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Neuron-intrinsic immunity to viruses in mice and humans

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Summary

Viral encephalitis is a major neglected medical problem. Host defense mechanisms against viral infection of the central nervous system (CNS) have long remained unclear. The few previous studies of CNS-specific immunity to viruses in mice *in vivo* and humans *in vitro* have focused on the contributions of circulating leukocytes, resident microglial cells and astrocytes, with neurons long considered passive victims of viral infection requiring protection from extrinsic antiviral mechanisms. The last decade has witnessed the gradual emergence of the notion that neurons also combat viruses through cell-intrinsic mechanisms. Forward genetic approaches in humans have shown that monogenic inborn errors of TLR3, IFN-α/β, or snoRNA31 immunity confer susceptibility to herpes simplex virus 1 (HSV-1) infection of the forebrain, whereas inborn errors of DBR1 underlie brainstem infections due to various viruses, including HSV-1. The study of human pluripotent stem cell (hPSC)-derived CNS-resident cells has unraveled known (i.e. TLR3-dependent IFN-α/β immunity) and new (i.e. snoRNA31- or DBRl-dependent immunity) cell-intrinsic antiviral mechanisms operating in neurons. Reverse genetic approaches in mice have confirmed that some known antiviral mechanisms also operate in mouse neurons (e.g. TLR3 and IFN-α/β immunity). The search for human inborn errors of immunity (IEIs) underlying various forms of viral encephalitis, coupled with mouse models *in vivo*, and hPSC-based culture models of

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Declaration of interests

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CNS and peripheral nervous system cells and organoids *in vitro*, should shed further light on the cell- and tissue-specific mechanisms of host defense against viruses in the human brain.

Keywords

neurons; cell-intrinsic immunity; viral infection; inborn errors of immunity; mouse models; Human pluripotent stem cells

Introduction

Many viruses can underlie severe infection of the central nervous system (CNS) in humans. The incidence of viral encephalitis is at least 1-2 cases per 100,000 individuals per year, corresponding to a prevalence of ~1/1,000 births [1]. Viral encephalitis is life-threatening and most frequently affects children and the elderly [2,3]. Its clinical course is typically acute, but some forms can be chronic. There are at least 20 causal viruses, some of which are common, such as herpes simplex virus 1 (HSV-1), varicella zoster virus (VZV), enteroviruses (EV), influenza viruses (IV), measles virus (MV), JC virus (JCV) and noroviruses (NV), and some of which are rare, such as rabies virus, the Nipah and Hendra viruses, and West Nile virus (WNV) [4]. Most viruses are thought to reach the CNS by crossing the blood-brain barrier, although some viruses, including HSV-1, HSV-2, VZV and rabies virus, are known to invade the CNS via the peripheral nervous system (PNS). It is becoming increasingly clear that many viruses, including IV, EV, and WNV, can use both routes [5]. Regardless of the route taken to reach the brain, the invasion of the CNS by a neurotropic virus can result in CNS inflammation and neurodegeneration, with significant mortality and neurological sequelae in survivors. Many of the causal viruses have been identified. Patients with known cellular immunodeficiencies, such as those with acquired immunodeficiency related to human immunodeficiency virus (HIV) or organ transplantation, display higher vulnerability to viral infection of the CNS by some viruses including Epstein-barr virus (EBV), cytomegalovirus (CMV) and human herpes virus 6 (HHV6) [6]. However, viral encephalitis typically affects otherwise healthy people, and the molecular and cellular mechanisms underlying the pathogenesis of viral encephalitis remain unclear.

