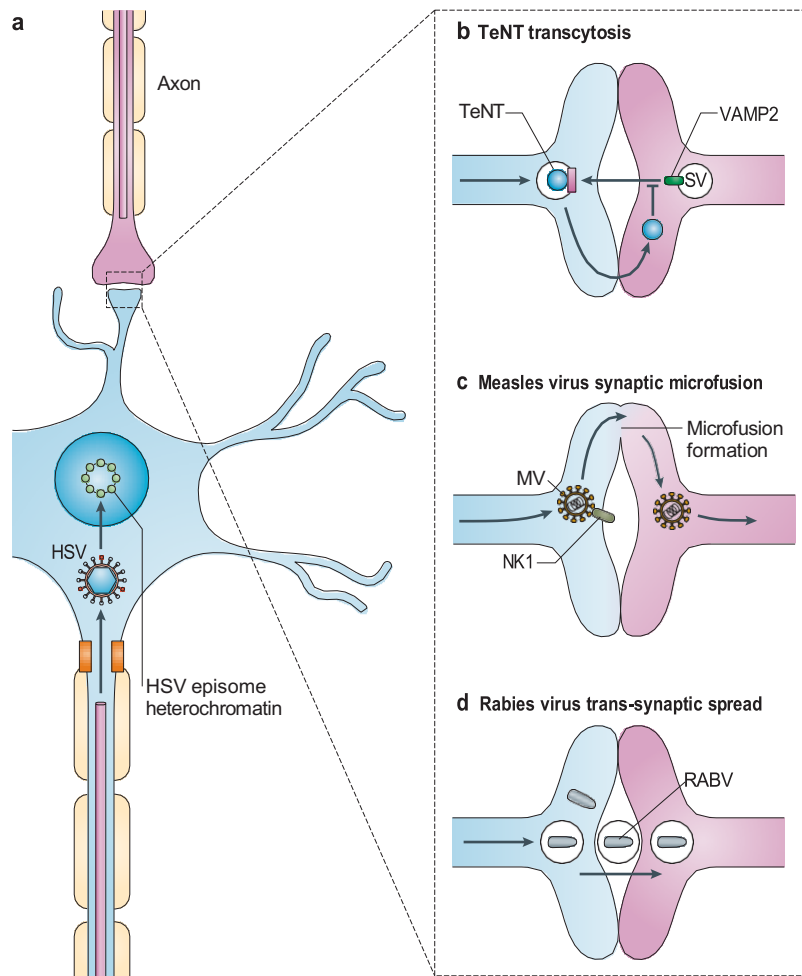


Figure 3- Somatodendritic sorting of pathogens and bacterial toxins.

Once in the cell body of neurons, microbial agents often have distinct fates. i) The DNA viruses of the *herpesviridae* family can package their genome in a chromatin-like structure and establish latency, ii) TeNT is transcytosed in adjacent inhibitory neurons where it cleaves locally components regulating neurotransmission, and iii) measles virus continues its journey in a new neuron by trans-synaptic spread possibly involving microfusion events that would allow its passage.

Box 1: The blood brain barrier and neurotropic pathogens.

The BBB is a neurovascular filtering system that allows the brain to be supplied with nutrients such as oxygen and glucose, while being protected from potentially toxic molecules present in the blood. It separates the cerebrospinal fluid from the bloodstream and under physiological conditions it does not allow agents such as bacteria to invade the CNS. Only small non-polar molecules can diffuse through the BBB, and because of this property, their chemical backbones represent a chemical template for many drugs designed to cross this barrier. The BBB is composed of microvascular endothelial cells that are firmly sealed with tight junctions, creating a physical barrier between the blood stream and the CNS. Other cell types such as astrocytes, microglia, pericytes and neurons provide molecular support to the BBB and form a functional neurovascular unit. Immune “privileged” however does not equal immune free: macrophages and leukocytes can actively migrate through this barrier and may in some case serve as a “Trojan horse” for pathogens entry in the CNS. For instance, HIV is often associated with neurological dysfunctions that result from virus invasion into the CNS via migration of HIV-infected leukocytes through the BBB. Ultimately, HIV will also lead to impairment of the BBB.

Box 2 –Viral vectors for gene transfer in the CNS.

There are more genes expressed in the mammalian CNS than in any other tissue, making the brain the most functionally and genetically complicated organ. This creates a formidable temporal and spatial complexity that confronts functional and therapeutic questions. The choice of vector, which can be from pathogens from numerous species, depends on many parameters including the duration of expression needed, the cell type targeted, cloning capacity, *etc*³⁹. Four major CNS vector platforms are briefly described.

Adenoviruses (Ads)

Ads are nonenveloped, DNA viruses. Adenovirus vector have cloning capacities of ~30 kb, can be purified into pure high titres ($>10^{13}$ pp/ml) and lead to long-term (years) expression *in vivo* without integration. *In vivo*, human serotype 5 (HAd5) vectors transduce ependymal cells oligodendrocytes, and neurons, but preferentially astrocytes (~60%) in the brain parenchyma. Limited retrograde transport has been reported. Vectors derived from CAV-2 preferentially transduce neurons *and* retrograde axonal transport is particularly efficient.

Adeno-associated viruses (AAVs)

AAVs are nonenveloped, single-stranded DNA viruses are in the family of Parvoviridae. Most serotypes have a cloning capacity of ~5 kb. Human and nonhuman primate serotypes transduce a wide variety of cells in the CNS, including neurons. The ability to be transported by axonal retrograde transport also varies depending on the serotypes. The duration of transgene expression also appears to vary depending on the serotype and target cells and host. The serotypes preferentially transduce neurons can lead to long-term expression *in vivo*.

Herpes simplex virus type 1 (HSV-1)

HSV type 1 is a nonenveloped DNA virus. Replication-incompetent vectors ($\sim 10^8$ infectious particles/ml) efficiently express transgenes in central and peripheral neurons and; iii) Amplicon vectors have a theoretical cloning capacity of 150 kb of DNA, efficiently express transgenes in central and peripheral neurons. The enormous potential of HSV vectors has been slow to come to fruition because of their immunogenicity and cytotoxicity.

Lentivirus

Lentiviruses are enveloped retroviruses belonging to the family *Retroviridae*. They have a cloning capacity of ~8 kb and can readily be pseudotyped with surface glycoproteins from other numerous viruses. VSVG, the vesicular stomatitis virus glycoprotein, is the most widely used glycoprotein to pseudotype vectors, leads to a preferential infection of neurons in the mouse brain. In general, if a specific pseudotyped lentivirus vector can be produced, its tropism and axonal retrograde transport will be determined by the Env and therefore similar to the tropism of the parent virus.