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Enterovirus Infections of the Central Nervous System Review

Ross E. Rhoades, **Jenna M. Tabor-Godwin**, **Ginger Tsueng**, and **Ralph Feuer**
Cell & Molecular Biology Joint Doctoral Program, Department of Biology, San Diego State University, San Diego, CA, 92182-4614, USA

Abstract

Enteroviruses (EV) frequently infect the central nervous system (CNS) and induce neurological diseases. Although the CNS is composed of many different cell types, the spectrum of tropism for each EV is considerable. These viruses have the ability to completely shut down host translational machinery and are considered highly cytolytic, thereby causing cytopathic effects. Hence, CNS dysfunction following EV infection of neuronal or glial cells might be expected. Perhaps unexpectedly given their cytolytic nature, EVs may establish a persistent infection within the CNS, and the lasting effects on the host might be significant with unanticipated consequences. This review will describe the clinical aspects of EV-mediated disease, mechanisms of disease, determinants of tropism, immune activation within the CNS, and potential treatment regimes.

Introduction

Enterovirus (EV) infections are a significant cause of morbidity and mortality throughout the world. The EV genus is part of the picornavirus family and includes such notable members as poliovirus (PV), coxsackievirus (CV), and enterovirus-71 (EV-71). EVs have been associated with many human diseases, including myocarditis (Klingel *et al*., 1992), pancreatitis (Ramsingh, 2008), and chronic inflammatory myopathy (Tam and Messner, 1999). Diseases caused by EV are not restricted to the well-known scourge of mankind throughout history, recognized as poliomyelitis. Many non-polio human EVs are quite common, causing an estimated 10–15 million or more symptomatic infections in the US alone. Non-polio EVs are known to target the central nervous system (CNS) and are responsible for numerous clinical manifestations, including encephalitis, and meningitis (Michos *et al*., 2007). The long-term consequences of EV infection upon the CNS are largely unknown. However, these viruses are known to persist, and the presence of viral RNA by itself is known to be potentially pathogenic in some cases. Also, EVs have been linked to autoimmune-like diseases, including diabetes, chronic inflammatory myopathy, and chronic myocarditis, perhaps in part due to the long-term presence of viral material. Therefore, EVs may be able to persist within the CNS potentially causing lasting neuropathology.

The original classification of enteroviruses included the four groups: Polioviruses (PVs), Coxsackie A viruses, Coxsackie B viruses, and ECHO (***E***nteric ***C***ytopathic ***H***uman ***O***rphan) viruses. However, the significant level of phylogenetic overlap among the four groups has

*Correspondence to: Dr. Ralph Feuer, Cell & Molecular Biology Joint Doctoral Program, Department of Biology, San Diego State University, 5500 Campanile Drive; San Diego, CA 92182-4614, Phone: 619-594-7377 Fax: 619-594-0777, rfeuer@sciences.sdsu.edu.

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led to a new classification system of consecutive numbers for the more recently isolated viruses (such as Enterovirus-71, Enterovirus-72, etc.) (Oberste *et al.*, 2002). Our review will cover the most common and more extensively studied types of enteroviruses. However, the large number of circulating strains in human populations alone suggests a potential role for these viruses for unknown or unappreciated human diseases (Victoria *et al.*, 2009). Also, vaccine design against enteroviruses may be difficult based on significant EV genetic variability.

Polioviruses (PVs)

Polioviruses (PVs) are perhaps the most studied and characterized of the enteroviruses, especially given the clinical consequences of infection which can infect motor neurons of the anterior horn of the spinal cord and lead to paralytic poliomyelitis in humans. Although the advent of polio vaccination has proven effective in greatly reducing the incidence of disease, some cases still occur and these cases appear to be on the rise; outbreaks following wild PV importations into previously polio-free countries continue to be an ongoing risk (MMWR, 2010). Over the years, basic research on PV has revealed the mechanics of protein translation and viral replication, immune activation, vaccine development, and viral population dynamics (Cameron *et al.*, 2010; Pfeiffer, 2010). These discoveries have provided valuable information for the ongoing research of non-polio enteroviruses which continue to cause encephalitis and meningitis in humans.

Coxsackieviruses (CVs)

Coxsackieviruses (CVs), members of the enterovirus genus, may cause severe morbidity and mortality, particularly in the very young (Romero, 2008; Tebruegge and Curtis, 2009). CV infection during pregnancy has been linked to an increase in spontaneous abortions, fetal myocarditis (Ornoy and Tenenbaum, 2006), and neurodevelopmental delays in the newborn (Euscher *et al.*, 2001). Infants infected with CV have been shown to be extremely susceptible to myocarditis, meningitis and encephalitis with a subsequent mortality rate as high as 10%. Also, a number of delayed neuropathologies have been associated with previous CV infection, including schizophrenia (Rantakallio *et al.*, 1997; Suvisaari *et al.*, 2003), encephalitis lethargica (Cree *et al.*, 2003), and amyotrophic lateral sclerosis (Woodall and Graham, 2004; Woodall *et al.*, 1994). Of note, few studies have been done to determine the lasting consequences of CV infection upon surviving individuals despite the relatively common occurrence of CV meningo-encephalitis in infants.

Enterovirus-71 (EV-71)

Enterovirus-71 (EV-71) is a major public health issue across Asia and of increasing concern globally, causing hand foot and mouth disease with potential neurological complications (Solomon *et al.*, 2010). The brain stem is the most likely target for infection, and infection may cause serious clinical disease and psychological disorders in young children (Lee *et al.*, 2009). New outbreaks of EV-71 have occurred across Asia, perhaps due to the ability of the virus to rapidly evolve (Solomon *et al.*, 2010).

Echoviruses (ECHO-Vs) and Parechoviruses (P-ECHO-Vs)

Echoviruses (ECHO-Vs) were first isolated from the feces of asymptomatic patients; however, these viruses are now recognized to be associated various human diseases, including aseptic meningitis. ECHO-Vs are highly infectious and preferentially target infants and young children. These viruses can cause a mild, nonspecific illness similar to that of CVs. Also, parechoviruses (P-ECHO-Vs) have been found to be associated with encephalitis and white matter injury in neonates (Gupta *et al.*, 2010; Verboon-Maciolek *et*

al., 2008a), with similarities to EV infections (Verboon-Maciolek *et al.*, 2008b). Although originally described as ECHO-V-22 and ECHO-V-23, their distinct genetic features have led to their re-classification (Harvala and Simmonds, 2009).

Theiler's Murine Encephalomyelitis Virus (TMEV)

Although the picornavirus, Theiler's murine encephalomyelitis virus (TMEV), is designated a cardiovirus by phylogenetic analyses, much can be learned from the existing literature on TMEV and CNS disease. Also, many similarities exist between EVs and TMEV in terms of their molecular biology, tropism, ability to persist, and induction of neuropathology. Therefore, we have included relevant information on TMEV and CNS pathology in this review. Two main subtypes of TMEV are studied in animal models. The strain GDVII produces acute encephalomyelitis quickly in mice. The DA, WW, or BeAn strains persist within the CNS and induce demyelinating plaques similar to those seen in multiple sclerosis. More detailed reviews of TMEV tropism, immune response, persistence, and demyelination has been published by other research groups (Brahic *et al.*, 2005; Lipton *et al.*, 2005; Oleszak *et al.*, 2004; Olson *et al.*, 2005; Rodriguez, 2007).

Molecular Biology of Enteroviruses

To better understand EV infection of the CNS and potential consequences upon glial or neuronal host cells, a full comprehension of EV structure and molecular biology is essential. EVs consist of icosahedral, non-enveloped viruses of approximately 30nm in diameter. These viruses generally have the proclivity to survive acidic environment, enabling their passage through the stomach following fecal/oral transmission and eventual invasion into the small intestine. The viral capsid contains the positive-strand RNA genome, ranging in size from ~7.2 – 8.5 kb. The viral genome includes several cis-acting RNA elements which play a role in replication and/or translation. These cis-acting elements include the 5′ and 3′ nontranslated regions (NTRs), the cis-acting replication element (CRE) and the 3′ poly(A) tail (Steil and Barton, 2009).