The CNS is widely considered an immunological sanctuary, although it is increasingly recognized that ‘immune responses’ mediated by circulating leukocytes and CNS-resident cells can take place in the CNS, either to protect the brain against damage due to autoimmune, infectious, neurodegenerative, and oncogenic processes, or to contribute to the autoimmune responses in this organ [7]. The mechanisms of host defense against viral infection of the CNS have long remained unknown. Most previous studies on CNS immunity have focused on the contribution of leukocytes and two populations of glial cells — microglia and astrocytes —with neurons generally considered to be passive victims of viral infections [8–13]. However, the last decade has witnessed the gradual emergence of the notion that neurons make use of intrinsic mechanisms to fight viral attacks. This notion emerged from initial studies testing the hypothesis that CNS-specific cell-intrinsic immunity, rather than leukocyte-mediated immunity, is crucial for host defense against

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HSV-1 in humans [14–16]. We review here recent progress towards understanding cell-intrinsic antiviral immunity in specific types of neurons, in both humans and mice.

Antiviral immunity in rat and mouse neurons: type I IFN-related mechanisms

Since the discovery of type I interferons (IFNs) in 1957 [17], tremendous efforts have been made to determine the role of these cytokines in defenses against diverse RNA and DNA viruses, in various mouse or rat models of infection *in vivo* and *in vitro.* Perhaps the first clue to the antiviral activity of type I IFNs in neurons came from the observation that the *in vitro* treatment of cultured isolated embryonic rat dorsal root ganglion neurons with recombinant IFN-α or IFN-β inhibited virus replication in these neurons in a dose-dependent manner [18]. Various studies have since shown that the protective effect of type I IFNs differs between types of neurons and viruses. IFN-α treatment in rat hippocampal neurons induces no protective response against Borna disease virus [19]. Similarly, IFN-β does not protect embryonic mouse hippocampal neurons against infection with Theiler's murine encephalomyelitis virus (TMEV) or vesicular stomatitis virus (VSV), possibly due to the lack of induction of certain antiviral IFN-inducible genes (ISGs), such as the ApoL9b gene, by IFN-β in these neurons [20]. Nevertheless, mouse hippocampal neurons seem to have high basal levels of the type I IFNs thought to be crucial for the early control of MV infection [21]. Major differences in IFN responsiveness have also been observed between the granule cell neurons of the cerebellum and the cortical neurons in mice, with granule cell neurons having higher basal ISG expression levels and responding more strongly to IFN-β treatment, consistent with the lower susceptibility of granule cell neurons and the cerebellum to WNV infection [22]. IFN-β treatment has also been shown to inhibit VSV replication strongly in mouse primary olfactory neurons [23]. All these observations suggested a neuron cell type-specific protective effect of type I IFNs against viral infections, for at least some of the viruses tested.

Type I IFNs are produced by CNS-resident cells, including neurons, during viral infections [24,25]. Mouse models with specific gene knockouts have therefore been used for *in vivo* and *in vitro* studies investigating the requirement for cellular IFN pathways in host defense against viral infections of the CNS [22,26]. The outcome of experimental viral infections in mouse models may ultimately be influenced by viral load, virus strain, route of infection and animal strain. However, studies performed over the last decade have provided evidence of type I IFN-mediated cell-intrinsic antiviral mechanisms in mouse neurons *in vitro* and *in vivo.* In cultured trigeminal neurons isolated from mice lacking Stat1, a key transcription factor of the cellular type I IFN response pathway, defective neuron axonal IFN-β signaling resulted in a failure to restrict HSV-1 infection, as shown by comparisons with wild-type (WT) neurons. These findings were consistent with the uncontrolled viral infection observed in the trigeminal ganglia and brain stem of Stat1-deficient mice *in vivo* [27]. Hippocampal neurons from mice lacking Irf1, an ISG-inducing transcription factor with an unclear role in type I IFN responses, were found to display higher levels of VSV replication than WT mouse neurons, consistent with the impaired control of a late-phase viral infection of the mouse brain, which was not restricted by type I IFNs and affected the cerebrum,

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cerebellum and brain stem [28]. In mice devoid of type I IFN signaling (due to disruption of the *Ifnar1* locus) specifically in calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα)-expressing neurons, infected with mouse hepatitis virus A59 (MHV-A-59), viral spread in Ifnar1-deficient mice was enhanced in the neurons of the forebrain, mid- and hindbrain, and the spinal cord, but not in the cerebellum [29]. Finally, type I IFN signaling in neurons seems to be essential for microglial activation and protection against lethal VSV encephalitis in mice, as shown by studies based on the conditional knockout of Ifnar1 (and therefore of type I IFN signaling) in neurons or microglia, which demonstrated the importance of type I IFN-dependent crosstalk between neurons, astrocytes and microglia [30]. Thus, studies of gene-targeted mouse models have provided clear evidence that neuron-intrinsic type I IFN-related immunity to viruses may be crucial for the brain region-specific control of viral infections.

Antiviral immunity in mouse neurons: other antiviral mechanisms

A role for apoptosis in antiviral immunity in neurons was first suggested by early investigations searching for mechanisms underlying the age-dependent morbidity and mortality of viral encephalitis [31]. In mouse *in vivo* and *in vitro* studies, an alphavirus, Sindbis virus (SV), caused acute encephalomyelitis in an age-dependent manner in mice, and this age-dependent susceptibility to fatal infection appeared to be due to a greater intrinsic susceptibility to the induction of apoptosis in the neurons of the dorsal root ganglion and spinal cord in young mice. The contributions of apoptosis and other programmed cell death pathways to host antiviral defense mechanisms have been studied in depth in many other organs and cell types [32], but relevant data for neurons and other CNS-specific cells remained limited. In one study published in 2017, cortical neurons isolated from mice lacking RIPK3, a key component of the cellular apoptosis and necroptosis pathways, produced abnormally low levels of chemokines upon infection with WNV [33]. This led the authors to suggest that the enhanced susceptibility to WNV of RIPK3-deficient mice *in vivo* was due to the suppression of neuronal chemokine expression, leading to lower levels of recruitment of T lymphocytes and myeloid cells to the CNS for the restriction of viral infection [33]. By contrast, in another study published in 2019 [34], the cell type-specific targeted expression or deletion of *RIPK3* in forebrain cortical neurons decreased and increased, respectively, the viral load in the mouse brain following infection with Zika virus (ZIKV). This study also showed, in cultured mouse cortical neurons, that ZIKV infection activated the nucleotide sensor ZBP1 and downstream RIPK1 and RIPK3 signaling for the restriction of viral replication, by altering cellular metabolism via the upregulation of IRG1, an enzyme, and the production of the metabolite, itaconate [34]. These two studies indicated that key molecular components of the cell apoptosis and necroptosis pathways could control mouse cortical neuron-intrinsic immunity to viruses through their canonical and non-canonical functions.

Autophagy is a highly conserved catabolic pathway that is crucial for maintaining the basal turnover of cellular components. Interestingly, the *in vivo* evidence for an antiviral role of autophagy in mammalian animal hosts is mostly restricted to viruses specifically targeting neurons, including SIV and HSV-1 [35]. DRG neurons from Atg5 knockout mice, which have a defective autophagy pathway, displayed uncontrolled HSV-1 replication [36]. This

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observation was further confirmed in DRG neurons from mice with conditional deletions of Atg5 limited to neurons, suggesting that autophagy governs a peripheral nervous system cell-intrinsic antiviral mechanism against HSV-1 infection, at least in mouse DRG neurons. The formation of autophagic clusters, in a type I IFN signaling-dependent manner, has also been reported in cultured mouse trigeminal ganglion (TG) neurons, following infection with HSV-1, HSV-2, pseudorabies virus (PrV) and, to a lesser extent, yellow fever virus, but not after infection with MHV68, VACV, TMEV or VSV, although the physiological relevance of this observation remains unclear [37]. Thus, in parallel to type I IFN responses, a number of alternative mechanisms for governing neuron-intrinsic antiviral immunity in mice have been proposed, principally involving host cell death pathways, including apoptosis, necroptosis and autophagy, although key cell death pathway molecules may also be involved, through their non-canonical functions [33].