A single open reading frame (ORF) within the NTRs encodes a long viral protein which undergoes post-translational cleavage into the mature viral proteins. These viral proteins include the four structural proteins, VP1-4 which comprise the viral capsid, and seven non-structural proteins (2A–C and 3A–D) including the primer- and RNA-dependent RNA polymerase ($3D^{Pol}$) (Kitamura *et al.*, 1981). In addition, one of the viral proteins 3B (also known as VPg) covalently attaches to the 5′ end of the viral genome. VPg plays an essential role in viral replication by acting as a primer for RNA synthesis. Utilizing PV as a model, VPg has been shown to undergo uridylylation to VPgpUpU by $3D^{Pol}$ using adenosine residues in the stem-loop structure of CRE, located in the 2C region of the EV ORF, as a template (Goodfellow *et al.*, 2000; Paul *et al.*, 2003). Recent elucidation by NMR suggests that VPg undergoes a structural change upon uridylylation which allows viral replication to be more efficient and selective (Schein *et al.*, 2010). Since uridylylation is not observed during host cell transcription, this process may be a unique therapeutic target against EV infections, given that point mutations that block uridylylation may prevent viral growth (Paul *et al.*, 1998). While VPg plays a role in both positive- and negative-strand RNA synthesis, it is still unknown how approximately 40 to 70 copies of positive-strand genome are produced for every negative-strand genome during an EV infection (Novak and Kirkegaard, 1991). Intriguingly, this ratio decreases upon the establishment of CVB3 persistence within the CNS suggesting that a double-stranded RNA genomic structure might assist in virus stability during persistence (Feuer *et al.*, 2009).

Despite the unanswered questions with regards to the ratio of EV positive to negative strand synthesis during both acute and persistent infection, many other aspects of their translation

and replication are well understood. For example, the well-characterized internal ribosome entry site (IRES) in the 5′ NTR directly interacts with host cell ribosomes to initiate translation of the viral genome upon entering the cell (Pelletier and Sonenberg, 1988). Immediate translation is necessary in order to produce the primer- and RNA-dependent RNA polymerase needed for viral replication, as well as the other viral proteins that facilitate replication. The viral genome is translated into a ~250-kDa polyprotein, which undergoes a series of cleavages at predetermined cleavage sites carried out by the viral proteases $2A^{pro}$ and $3CD^{pro}$. During proteolytic processing, several precursor protein intermediates also play important roles during viral infection.

Only after viral protein translation is completed does negative-strand replication begin since translation and replication cannot occur at the same time (Gamarnik and Andino, 1998). A ternary complex then forms to facilitate both positive- and negative-strand synthesis at the 5′ cloverleaf structure of the 5′ NTR that includes the host poly(rC) binding protein (PCBP) and the viral precursor protein 3CD (Vogt and Andino, 2010). This complex associates with poly(A) binding protein 1 (PABP1), which is bound to the poly(A) tail of the genome, and causes the genome to circularize during negative-strand synthesis (Herold and Andino, 2001). Further analysis of the crystal structure of 3CD has shown that the N-terminus is in close proximity to the VPg binding site, and that 3CD may be involved in the uridylylation reaction as part of the replication complex (Marcotte *et al.*, 2007). Moreover, 3AB (also the VPg precursor protein) is a membrane bound protein (Fujita *et al.*, 2007), thus providing a spatial link between viral replication on host membranes, viral priming and the replication complex.

Enteroviruses and Autophagy

Interestingly, EVs have been shown to benefit from, and induce the cellular degradation process known as autophagy (Huang *et al.*, 2009; Suhy *et al.*, 2000; Wong *et al.*, 2008). It is thought that EVs use the autophagosome membrane as a scaffold for viral replication. A recent publication has shown an increase in viral replication linked to the induction of autophagy during CV infection in rat primary neurons (Yoon *et al.*, 2008). Autophagy is known to play an important role in preventing cellular damage in neurons, and this normal cellular process has recently been shown to be highly upregulated in neurons during short-term fasting (Alirezaei *et al.*, 2010; Simonsen *et al.*, 2008).

However, it still remains to be determined if EVs use autophagy for viral replication in all cells type within the CNS. For example, given their unique and critical role in development and CNS cellular homeostasis, neural stem cells might respond differently and uniquely to microbial infection. Furthermore, it will be important to elucidate whether autophagy plays a role in the establishment of viral persistence in the CNS. The induction or inhibition of autophagy in cells harboring viral material may alter EV persistence. Autophagy inhibitors have been shown to preferentially decrease extracellular as compared to intracellular PV production, thus providing an attractive model for non-cytolytic virus release from the cell (Jackson *et al.*, 2005). Also, one might expect that an increase in the level of autophagy which might recycle organelles or other cellular components associated with EV persistence may hasten viral RNA degradation.

Host Cell Translational Shutdown Following Enterovirus Infection

In addition to their roles in viral replication and post-translational processing, several viral proteins have profound effects on the host cell during the course of infection effectively modulating cellular transcription, translation and protein secretion. Host cell transcription is suppressed by the viral protease 3C, which is carried into the nucleus by the nuclear localization signal located on 3D when in its precursor form (Sharma *et al.*, 2004).

Additionally, the 3C protein of EV-71 has recently been shown to block polyadenylation of host mRNA by cleaving a critical factor needed for this process (Weng *et al.*, 2009). In turn, viral protease 2A halts host cell cap-dependent translation by cleaving eIF4G, which in a clever twist also enhances the translation of viral mRNA by stabilizing polysomes (Etchison *et al.*, 1982; Kempf and Barton, 2008). Furthermore, viral proteins 2B and 3A inhibit host cell protein secretion, as plasma membrane and secretory protein transport are halted (Doedens and Kirkegaard, 1995).

In general, the host cell most likely faces a grim ultimate fate due the cytolytic nature of EVs. Viral protein 2B is a highly efficient viroporin which can permeablize the host cell membrane, as well as those of nearby cells (Madan *et al.*, 2010). However, the pathways activated in the cell during infection can be somewhat contradictory. In what is thought to be an attempt by the virus to keep the cell alive long enough to complete viral replication and eventually escape its cellular confinements. EVs induce both anti-apoptotic (3A and 2B proteins) and pro-apoptotic effects (VP2, 2A and 3C proteins) on the host cell (Whitton *et al.*, 2005). Furthermore, viral protein synthesis, which is boosted by 2A, has been shown to be necessary for apoptosis (Shih *et al.*, 2008). With several reports demonstrating apoptosis in neurons after EV infection, and the role of 2A in regulating translation, it is interesting to speculate that shutoff of host cell translation by EVs may directly cause apoptotic cell death in neurons. We have shown that CVB3 induced apoptosis within pyramidal neurons in the hippocampus (Feuer *et al.*, 2003). In contrast, TMEV may indirectly induce apoptosis of nearby pyramidal neurons in the hippocampus in non-cell autonomous manner (Buenz *et al.*, 2009).

Virus Entry – Cell Receptors

EVs use a wide array of receptors and entry mechanisms to invade the host cell. In particular, EV-71 has been shown to use several receptors, including sialylated glycans, P-selectin glycoprotein ligand-1 and Scavenger receptor B2 (Nishimura *et al.*, 2009; Yamayoshi *et al.*, 2009; Yang *et al.*, 2009). CVB can utilize both decay accelerating factor (DAF) and the coxsackievirus and adenovirus receptor (CAR) for viral entry, depending on the target cell. For example in polarized cells, CVB elicits a unique strategy by binding to DAF at the apical surface of the cell, followed by CAR binding within the tight junctions in a caveolin-dependent, dynamin-independent manner (Coyne and Bergelson, 2006). Whereas in non-polarized cells, CVB only appears to utilize CAR for entry and undergoes dynamin-dependent and caveolin-independent entry (Patel *et al.*, 2009).

While some EVs are capable of utilizing multiple receptors, PV uses only one, CD155, an adhesion molecule also known as the human PV receptor (hPVR). In an *in vitro* blood-brain barrier (BBB) model, PV has been show to enter human brain microvascular endothelial cells (hBMECs) using hPVR in a dynamin and caveolin-dependent manner (Coyne *et al.*, 2007). However, when using non-CNS specific immortalized cells lines, PV entry was found to be caveolin-independent. Both reports found tyrosine kinases to be essential for entry (Brandenburg *et al.*, 2007). These studies illustrate the importance of characterizing EV entry in multiple CNS cell types, since key differences may exist in different model systems of infection. Similarly, care must be taken in making generalized conclusions of virus entry based on *in vitro* cell culture models.

Undoubtedly, receptor expression on potential target cells defines the first barrier to virus entry. A recent study has shown a link between decreased CAR expression in differentiated primary neurons and a reduction in CVB3 infection, suggesting that susceptibility to infection may diminish as the level of virus receptor decreases during the differentiation process (Ahn *et al.*, 2008). Similarly, PV infection and tissue tropism was largely found to

be restricted by hPVR expression in the CNS of hPVR transgenic mice (Gromeier *et al.*, 1995). Analysis of receptor distribution in hPVR transgenic mice during development showed high levels of protein expression in the spinal cord anterior horn motor neurons which are known to harbor infection in the mature CNS (Gromeier *et al.*, 2000). Although some EV receptors may be widely expressed in tissues including the CNS, additional cellular determinants may ultimately control tropism. For example, CAR expression is relatively widespread in the murine neonatal CNS (Honda *et al.*, 2000; Hotta *et al.*, 2003; Venkatraman *et al.*, 2005) and utilized by adenovirus - a DNA virus with substantial tropism differences as compared to CVB3. Yet, early CVB3 infection is largely restricted to neural progenitor and stem cells (NPSCs) (Figure 1) or infiltrating myeloid cells during early infection (Feuer *et al.*, 2005) (Figure 2). The proliferative status of these cells may provide an additional level of susceptibility to infection (Feuer *et al.*, 2002; Feuer *et al.*, 2003; Feuer *et al.*, 2005; Feuer and Whitton, 2008).