Neuron-intrinsic antiviral immunity in humans: TLR3-dependent type I IFN responses

Cell-intrinsic antiviral immunity had never been studied in human neurons before we used human pluripotent stem cell (hPSC)-derived CNS cells to dissect the cellular basis of the pathogenesis of HSV-1 encephalitis (HSE). Over the last 15 years, we have tested the hypothesis that childhood HSE results from single-gene inborn errors of immunity to HSV-1 in the human CNS [38,39]. Our human genetic studies have progressively led to the dissection of neuron-intrinsic antiviral immunity in humans, through genetically defined, patient-specific hPSC-based disease modeling [40]. HSV-1 enters the human body via the oral or nasal epithelium and infects neurons, subsequently establishing latency in the TG sensory ganglia [41]. More than 85% of adults worldwide have antibodies against HSV-1, which causes asymptomatic infection or benign, self-healing disease in most individuals. In about 1~2/10,000 infected individuals of all ages, HSV-1 invades the CNS via the olfactory bulb, causing forebrain HSE (~95% of cases), or via the TG nerves, causing brainstem HSE (~5% of cases) [3,42]. HSE is fatal in more than 70% of patients if left untreated, and most acyclovir-treated survivors develop mild to severe neurological sequelae [43]. Typically, HSE is isolated, and affects otherwise healthy individuals who are not particularly susceptible to other clinical forms of HSV-1 infection [3,44]. Remarkably, HSE has not been reported in children with AIDS or conventional primary immunodeficiencies of hematopoietic cells, suggesting that inborn errors of leukocytes are unlikely to be causal [6]. A definitive link between HSV and HSE was established in 1941 [45]. The pathogenesis of HSE has long remained unclear, despite identification of the causal virus. However, very rare cases of “syndromic” HSE combined with mycobacterial diseases have been observed: in a child with autosomal recessive (AR) STAT1 deficiency with impaired cellular responses to IFN-α/β, IFN-λ and IFN-γ [46–48], and a child with a *IKBKG* (*NEMO*) mutation whose cells displayed poor IFN-α/β and IFN-λ production [49]. Both disorders involved an impairment of cell-intrinsic immunity in leukocytes and, probably, in all other cell types. These observations suggested that human genetic defects affecting intrinsic immunity in the CNS, probably related to IFN-mediated antiviral defense, may underlie HSE, in at least some children.

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Genetic studies of isolated forebrain HSE led to the discovery of single-gene inborn errors of the Toll-like receptor 3 (TLR3)-dependent pathway of interferon (IFN)-α/β and -λ production, with mono- or biallelic mutations of six TLR3 pathway genes (*TLR3, UNC93B1, TICAM1 (TRIF), TRAF3, TBK1* or *IRF3*) [14,50–57] and one gene of the type I IFN response pathway (IFNAR1) [58] (Figure 1). Toll-like receptor 3 (TLR3) is an endosomal receptor of dsRNA [59], governing a pathway of IFN-α/β and IFN-λ induction. IFNAR1 is one of the two subunits of the receptor of type I IFNs. It governs activation of the downstream pathway by all type I IFNs. These findings, together with the previous observation of syndromic HSE in patients with X-linked recessive (XR) NEMO deficiency [49] or AR complete STAT1 deficiency [46], suggested a crucial role for TLR3-dependent IFN-α/β immunity in host defense against HSV-1 in the CNS. It has also been suggested that other mutations of these and otherTLR3 or IFN pathway genes may underlie HSE in children or adults [60–64]. Interestingly, a minority of patients with mutations of *TLR3* have developed pneumonia due to influenza A virus or SARS-CoV-2, or ophthalmic zoster [65,66]. Nevertheless, TLR3-mediated responses to dsRNA and antiviral immunity seem to be redundant in most TLR3-expressing cell types, including leukocytes in particular, probably accounting for the lack of viral dissemination during the course of HSE [51,55]. TLR3- and type I IFN-mediated cell-intrinsic immunity was initially studied with dermal fibroblasts as surrogate cells, and then with hPSC-derived CNS- and PNS-resident cells from patients with forebrain HSE and mutations of TLR3 pathway genes. Consistent with the findings of studies showing that mouse Tlr3 is required for responses to HSV in neurons and astrocytes [67,68], TLR3 pathway-deficient human fibroblasts [14,51–55,57] and iPSC-derived cortical neurons and oligodendrocytes [69] were found to be much more susceptible to HSV-1 infection than control cells. More recently, the molecular basis of TLR3-dependent cell-intrinsic antiviral immunity was attributed to a role as the rheostat in controlling basal cellular levels of IFN-β immunity in fibroblasts and cortical neurons [70]. By contrast, *in vitro*-differentiated human UNC-93B-deficient astrocytes or neural stem cells, and TLR3-deficient peripheral TG neurons are as susceptible to infection as control cells [71], probably due to insufficient basal levels of IFN immunity, as pretreatment with IFN-α or poly(I:C) protected TG neurons from healthy controls, but not those from TLR3-deficient patients, against HSV-1 infection [71]. These data provided a plausible cellular basis for the pathogenesis of genetically driven forebrain HSE, suggesting that TLR3-dependent, type I IFN-mediated cortical neuron-intrinsic anti-HSV-1 immunity, as opposed to the innate and adaptive immunity mediated by leukocytes and related cells, was crucial for host defense against HSV-1 in the human forebrain [14,55].

Neuron-intrinsic immunity in humans: new antiviral mechanisms

An unbiased genome-wide search of new HSE-causing genes via whole-exome sequencing led to the discovery of rare heterozygous variants of *SNORA31*, which encodes small nucleolar RNA 31 (snoRNA31), in five unrelated patients with forebrain HSE [72]. SnoRNA31 is a 130-nucleotide snoRNA of the H/ACA box class. *SNORA31* is highly conserved in the general population, and ubiquitously expressed in various human cell types [73]. SnoRNA31 had only one predicted function: directing the isomerization of uridine residues to generate pseudouridines in position 218 of the 18S ribosomal RNA (rRNA) and

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position 3,713 of the 28S rRNA [74]. Studies with hPSC-derived cortical neurons showed that SnoRNA31 was produced and functional in human cortical neurons, as a CRISPR/Cas9-introduced biallelic deletion within *SNORA31* impaired the pseudouridylation of the uridine residue in position 218 of the ribosomal RNA 18S in isogenic hPSC-derived CNS neurons. Moreover, snoRNA31 is a CNS neuron-intrinsic HSV-1 restriction factor, as CRISPR/Cas9-introduced biallelic and monoallelic *SNORA31* deletions render neurons highly susceptible to HSV-1. Accordingly, CNS cortical neurons derived from the iPSCs of patients with *SNORA31* mutations are highly susceptible to HSV-1, like those from TLR3- or STAT1-deficient patients. Exogenous IFN-β renders neurons with *SNORA31* and *TLR3* mutations, but not those with *STAT1* mutations, resistant to HSV-1 infection. Finally, transcriptomic analyses of *SNORA31*-mutated hPSC-derived cortical neurons have shown these cells to have normal responses to stimulation with TLR3 and IFN-α/β, but abnormal responses to HSV-1, suggesting that AD snoRNA31 deficiency impairs intrinsic immunity by a distinctive mechanism *in vitro*, and, by inference, underlies HSE *in vivo* by a distinctive mechanism. The modified transcriptome-level response to HSV-1 associated with snoRNA31 deficiency may affect the expression of one or more of the effectors induced by TLR3 or IFN-α/β, thereby impairing anti-HSV-1 immunity in these cells. Alternatively, snoRNA31 may interfere with HSV-1 propagation directly, by interacting with viral transcripts. Future studies should address the fine molecular mechanism by which snoRNA31 contributes to the control of HSV-1 in CNS cortical neurons and, potentially, in other CNS-resident cell types. The discovery of AD snoRNA31 deficiency as a genetic etiology of forebrain HSE demonstrated that snoRNA31 is a new CNS neuron-intrinsic HSV-1 restriction factor. It also provided evidence that snoRNAs can be essential for host defense.