Coxsackievirus Infection of Neural Progenitor and Stem Cells

What might be the benefit of CVB3 targeting NPSCs for infection? We expect that the benefits may be many fold in terms of viral replication, persistence, dissemination, and transmission. First, NPSCs actively proliferate not only during neonatal development, but also occasionally within the adult host. Infection of NPSCs may assist in expanding the tropism of CVB3 upon their differentiation into the three neural lineages - neurons, astrocytes, and oligodendrocytes. Previously, we determined that CVB3 preferentially replicates within actively proliferating cells (Feuer *et al.*, 2002). Hence, NPSCs may be considered ideal host cells for infection.

Second, NPSCs migrate upon their differentiation into particular regions within the CNS, including the hippocampus and the olfactory bulb. The olfactory bulb might be considered an escape route for the virus upon anterograde transport across the olfactory neuroepithelium. Third, NPSCs might be altered upon their differentiation into neurons. Alterations in neuronal function may lead to behavioral modifications within the host, which may act to maximize virus transmission. Fourth, targeting of NPSCs might be a strategy of CVB3 to establish persistent infection with sporadic reactivation whenever quiescent stem cells become activated to generate downstream progenitor cells (Figure 3). The majority of primary neural stem cells at any moment in time might be expected to remain within a quiescent state. Therefore, CVB3 replication may be temporarily arrested in the host cell until a later point in time when the production of virions may be of benefit to viral transmission, perhaps through the olfactory neuroepithelium. Finally, normal immune responses may be suppressed within neurogenic regions, either due to the immunopriviledged status of the CNS, or potential reduced antiviral responses in these crucial neural stem cells.

Enterovirus Dissemination – Hitch-Hiking on Migratory Immune Cells

Perhaps surprisingly, polarized epithelial cells are not considered primary target cells for CVB3 infection *in vivo*, despite their expression of intrajunctional proteins. Rather, the major targets of infection have been found to include the acinar cells of the pancreas, cardiomyocytes, activated lymphocytes within the marginal regions of the spleen, infiltrating nestin^{+} myeloid cells, proliferating NPSCs, and immature neurons of the CNS. Instead, targeting of intrajunctional proteins such as CAR by CVB3 might be a sophisticated viral strategy to specifically infect migratory immune cells as they undergo diapedesis through the tight junctions. In this regard, we have recently shown the ability of CVB3 to infect nestin^{+} myeloid cells upon their infiltration across the tight junctions of the choroid plexus epithelial cells comprising the blood-cerebral spinal fluid-barrier in response to early

CNS infection (Tabor-Godwin *et al.*, 2010). These infected myeloid cells eventually migrate into the parenchyma of the brain, thereby assisting in virus dissemination.

The benefits to virus dissemination by targeting intrajunctional proteins may be many fold; first, virions temporarily attached to sequestered intrajunctional proteins might find themselves in a perfect location to "hitch-hike" onto a migratory immune cell as these cells enter sites of inflammation or injury in response to early infection. Second, immune cells responding to inflammation or injury tend to be highly activated or undergoing proliferation, and each virus may induce unique chemokine profiles early after infection to attract their immune cells of choice. CVB3 has been shown previously to preferentially replicate within activated or proliferating cells. Third, virions carried internally by immune cells might temporarily shield the virus from neutralizing antibodies upon dissemination. Neutralizing antibodies are thought to play a major role in limiting EV infections, and hypogammaglobulinemic patients deficient in humoral immunity are highly susceptible to EV infection. Perhaps not surprisingly, other viruses have been shown to bind to intrajunctional proteins (Bergelson, 2009), suggesting that the proposed mechanism above might be a general feature of virus dissemination within the host. Therefore, rather than being a hindrance to virus entry - which might be expected for receptors sequestered within highly inaccessible areas - virus evolution may have optimized virion attachment to intrajunctional proteins to maximize viral spread using immune target cell "Trojan horses" which become infected during their migration across tight junctions. Supporting this hypothesis, evidence for viral targeting of lymphocytes or monocytes for spread into primary target organs and maximal viral replication has been described for CVB3 (Mena *et al.*, 1999) and other viruses (Noda *et al.*, 2006).

Routes of Enterovirus Entry into the CNS

Since EVs, such as PV, are transmitted via the fecal-oral route of infection, a detailed understanding of how the virus travels from the gut to the brain is essential in designing strategies to limit EV disease. Despite the extensive research that has been performed to understand CNS invasion, many questions remain. Multiple pathways may actually work in combination to complete this phenomenon. The two main models of PV entry into the CNS both involve viremia, or the presence of virus in the blood, after the virus has replicated in the lymphatic tissues of the gastrointestinal tract. One hypothesis simply proposes the direct movement of the virus through the blood-brain-barrier (BBB) in an hPVR-independent manner (Yang *et al.*, 1997). Perhaps direct entry across the BBB via early PV infection of hBMECs may occur first, thus weakening or exposing the BBB and allowing for secondary virus entry into the CNS regardless of receptor expression.

A second model of EV entry proposes PV spread from muscle to the CNS along neural pathways (Ren and Racaniello, 1992). These neural pathways were more clearly defined by Gromeier and Wimmer in 1998 as retrograde axonal transport of the virus from damaged muscle to the CNS (Gromeier and Wimmer, 1998). Since muscle injury has been shown to induce neurological PV infection, the model is typically referred to as "provocation poliomyelitis". Initially, it was proposed that hPVR was necessary for retrograde axonal transport of the virus along microtubules (Ohka *et al.*, 2004). However more recently, retrograde transport has been shown to occur independently of the hPVR (Ohka *et al.*, 2009).

Another emerging model of CNS entry involves the use of EV-infected immune cells invading the CNS in a "Trojan horse" fashion, as described above. Of note, PV has been shown to infect monocytes, macrophages and dendritic cells in an hPVR-dependent manner (Freistadt *et al.*, 1993; Wahid *et al.*, 2005). In addition, lymphocytes may play a role in

monocyte infection by inducing their activation and facilitating viral replication (Eberle *et al.*, 1995). Further investigation has revealed a possible link between PV pathogenesis in the CNS and monocyte infection, as neurovirulent strains were found to replicate more efficiently in monocytes (Freistadt and Eberle, 1996). As described above, CVB3 and also EV-71 have been shown to replicate in immune cells, B-lymphocytes, T-lymphocytes and cells of the myeloid lineage (Haddad *et al.*, 2004; Vuorinen *et al.*, 1996). Our laboratory has recently revealed the rapid infiltration of a novel population of nestin^{+} myeloid cells into the neonatal CNS following CVB3 infection (Tabor-Godwin *et al.*, 2010). These cells are highly susceptible to CVB3 infection and move into the brain parenchyma over time, suggesting a role in viral dissemination. The growing number of studies demonstrating EV infection of immune cells suggests that furthers investigation of the "Trojan horse" model of CNS invasion may be warranted.

Enterovirus Quasispecies

Another essential factor in CNS invasion may be the involvement of EV quasispecies, or genotypic variants of virus populations, due to mutational errors accumulated during viral replication. These errors result from the low fidelity of the RNA-dependent RNA polymerase whereby at least one incorrect nucleotide is incorporated per genome duplication (Ward and Flanegan, 1992; Ward *et al.*, 1988). Other contributing factors during viral replication may also increase viral diversity. For example, a recent *in vitro* study performed with PV 3D^{Pol} found that the replication fidelity may be relatively high; yet other events during replication, such as template switching, may actually contribute to the errors found in the genome (Freistadt *et al.*, 2007).

Regardless of the nature of mutation rate, the existence of a swarm of viral variants, or a quasispecies cloud, has been shown to contribute to EV spread to the CNS. Recent elegant work utilizing a PV variant encoding a mutation in 3D^{Pol} isolated during serial passage in the presence of ribavirin has revealed the critical nature of viral quasispecies in contributing to invasion of the CNS. The PV isolate (G64S) was shown to increase the fidelity of the viral polymerase and decrease quasispecies diversity (Vignuzzi *et al.*, 2006). The G64S PV isolate was less neurovirulent than wild type virus, and CNS invasion failed to occur unless the isolate was co-inoculated in combination with a diverse quasispecies population. These intriguing results demonstrated that cooperation between variants within a viral population was necessary for CNS invasion and the ensuing neuropathology. Cooperation among viral genotypes might include evasion of the immune response simultaneously with CNS invasion.