The search for single-gene inborn errors of immunity underlying brainstem viral encephalitis (BVE) led to the discovery of another new cell-intrinsic antiviral mechanism, mediated by debranching enzyme 1 (DBR1). AR partial DBR1 deficiency was reported in 2018, in otherwise healthy children with BVE caused by various viruses, including HSV-1, influenza B virus (IBV), and norovirus [75]. DBR1 is the only known RNA lariat-debranching enzyme in humans. As inferred from studies performed in yeast, DBR1 hydrolyzes 2’5’-phosphodiester linkages at the branch points of intron lariat RNAs, facilitating their rapid turnover [76]. No connection between DBR1 and host immunity to infection was previously known. In most patients with devastating BVE, the brainstem is the only region of the CNS affected, suggesting that, if there is an inborn error of immunity underlying BVE, it may affect brainstem-specific immunity. Two of the five DBRl-deficient children developed brainstem HSE. DBR1 protein levels are highest in the brainstem and spinal cord, suggesting that DBR1 deficiency disrupts immunity in brainstem-resident cells [75]. DBRl-deficient fibroblasts from the patients, whose TLR3 and IFN-α/β response pathways were intact, were found to contain higher RNA lariat levels than control cells, this difference becoming even more marked during HSV-1 infection. Moreover, DBR1-deficient fibroblasts were highly susceptible to HSV-1 and VSV, like TLR3- and STAT1-deficient fibroblasts [75]. The underlying molecular mechanism remains elusive. The accumulation of RNA lariats may impair virus recognition by host cells, thereby damaging cell-intrinsic defenses against viral invasion. DBR1 may also regulate the processing of some host protein-

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coding RNAs, non-coding RNAs (ncRNAs) [77–79], or viral RNA lariats [80–83], thereby controlling cell-intrinsic defense against intracellular virus replication. It could be speculated that inherited DBR1 deficiency probably underlies viral infection of the brainstem through the disruption of brainstem-specific and cell-intrinsic immunity to viruses, including HSV-1. Future investigations should test these hypotheses in hPSC-derived brainstem neurons and other CNS cell types.

Concluding remarks

The human brain contains ~10^{11} neurons, of diverse subtypes, with only a limited regeneration capacity. Based on the large body of studies on cultured rat and mouse primary neurons and reverse genetic studies *in vivo* and *in vitro*, it has been possible to establish the roles of antiviral pathways (i.e. type I IFNs, cell death pathways) in various types of neurons, against multiple neurotropic viruses. Studies of the human inborn errors of immunity underlying viral encephalitis led to the discovery of HSE-causing gene mutations disrupting known (TLR3-dependent IFN-α/β immunity) and new (dependent on DBR1 or snoRNA31) antiviral mechanisms, providing opportunities for the use of hPSC-based disease modeling for experimental testing of the hypothesis that CNS-specific cell-intrinsic immunity, rather than leukocyte-mediated immunity, is crucial for host defense against HSV-1. It is now clear that neurons use direct mechanisms to fight viral attacks, with different molecular pathways used by different types of neurons. The added value of human forward genetic studies is that such studies provide information about host defense genes under natural conditions of infection, through an unbiased, genome-wide approach. Future studies making use of next-generation sequencing technologies and genome-wide computational tools [84–89] to search for inborn errors of immunity conferring predisposition to various types of isolated viral encephalitis, such as BVE due to IBV or norovirus [75], are likely to discover additional, key molecules controlling CNS-specific immunity to viruses. Such discoveries will, in turn, pave the way for further studies of CNS tissue-specific, cell-intrinsic, as opposed to leukocyte-mediated, innate or adaptive immunity to viruses in humans [16]. Known and new antiviral mechanisms are likely to be discovered. Each of the various types of neurons may be crucial for antiviral immunity in a specific region of the brain, for the control of one specific virus or a small subset of viruses, either as the master ‘protector’ or in coordination with other cells resident in or infiltrating into the brain during infection [30,69,90–95]. Both mouse *in vivo* models and human hPSC-mediated CNS- and PNS-specific cell and organoid models will be powerful tools for unraveling cell-intrinsic immunity to viruses in neurons and other cell types, in great breadth and depth.