Genetic bottleneck effects have also been observed as viral variants enter the CNS. As the first few viruses enter cellular routes into the CNS, antiviral responses become initiated which block entry for the remaining variants (Pfeiffer and Kirkegaard, 2006). This antiviral genetic bottleneck effect, also referred to as the "burned-bridge" model, may contribute to the rare transmission of PV into the CNS which occurs in approximately 1 to 2% of cases (Gromeier and Wimmer, 1998). Later studies have added to the "burned-bridge" model whereby multiple barriers may contribute to genetic bottleneck effects of quasispecies. These barriers which may limit neurovirulence include the integrity of the gut, the induction of a protective innate immune response, and inefficiency in neuronal transport of virus (Kuss *et al.*, 2008; Lancaster and Pfeiffer, 2010). Thus, EV entrance into the brain appears to be a complicated interplay not only between the virus and the host, but also among viral variants within the quasispecies population.

Enterovirus Tropism

EVs are inherently neurotropic, yet each member of the virus genus targets different regions of the CNS (Table 1). A recent *in vivo* study by Nagata et al. examined the localization of PV and EV-71 following intravenous infection of monkeys. The authors identified PV-induced lesions primarily within the pyramidal tract of the CNS causing a total loss of motor neurons in the anterior horn of the spine. In contrast, EV-71 induced only limited damage in both the extrapyramidal and pyramidal tracts, leaving many motor neurons in the anterior horn of the spine intact (Nagata *et al.*, 2004). Furthermore, the authors were able to isolate PV from the entire CNS, including the dorsal root ganglia and the trigeminal. In contrast, EV-71 could not be isolated from either of the latter two regions of the CNS. These results illustrate the broad range of neuronal target cells for PV, as compared to the more restricted neuronal targets for EV-71.

In yet another example of neuronal targeting of a picornavirus, TMEV was shown to initially infect gray matter regions of the CNS, yet virus persisted in the white matter following acute infection (Roussarie *et al.*, 2007). Echovirus type 1 (ECHO-1-V) has been shown to cause cellular necrotic lesions in the cerebral cortices of transgenic mice expressing human integrin very late antigen 2, leading to paralysis and wasting (Hughes *et al.*, 2003). In contrast, early CVB3 infection was found to be localized to specific neurogenic regions of the brain, including the subventricular zone (SVZ) (Feuer *et al.*, 2005).

Each EV genus member clearly prefers distinct regions of the CNS. Yet, what factors determine preferential tropism at the cellular level? Tropism at the cellular level can viewed as an interplay of at least three general factors: the ability of the virus to infect and replicate in the specific host tissues; the capability of the host cell to clear the virus; and the capacity of the virus to avoid clearance by the host. Potential differences in the magnitude of the interferon α/β response may greatly influence PV tissue tropism (Ida-Hosonuma *et al.*, 2005; Yoshikawa *et al.*, 2006). The tropism of PV for neurons has previously been shown to be also determined by the PV Internal Ribosomal Entry Site (IRES) (Kauder and Racaniello, 2004). Kauder et al. initially found that the tropism of PV towards nervous tissues could not be fully explained by organ-specific differences in IRES-mediated translation within age-matched samples. They investigated alterations in PV tropism by generating recombinant viruses engineered to contain the IRES from human rhinovirus (Kauder *et al.*, 2006). In this manner, the investigators observed IRES-mediated, organ-specific translational differences between EVs in adult versus neonatal infection. They concluded that these differences might be attributed to IRES *trans*-acting Factors (ITAFs) differentially expressed in various cell types, including neurons.

Ires Trans-Acting Factors (ITAFs)

ITAFs have been found to play a key role in EV IRES-mediated translation initiation. Some IRESs require specific ITAFs, while others require few additional proteins (Fitzgerald and Semler, 2009). Hence, tissue specific ITAFs may help to explain the tropism of EVs towards particular tissue types. Neurons may be more susceptible to infection due to the availability of specific ITAFs needed to initiate IRES-mediated translation. Investigation of TMEV and FMDV IRESs suggested that cell-specific differences in ITAFs may explain why some viruses are able to replicate in neurons, while others cannot (Pilipenko *et al.*, 2000). Furthermore, translation initiation of different EVs IRES may depend on a combination of different ITAFs and eukaryotic initiation factors which may have different cell-specific levels of abundance (Boussadia *et al.*, 2003). Recently, the Far Upstream Element (FUSE) binding protein (FBP2), originally identified to associate with a protein complex involved in

intronic c-src neuronal specific silencing enhancer, was discovered as a critical ITAF for EV-71 infection (Lin *et al.*, 2009a).

While the IRES and ITAFs have largely been associated with viral replication in neurons, EVs are capable of infecting other cell types in the CNS. For example, PV can infect astrocytes and oligodendrocytes (Couderc *et al.*, 2002). Also, it has been known for some time that TMEV can infect astrocytes, yet persist in oligodendrocytes (Rodriguez *et al.*, 1983). EV-71 has been found to readily infect and replicate in astrocytoma cultures (Kung *et al.*, 2007) and cultures of rat brain astrocytes (Tung *et al.*, 2010). We have shown that CVB3 preferentially infects NPSCs (Feuer and Whitton, 2008), and more recently, a novel population of nestin$^+$ myeloid cells infiltrating into the CNS (Tabor-Godwin *et al.*, 2010). Parechoviruses (P-ECHO-Vs) are suspected to be taken up by microglia which leads to the activation of these resident immune cells (Volpe, 2008). Also, microglia, and glial cells may be potential sites of EV persistence. For example, macrophages and microglia have been found to be the main reservoir for harboring TMEV during persistent infection (Roussarie *et al.*, 2007). Respiratory EV, including rhinoviruses (RVs), have not been historically found infect the CNS. However, a recent study suggested that recombination of respiratory EV to genetically similar viruses, such as CV and PV, may eventually lead to respiratory EVs with tropism for the central nervous system (Tapparel *et al.*, 2009). This possibility is not implausible, especially with the recent findings suggesting the contribution of viral variants or quasispecies to PV spread into the CNS (Vignuzzi *et al.*, 2006).

Immune Responses to Enteroviruses

The immune response plays a critical role in protecting the host from viral pathogens by both modulating the release of chemokines and inflammatory cytokines for leukocyte recruitment, and by directly fighting infection via the interferon response. An informative review of chemokine induction in response to neurotropic infections has recently been published by Hosking et al (Hosking and Lane, 2010). These chemoattractant molecules may be especially critical to combating microbes within immunopriviledged sites, such as the CNS. We have demonstrated the induction of numerous chemokines in the CNS following CVB3 infection. One particular chemokine, CCL12, may play an essential role in the recruitment of nestin$^+$ myeloid cells that infiltrate across the blood-CSF-barrier and become infected upon their entry through the choroid plexus (Tabor-Godwin *et al.*, 2010). “Bystander” casualties suffered upon activation of cytolytic T cells during an adaptive immune response within the CNS may be potentially more devastating to neurons than a viral infection itself. Thus, the host may restrict or control immune responses in response to a viral infection within immunopriviledged sites; this restriction may partly explain why certain viruses may preferentially target the CNS.

Innate Immune Response Following Enterovirus Infection

The innate immune response begins with the recognition of the virus by cytoplasmic sensors. EVs have been shown to activate essential innate immune response molecules, including Toll-like Receptor 3 (TLR3), Retinoic Acid Inducible Gene I (RIG-I), and Melanoma Differentiation-Associated Gene 5 (MDA5). TLR3 is located on the membrane of endosomes or the plasma membrane and recognizes double-stranded RNA, a structure generally observed during viral replication of all RNA viruses. Recent studies suggest that TLR3 plays a strong role in cardioprotection against EV infections and may depend on autophagic processes (Gorbea *et al.*, 2010). Studies of the recognition of EVs by cytoplasmic sensors of the innate immune response have largely centered on DExD/H-box-containing RNA helicases, such as RIG-I and MDA5. In examining encephalomyocarditis virus (EMCV) induction of the type I interferon (IFN) response in mouse embryonic

fibroblasts, Kato et al concluded that MDA5, but not RIG-I, was critical for picornavirus detection (Kato *et al.*, 2006). However, this does not mean that RIG-I is excluded from picornavirus detection. By Utilizing MDA-5 knockout mice, Papon et al observed that RIG-I was also useful in detecting EMCV (Papon *et al.*, 2009). Both of these sensors may play important roles in EV detection by the host. EVs may circumvent the action of these molecules by encoding proteins that affect their signaling pathways.

Once a pathogen is recognized, the innate immune response may play a substantial role in initiating virus-mediated neuropathology following EV infections. For example, the severity of echovirus and EV infections in the CNS have been associated with higher systemic levels of proinflammatory cytokines like IL-6, IL-1β, and TNF, which can lead to greater cytokine-induced tissue destruction (Lin *et al.*, 2003; Liu *et al.*, 2005). Also, CV activation of the inflammatory response has been shown to cause extensive infiltration of leukocytes into the CNS thereby causing inflammatory lesions and contributing to neuropathology associated with the virus (Feuer *et al.*, 2009).

In addition to the potentially harmful inflammatory responses triggered within the CNS, EV infections may also initiate the type I IFN response pathway. TLR-3, RIG-1, and MDA5 are all able to induce the type I IFN response. TLR-3 induces the production of type I IFNs by activating the transcription factors Interferon Regulatory Factor 3 (IRF3), NF-κB, and AP-I through the adaptor protein TIR-domain-containing Adapter Inducing Interferon β (TRIF) (Matsumoto and Seya, 2008; Wilkins and Gale, Jr., 2010). The RIG-I and MDA-5 pathways are distinct and independent of the TLR-3 pathway. RIG-I and MDA-5 both interact with the adaptor molecule IPS-1 through their CARD domains. IPS-1 transfers the activation signal to downstream kinases, which in turn activate IRF-3 and other transcription factors involved in type I IFN induction (Kato *et al.*, 2006). Malathi et al determined that the helicase activity of RIG-I and MDA5 may have important autoregulatory roles and work in concert with Endoribonuclease L (RNase L) to produce small self RNA cleavage products that can interact with RIG-I or MDA5, thereby amplifying the signal (Malathi *et al.*, 2007).

Following their induction, type I IFNs bind to their receptors either on the same cell or on neighboring cells. Binding of type I IFNs to their receptor induces the transcription of antiviral miRNAs and IFN Stimulated Genes (ISGs) through the JAK/STAT pathway. Many of these ISGs encode antiviral products or serve to further upregulate the type I IFN pathway. For example, RNase L is an ISG that contributes to the amplification of the type I IFN response, but also cleaves viral RNA and inhibits their translation. Furthermore, the antiviral activity of RNase L has been found to have a protective role in the CNS against coronavirus-induced demyelination. In comparing wildtype and RNase $L^{-/-}$ mice, Ireland et al found that RNase L deficiencies increased demyelination and axonal damage in the brain following infection, and affected the regulation apoptosis (Ireland *et al.*, 2009). Additional ISGs encode products that can inhibit specific viruses. Additionally, IFN-β has been found to modulate the expression of antiviral microRNAs in response to RNA viruses, including hepatitis C (Pedersen *et al.*, 2007). Hence, it is reasonable to suspect that type I IFN-induced antiviral miRNAs might play an important role in host defense against EVs in the CNS.

EVs have evolved a surprising number of mechanisms for undermining the host innate immune response, attacking at different stages of the IFN induction and response pathways. EV and cardiovirus (such as TMEV) proteins may counter-act host defenses (Agol and Gmyl, 2010). The ability to evade the host antiviral response may be an important contributing factor not only for maximizing acute infection, but also for the successful establishment of viral persistence. PV, rhinovirus (RV), and EV have been found to reduce RIG-I levels and disrupt RIG-I mediated interferon signaling (Barral *et al.*, 2009). A reduction in RIG-I levels has been attributed to the activity of the PV 3C protease. PV and

RV Type-1a have also been observed to cleave MDA5 through a caspase and proteasome-dependent mechanism (Barral *et al.*, 2007). Since RIG-I signaling induces further RIG-I expression, and MDA5 provides secondary protection against viral infection in the host cell, EVs may also target the downstream signaling pathways for these molecules. The 3C protease of EV-71 has also been found to disrupt RIG-I signaling through a protease-independent fashion. Instead of cleaving, 3C protease binds to RIG-I and prevents the recruitment of IPS-1 (Lei *et al.*, 2010). In contrast, RV-14 attenuates the type I interferon response by targeting the activation of IRF-3 upon identification by MDA5 (Kotla *et al.*, 2008). Similarly, TMEV has been found to inhibit IRF-3 dimerization (Ricour *et al.*, 2009).

Once the antiviral response has been initiated, viruses have developed ways to combat these protective actions. PV and RV have been found to cleave Nup62 (Park *et al.*, 2010) and degrade Nup153 and Nup98 (Park *et al.*, 2008). By altering the nucleus by degrading nuclear pore complex proteins, viruses can affect host mRNA and protein localization, and shut down host protective factors. The hyperphosphorylation of Nup98 by the L protein during TMEV infection has been shown to block host mRNA export from the nucleus (Ricour *et al.*, 2009). Herpesvirus genomes have been found to encode miRNAs suggesting that they exploit the host miRNA machinery to regulate the expression of host and viral genes (Pfeffer *et al.*, 2005). Although this mechanism has not been demonstrated for EVs, Pelletier et al have found that cells exposed to and cured of PV using RNAi respond more quickly to RNAi treatment, as compared to cells never having been exposed to PV (Pelletier *et al.*, 2010). These intriguing results suggest that cellular miRNA processing machinery plays an antiviral role during EV infections, as well. Furthermore, cells which have successfully cleared the virus may be permanently altered to better respond to future viral infections.

Adaptive Immune Response Following Enterovirus Infection

Numerous clinical case reports involving patients suffering from encephalitis due to EV document the importance of neutralizing antibodies in controlling infection (Xie *et al.*, 2010). The significance contribution of a neutralizing antibody response in controlling infection is also shown by studies describing individuals suffering from agammaglobulimia. The absence of neutralizing antibodies in agammaglobulimic patients results in an increased susceptibility to EV infections of the CNS that can lead to chronic neuropathies (Misbah *et al.*, 1992). Experiments evaluating CVB3 infection in mice lacking B cells (BcKO mice) indicate that antibodies are critical for viral clearance (Mena *et al.*, 1999). The role of B cells in controlling CVB3 infection may be more complex by evidence suggesting B cells may contribute to virus dissemination via the “Trojan horse” hypothesis. Early after infection in normal mice, high levels of viral RNA was observed in proliferating lymphocytes located within the marginal zone of the spleen.

Macrophages and microglia may be involved in EV clearance within the CNS. These phagocytic cells are thought to be early responders to viral infection. The presence of activated microglia and macrophages in response to CVB3 infection has been observed in our neonatal mouse model (Feuer *et al.*, 2009). By confocal microscopy, $Iba1^+$ macrophages/microglia within the CNS were shown actively engulfing virally-infected cells.

T cells also play a critical role in the adaptive immune response to EV infection in the CNS; however, bystander damage following their activation may be significant. Much work in understanding T cell responses to picornavirus infection in the CNS and the ensuing immune-mediated pathology has been described for TMEV during acute and persistent infection. A highly detailed and informative review on TMEV-induced molecular mimicry model of multiple sclerosis has been published (Olson *et al.*, 2005). Infiltration of $CD4^+$ and

$CD8^+$ lymphocytes within the CNS has also been shown following EV infection (Lin *et al.*, 2009b). Exacerbation of virally-induced morbidity may occur in mice deficient in $CD4^+$ and $CD8^+$ lymphocytes, indicating that T cells are necessary to combat infection. In contrast, some EVs such as CVB3, have evolved ways to escape detection by $CD8^+$ T cells via the inhibition of antigen presentation by the MHC class I pathway (Kemball *et al.*, 2009). Therefore, activation of the T cell response may vary greatly, depending upon the EV genus (Slifka *et al.*, 2001).

Enterovirus Persistence

Although EV possess multiple mechanisms by which they can evade the host immune response, their success is not absolute, and the struggle between the host and virus can last for very long periods of time. EV persistence appears to be the product of the ongoing attempt of the host to eliminate or suppress virus replication, and the virus' struggle to remain intact in a hostile cellular environment. Under the selective pressures generated by a successful host immune response, sometimes the best defense for the virus is simply to mutate. As described above, errors in EV replication can lead to the existence of viral quasispecies (Domingo *et al.*, 2008) which may broaden the tropism of EV (Vignuzzi *et al.*, 2006), assist in evasion from the immune response, and lead to persistence. Thus, perhaps it comes as no surprise that EVs may persist in the CNS and avoid clearance by the host.