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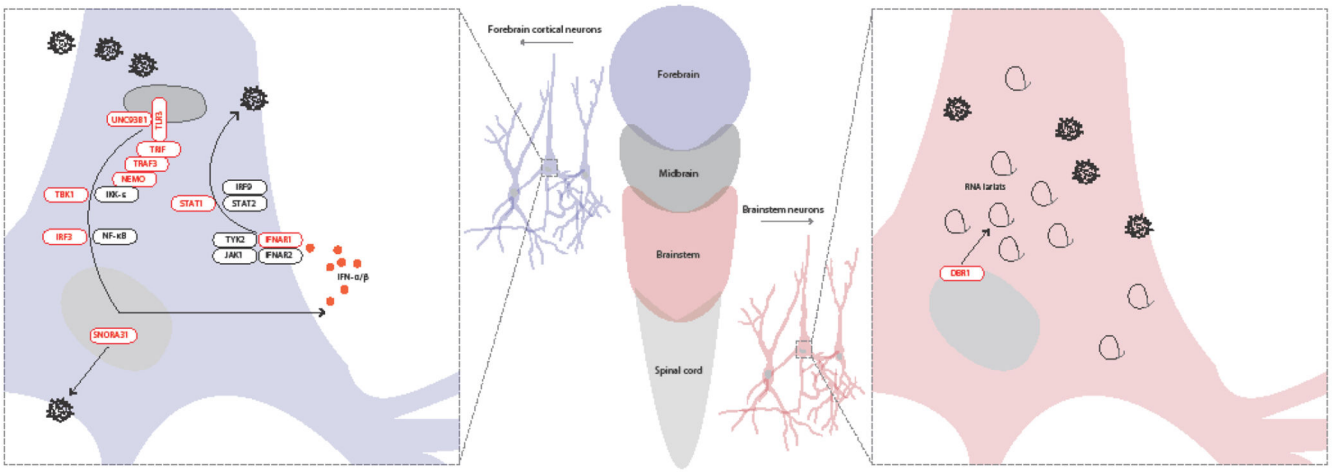

Figure 1. Illustration of neuron-intrinsic type I IFN immunity or new antiviral mechanisms crucial for defense against viral infection of the central nervous system in humans.

TLR3-type I IFN circuit and snoRNA31 immunity in forebrain HSV-1 infection (left), and DBR1-mediated RNA lariat metabolism in brainstem viral infection (right). TLR3 controls basal levels of IFN-β-mediated anti-HSV-1 immunity in cortical neurons and oligodendrocytes. DBR1 controls brainstem-specific immunity to viruses (HSV-1, influenza virus, norovirus), presumably in neurons and other cells of the brainstem, which is composed of the midbrain and hindbrain. In red: molecules shown to be involved in TLR3-IFN-α/β-mediated or snoRNA31-mediated immunity to HSV-1 in cortical neurons (left), or involved in DBR1-mediated RNA lariat metabolism controls brainstem viral encephalitis due to HSV-1, influenza virus and norovirus (right). In dark gray: other key molecules of the TLR3-type I IFN circuit that have not been shown to be involved in central nervous system antiviral immunity in humans.