EV-71, PV, CVB3, and TMEV all have mechanisms for mitigating the host innate immune response. These viruses have been observed to persist long after the initial infection. Evidence of EV-71 persistence in a clinical setting has been observed by the continued detection of virus sequences in the excretions and secretions of patients long after the detection of the initial infection (Han *et al.*, 2010). Whether or not the CNS is a potential site of the EV-71 persistence has not yet to be determined. In the case of TMEV, virus has been found to persist in oligodendrocyte cell cultures, but the main reservoir for persistent TMEV may be macrophages (Roussarie *et al.*, 2007). Upon infection, TMEV is transferred from the neuronal axon to the oligodendrocyte in a myelin-mediated fashion; following the ingestion of infected oligodendrocytes, macrophages become the primary reservoir for TMEV persistence.

PV has previously been demonstrated to persist in primary neural cell cultures (Colbere-Garapin *et al.*, 1998). PV persistence and reactivation has been a suspected cause of Post-Polio Syndrome (PPS) (Baj *et al.*, 2007). Indeed PV RNA could be detected in some patients with PPS, though the persistent virus may be very different from the wild type strain (Baj *et al.*, 2007). The low viral load and the unclear mechanism of reactivation for mutated virus in PPS patients most likely fuel the controversy regarding the potential contribution of persistent PV material to recurring disease. Perhaps PV persists in an attenuated form in peripheral nervous tissues of the host at low replication rates; a reactivation event, such as injury, might trigger higher levels of viral replication with ensuing neurological sequelae. Injury-mediated retrograde axonal transport of PV has been demonstrated to carry PV into the CNS (Gromeier and Wimmer, 1998), and may be a mechanism by which PV can be transported from sites of persistence back to the CNS.

CV persistence in the heart has been the subject of much investigation, and its persistence in the CNS has only recently been described (Feuer *et al.*, 2009). In the heart, CV persistence is associated with chronic myocarditis and dilated cardiomyopathy (Chapman and Kim, 2008). The host innate immune response, in particular the type I IFN response, is extremely critical for controlling CV infection of the heart (Deonarain *et al.*, 2004). The type I IFN response might also create selective pressures resulting in viral genome mutations (Kim *et al.*, 2005b), attenuation of CV, and persistence in the heart. Genome deletions in the 5′ UTR

of CVB3 was recently observed upon passage of CVB3 in primary heart cell cultures. These deletions were associated with a reduction in cytopathic effects (cpe) without reductions in viral titers (Kim *et al.*, 2008). Similarly, persistent CVB3 RNA was detected in the CNS of mice following neonatal infection for up to 90 days post-infection (PI), as determined by nested RT-PCR, in the absence of infectious virus by plaque assay (Feuer *et al.*, 2009). Our most recent results suggest that CVB3-infected neurospheres, or NPSCs grown in culture as free-floating structures, may support a persistent or carrier-state infection (manuscript submitted). Based on these results, we hypothesize that neurogenic regions of the CNS may be potential sites of CVB3 *in vivo*. Perhaps CVB3 reactivation is induced upon occasional proliferation and asymmetric division of resting or quiescent type B neural stem cells harboring persistent virus within the CNS (Figure 3), thereby leading to intermittent reactivation of virus.

The possible persistence and reactivation of EV in a clinical setting is of profound importance, especially with the addition of immunosuppressive drugs, such as Rituximab, given to patients suffering from B cell lymphomas. Immunosuppression, especially with drugs targeting the humoral immune response, may lead to reactivation/increased replication of persistent EV in the CNS with potentially dangerous neurological complications (Schilthuizen *et al.*, 2010; Servais *et al.*, 2010).

Pathophysiology of Enterovirus Infection

EV infection of the CNS have been associated with acute flaccid paralysis (Solomon and Willison, 2003), acute disseminating myelitis and acute transverse myelitis (Agin *et al.*, 2010; Minami *et al.*, 2004). Aseptic meningitis and encephalitis can also result from EV infection (Dalwai *et al.*, 2010; Lewthwaite *et al.*, 2010). Further evidence indicating the extensive distribution and diversity of EVs associated with human disease is demonstrated by a recent informative publication by Victoria et al (Victoria *et al.*, 2009). Their metagenomic analyses suggested the presence of circulating human EV species A (HEV-A) through HEV-C, including other members of the *Picornaviridae*, such as P-ECHO-Vs, rhinoviruses, and human cardioviruses in South Asian children suffering from acute flaccid paralysis. Metagenomic analysis may be a highly informative technique in determining the distribution and number of circulating EVs in human populations.

Clinical Diagnosis of Enterovirus Infection of the CNS

A variety of differing techniques have been used in the clinical diagnosis of EV infections of the CNS (Table 2); a comprehensive description of which is beyond the scope of this review. The GreeneChip pioneered by Ian Lipkin represents the technological forefront of pathogen identification, but has yet to be employed in the context of EV infections of the CNS (Hunter, 2008). Diagnosis via selective culture using transgenic cell lines is limited and hindered primarily by the absence of cell lines supporting strain-specific replication (Leland and Ginocchio, 2007). In contrast, molecular techniques, such as RT-PCR, represent a sensitive and precise methodology for identification of EV CNS infection. Cerebrospinal fluid (CSF) from afflicted patients is the preferred source of clinical sample upon which these techniques can be performed. Genomic analysis using RT-PCR can be completed within 24 hours of sample collection (Romero, 1999). RT-PCR diagnosis of enteroviral meningitis has been performed using primers recognizing a conserved region within the 5′ UTR (Rotbart, 1990). While genus level identification of EVs is clinically relevant and can be used to guide antiviral regimes, strain-specific genotyping is required in some instances. RT-PCR characterization of the VP1 region has lead to strain-specific phylogenetic classification (Mirand *et al.*, 2008). Sequencing of the VP4 and VP2 regions was performed in instances where molecular typing using the VP1 region was inconclusive. These studies

indicate that RT-PCR is an effective and clinically feasible technique for the identification of the specific EV strain present in clinically obtained CSF samples.

Quantitative Real-Time PCR (qRT-PCR) may be a more sophisticate molecular method utilized to detect the amount of EV in clinical CSF samples (Dierssen *et al.*, 2008). The majority of qRT-PCR assays identifying EV use the primer pair designed by Robart et al. in 1990, although variations of these primers do exist (Rotbart, 1990). A limitation of the technique involves the absence of amplification curves for samples containing mutations in the probe binding region (Hymas *et al.*, 2008).

Contributions of Apoptosis to Enterovirus Disease in the CNS

We have previously described the induction of CNS lesions (Figure 4), inflammation, and apoptosis within CVB3-infected neurons in the cortex and hippocampus (Feuer *et al.*, 2003). TUNEL staining showed colocalization of infection and apoptosis within the CNS following IC inoculation of our neonatal mouse model with a recombinant CVB3 expressing eGFP (eGFP-CVB3). Also, a direct overlap of infection and activated caspase-3 protein revealed infected cells undergoing early stage apoptosis. Also, infection of virally-infected neuronal precursor cells contributed to the generation of lesions within the CNS. We also recently revealed the induction of apoptosis in the choroid plexus (Tabor-Godwin *et al.*, 2010) following CVB3 infection. These results suggest that EV may compromise the function of the choroid plexus, an essential organ involved in CSF production and immune regulation. The observation of hydrocephalus in mice following CVB3 infection may be related to choroid plexus dysfunction following infection. Reports also indicate that PV infection may be associated with induction of apoptosis in the CNS (Girard *et al.*, 1999). DNA oligonucleosomal laddering and enzyme-linked immunosorbent (ELISA) assays performed upon CNS samples obtained from mice exhibiting paralytic poliomyelitis indicated the presence cells undergoing apoptosis. These studies indicate that EVs are capable of inducing apoptosis within the CNS.

Potential Behavior and Memory Dysfunction Following Enterovirus Infection

The observation of infected neurons and ongoing apoptosis in the hippocampus and other regions of the CNS is highly suggestive that memory dysfunction or behavior changes might be expected following EV infection. Previous studies have suggested a link between EV infection early in childhood and the development of schizophrenia (Rantakallio *et al.*, 1997; Suvisaari *et al.*, 2003). EV infection of the CNS occurring during childhood is also associated with long term neuropathies including delayed neurodevelopment and reduced cognition (Chang *et al.*, 2007). Clinical examination of placental samples from newborns exhibiting neurodevelopmental defects has revealed the presence of EV within the tissue (Euscher *et al.*, 2001). EV-71 has specifically been implicated as the causative agent of developmental defects in the CNS (Huang *et al.*, 2006). Infection of pregnant mice with polyinosinic-polycytidylic acid, a compound that mimics replicating virus, may lead to inhibition of embryonic neuronal stem cell development (De *et al.*, 2010). Therefore, developmental defects observed following EV infection of the CNS might be attributed, at least in part, to the abrogation of normal stem cell replication within the brain.

Experiments using the Morris water maze as an indicator of memory formation found that mice infected with TMEV which also can target the hippocampus exhibited a spatial learning deficit (Buenz *et al.*, 2006). The deficit was correlated with the extent of damage to the hippocampus resulting from TMEV infection. We are currently examining the consequences of CVB3 infection on CNS development and spatial memory dysfunction. We

expect that lasting consequences on brain function may result following persistent CVB3 infection. These studies may be highly significant in a clinical setting, especially given that EV infections comprise the great majority of aseptic encephalitis infections in humans.

Antiviral Drugs to Treat Enterovirus Infections - RNAi Based Approaches

The idea that RNAi may act as a potent natural antiviral against RNA viruses in mammalian cells was first shown in 2003 (Gitlin and Andino, 2003). Since then, RNAi has proven to effectively inhibit EV reproduction both *in vitro* and *in vivo* in a variety of cell and mouse lines Table 3. Mechanistically, siRNAs inhibit the production of infectious virions via Dicer recognition of the viral dsRNA formed during genomic replication (Aliyari and Ding, 2009). Targeted RNAs are subsequently degraded, effectively hindering the formation of nascent virions.

The vast majority of siRNAs developed against EV focus upon the degradation of mRNAs coding for the viral RNA-dependent RNA polymerase (RdRp) and proteases. For example, siRNAs directed against protease 2A were the most effective in the inhibition of CVB3 infection in HeLa cells and murine cardiomyocytes (Yuan *et al.*, 2005). Interferon receptor knock-out mice transfected with siRNAs targeting CVB3 protease 2A exhibited increased survival time and attenuated viral replication when challenged with CVB3 (Merl *et al.*, 2005). The 19-mer siRNAs targeting the viral $3C^{pro}$ region of coxsackievirus B4 (CVB4) were effective in decreasing viral replication in rhabdomyocarcoma cells (Tan *et al.*, 2010). siRNAs targeted against the $3D^{pol}$ of EV-71 has also proven protective against this virus *in vivo* (Tan *et al.*, 2007). Prophylactic treatment of suckling mice with siRNAs prior to viral inoculation decreased the level of hind limb paralysis and weight loss associated with EV-71 infection. Also, RT-PCR and western blots demonstrated a decrease in viral replication and viral protein expression levels within the intestines of siRNA-treated mice.

EVs are especially prone to the formation of escape mutants due to the absence of an RdRp proofreading mechanism. The low fidelity of the RdRp increases the likelihood of mutants escaping the therapeutic effects of RNAi. The formation of escape mutants resistant to anti-CV siRNAs has been documented (Merl and Wessely, 2007). PV escape variants against siRNAs evolve to incorporate a single nucleotide mutation located in the center of the targeted RNA (Gitlin *et al.*, 2002). One strategy to combat this phenomenon may be the simultaneous administration of multiple siRNAs. The administration of a cocktail consisting of three distinct siRNAs may reduce the number of escape mutant progeny to extremely low levels (Merl and Wessely, 2007). Targeting receptors of viral entry with RNAi may be another strategy of effectively inhibiting EV replication. siRNAs directed against murine CAR (mCAR) decreased CVB3 titers in mice (Werk *et al.*, 2005). RNAi degradation of host genes constitutes a unique approach which may mitigate the formation of potential viral escape mutants by affecting cellular targets, as opposed to viral targets.

The development of therapeutic siRNAs symbolizes a novel and as of yet, undeveloped candidate for the treatment of EV infections of the CNS (Vaishnaw *et al.*, 2010). However, RNAi as antiviral therapy has yet to be employed within the CNS. Once the hurdle of delivering RNAi therapeutic constructs to the CNS has been overcome, siRNAs could potentially be used to treat life-threatening EV infections. EV infections of the CNS rarely occur in the absence of infection of peripheral tissues outside the brain. Therefore, even if a therapeutic agent has not been demonstrated to hinder virus production within the CNS *per se*; by inhibiting replication in other tissues, an siRNA-based drug might impede virus growth and the eventual progression of disease into the CNS. In summation, RNAi represents an immature yet powerful tool that should not be overlooked in the development of therapeutics designed to treat EV infections of the CNS.

Antiviral Drugs to Treat Enterovirus Infections - Ribavirin

Ribavirin (1-(_-d-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide) was first synthesized in 1972 by ICN pharmaceuticals. Currently under clinical development as a broad-spectrum antiviral, ribavirin has been shown to inhibit the replication of a variety of EVs. Ribavirin, a nucleoside analog, may act as a mutagen via incorporation into the viral RNA genome (Crotty and Andino, 2002; Crotty *et al.*, 2001; Crotty *et al.*, 2000). The presence of ribavirin may force the afflicted virus into “Error catastrophe” by generating a highly variable noninfectious quasispecies swarm and thereby causing lethal mutagenesis (Vignuzzi *et al.*, 2006). Ribavirin has been found to inhibit both *in vitro* and *in vivo* EV-71 replication (Li *et al.*, 2008). Human neuronal and mouse neuronal cell lines treated with ribavirin showed decreased signs of cpe following EV-71 infection. Also, ribavirin-treated mice exhibited decreased mortality, morbidity, and paralysis rates when challenged with EV-71 (Li *et al.*, 2008).

There are conflicting reports on the ability of ribavirin to cross the BBB; a necessary criterion when dealing with the treatment of EV infections of the CNS. Upon treatment of subacute sclerosing panencephalitis (SSPE), a neurological disease caused by persistent measles infection, only direct intracranial (IC) and not IP administration of RBV diminished the effects of this virus (Honda *et al.*, 1994). Combining the drug with the lipophylic carrier molecule, cyclodextrin, may increase the concentration within the CNS following IP injection (Jeulin *et al.*, 2009). Ribavirin has also been combined with IFN-α therapy to treat SSPE (Hosoya *et al.*, 2001). Patients received intravenous (IV) ribavirin, as well as intraventricular IFN-α treatment. Upon treatment with ribavirin (20 mg/kg), high performance liquid chromatography quantification indicated that the compound was present within the cerebral spinal fluid of one patient at a concentration of 7.5 μg/ml, a concentration shown to inhibit the replication of SSPE in both tissue culture and mouse studies (Hosoya *et al.*, 1989; Ishii *et al.*, 1996). Therefore, ribavirin may be capable of crossing the blood brain barrier (BBB) in sufficient quantities and to inhibit viral replication. In our neonatal model of CVB3 infection, IP administration of ribavirin led to brain wet weight recovery (manuscript in preparation) indicating that the compound may cross the BBB in effective quantities to reduce CVB3 replication during persistent infection.

EV resistance may necessitate the development of new antiviral drugs to combat these nefarious pathogens. Resistance against ribavirin by PV has been recently observed (Vignuzzi *et al.*, 2006). PV may combat the effects of ribavirin via a single point mutation in the RdRp, effectively increasing the fidelity and lowering the viral genomic mutation rate (Pfeiffer and Kirkegaard, 2003). Intriguingly, the emergent ribavirin-resistant quasispecies swarm may be less adaptable to a changing environment.

Antiviral Drugs to Treat Enterovirus Infections - Pleconaril

Pleconaril,3-(3,5-dimethyl-4((3-(3-methyl-5-isoxazolyl)propyl]oly)phenyl)-5-(trifluoromethyl)-1,2,4-oxadiazole is currently licensed by Schering Plough and has been developed as an anti-picornaviral drug with demonstrated efficacy against many EVs (Pevear *et al.*, 1999). Pleconaril is able to cross the BBB and remain within the CNS at concentrations that inhibit EV replication (Schmidtke *et al.*, 2009). The mechanism of action for pleconaril against viral pathogens is two-fold; the compound inhibits both viral attachment to the cognate receptor and uncoating of the nucleocapsid during replication (Chen *et al.*, 2008b). Pleconaril inhibits viral attachment by binding to the VP1 protein present in the canyon floor or ‘pocket’ of the nucleocapsid and inducing a conformational change in the protein. This conformational change synergistically inhibits the release of the viral RNA from the nucleocapsid, effectively hindering replication. Tissue culture and

animal challenge studies testing the susceptibility of clinical EV isolates to the drug have demonstrated antiviral activity (Pevear *et al.*, 1999). Also, numerous case studies evaluating the efficacy of pleconaril to treat clinically ill patients including immunocompromised individuals have been described (Desmond *et al.*, 2006; Webster, 2005). A previous study has suggested that the Nancy strain of CVB3 may be resistant to pleconaril (Schmidtke *et al.*, 2005). This resistance was found to be due to a point mutation at amino acid 1092 of the CVB3 open reading frame. Resistance was associated with a leucine at this position, whereas isoleucine and valine were associated with susceptibility to pleconaril. In summation, these studies demonstrate that pleconaril may be a valuable compound in the treatment of some EV infections of the CNS.

Intra-venous Immunoglobulins to Treat Enterovirus Infections

The standard therapy for aseptic meningitis caused by EV infection continues to be intra-venous immunoglobulin (IVIg) treatment, although the efficacy has not been proven (Abzug, 2004). IVIg is typically prepared from pools of plasma samples from healthy donors (Cheng *et al.*, 2008). This passive immunization-based therapy might neutralize infectious virus circulating within the host, in addition to other non-specific inflammatory mechanisms (Ooi *et al.*, 2010). Figure 5 illustrates the mechanism of action for each of the antiviral compounds and IVIg, described above. In addition, novel antiviral therapies against EVs continue to be developed (Wu *et al.*, 2010).

Vaccines Against Enteroviruses

The historical Salk and Sabin vaccines against PV demonstrate the effectiveness of immunization in protecting the host against EV infections. Many effective vaccines stimulating both the innate and adaptive immune response have been designed to combat the remaining clinically relevant EVs. To combat EV infection, the adaptive immune response employs both T cells and antibodies in the clearance of the virus. Therefore, the most successful vaccines activate both humoral and cell-mediated immunity and induce lasting viral immunity. The administration of novel vaccines to patients comes with potential risks, extensive and costly clinical studies, and a potentially apprehensive public. Unfortunately, acceptance of EV-based vaccines to the public may not materialize unless the potential diseases caused by these pathogens become much more widely appreciated. That said, ongoing EV vaccine studies may help us improve the efficacy and safety of potential vaccine candidates, especially if EV transmission and/or disease manifestations increase in the general population in the future. Also “therapeutic vaccines”, those vaccines given after initial infection, might be more readily accepted in patients suffering from EV-mediated disease. “Therapeutic vaccines” could be envisioned which may enhance or redirect an ongoing immune response in order to reduce viral load during early infection, or after the establishment of viral persistence.

Poliovirus Vaccines

Attenuation of EVs through mutations in the genome has historically led to efficient vaccine production. Perhaps the most well-known attenuated form of PV is that used for the Sabin vaccine, which has decreased neurovirulence in part controlled by two stem loops in the viral IRES (Gromeier *et al.*, 1999). Another mutation that can cause CNS attenuation is located between the 5′ NTR cloverleaf and IRES and reduces the binding of polypyrimidine tract-binding protein (Guest *et al.*, 2004). Other studies have made recombinant viruses that use the IRES from human rhinovirus type 2 (HRV2) to attenuate neurovirulence in the Sabin vaccine strain of PV as well as in herpes simplex virus type 1 (Campbell *et al.*, 2007; Gromeier *et al.*, 1996). Somewhat alarmingly, a vaccine-derived PV and coxsackievirus A17

recombinant has been generated in the laboratory, thus illustrating the possibility of such events occurring naturally (Jegouic *et al.*, 2009).

Mutations causing changes in virulence and/or attenuation can also exist in other regions of the genome. Recent publications have revealed Sabin vaccine strains that in rare instances caused paralytic poliomyelitis, have mutations in the capsid protein VP1 representing a mutational hot-spot (Blomqvist *et al.*, 2010; Rahimi *et al.*, 2007). Furthermore, defective interfering particles generated through mutations in the capsid region of PV *in vitro* have been used to investigate viral replication (Hagino-Yamagishi and Nomoto, 1989). Newer studies have used cleavage site mutants to learn more about PV replication and encapsidation, which appear to be separate steps in the viral life cycle (Oh *et al.*, 2009).

Both inactivated and attenuated forms of the historical PV vaccines have been used to induce confer immunity to the virus. The original formalin-inactivated form of the vaccine (IPV) was developed by Jonas Salk and licensed in 1955 (SALK *et al.*, 1954). In 1963, an orally administered live attenuated PV vaccine (OPV) was formulated (SABIN, 1957). The usage of these vaccines and optimized versions has severely reduced the incidence of poliomyelitis, although the pathogen has yet to be eradicated worldwide. Preference for the administration of the IPV over the OPV is due to the discovery of circulating vaccine-derived PVs (cVDPV) associated with the OPV. cVDPVs are virulent PVs derived from the OPV that occur in a small minority of vaccine recipients. The reversion of cVDPVs from attenuation to virulence is a direct consequence of the genetic instability of the vaccine. In 2000, cVDPVs were responsible for a polio outbreak in the Dominican Republic and Haiti (Olen Kew 2002). Sequencing determined that the cVDPVs were recombinant viruses. The derivation of neurovirulent polio strains from the OPV has led to the “OPV paradox,” which is based upon the idea that complete eradication of poliomyelitis is contingent upon the elimination of the attenuated form of the vaccine (Dowdle *et al.*, 2003).

Enterovirus-71 Vaccines

There have been a variety of effective vaccines designed against EV-71. A formaldehyde-inactivated whole virus preparation has been shown to protect mice from encephalomyelitis caused by EV-71 infection (Ong *et al.*, 2010). Histopathological examination using EV-71 specific antibodies revealed an absence of the virus within the brain stem of immunized mice. Plaque assays using brain stem and spinal cord homogenates confirmed the absence of infectious virus within these tissues. Using a unique strategy, transgenic mice were developed to secrete the VP1 capsid protein of EV-71 into milk secretions. When the milk from transgenic animals was fed to neonatal mice, these neonates developed serologically specific antibodies against EV-71 and did not exhibit weight loss when challenged with the virus (Chen *et al.*, 2008a). Also, virus-like particles, which resemble EV-71 in capsid structure and protein composition, have been found to elicit a protective humoral antibody response in animals (Chung *et al.*, 2008). In addition, passive immunization protected neonatal mice from EV-71 associated morbidity when confronted with a lethal dose of virus. A DNA vaccine coding for VP1 has also been developed (Wu *et al.*, 2001). But in contrast to the aforementioned vaccines, the VP1-based vaccine was only partially protective, as shown by a 40% survival rate of mice inoculated with a lethal dose of EV-71 following vaccination.

Coxsackievirus Vaccines

As with EV-71, many researchers have developed effective vaccines against CV, although no clinically available vaccine currently exists. Progress has been made using a variety of vaccines strategies utilizing either DNA plasmids expressing viral proteins, inactivated virus, or live attenuated forms of virus. A safe and effective RNA vaccine against CVB3 that

produces noninfectious virus has been recently produced (Hunziker *et al.*, 2004). Genomic mutation via PCR mutagenesis of the $3CD^{pro}$ cleavage site present between CVB3 proteins 2A and 2B resulted in an RNA viral genome incapable of producing infectious virions, yet conferring partial protective immunity. RNA-immunized mice exhibited lower viral titers within the pancreas and prolonged survival when challenged with CVB3. RNA-based vaccines are advantageous as compared to DNA vaccines due to their inability to integrate into host DNA.

Attenuating mutations have also been found in CVB viral capsid proteins, and administering these attenuated viruses was found to be protective against lethal re-challenge in the pancreas and heart; although the CNS was not examined (Dan and Chantler, 2005). Another successful vaccine in mice designed against CVB3 involved the generation of recombinant plasmids expressing capsid proteins. Two independent plasmids encoded the VP1 and VP3 capsid epitopes induced the production of virally specific antibodies following *in vivo* electroporation (Park *et al.*, 2009). Although vaccinated mice displayed a reduction in virally induced heart injury, the induced antibodies failed to neutralize infectious CVB3 in culture. Attenuated CVB3 strains have also been used as potential vaccines and may represent the forefront of the field (Kim *et al.*, 2005a). Whether any of the aforementioned vaccine candidates are capable of preventing CV pathology within the CNS has yet to be confirmed.

Studies using EVs have also been used as a model system for the development of innovative vaccine strategies. “High fidelity” RNA viruses isolated upon serial passage in the presence of low concentrations of ribavirin might be a general strategy for viral attenuation by decreasing the level of viral quasispecies (Vignuzzi *et al.*, 2008). Another original approach to vaccine design involved encoding a mutant PV that contained hundreds to thousands of under-represented codon pairs in the PV non-structural proteins (Coleman *et al.*, 2008). This codon-altered PV had the same amino-acid sequence as wild-type PV, yet suffered from a decrease in the virus protein translation rate. Thus, the codon-altered PV could illicit a protective immune response despite its attenuation (Wimmer *et al.*, 2009). Both of these approaches greatly decreased the ability of the attenuated “vaccine strain” to revert to wild-type PV. Importantly both strategies potentially could be developed quickly for emerging RNA viruses, or for other viruses whereby vaccine development has been deemed problematic.

Gene Delivery in the CNS Utilizing Enterovirus-Based Vectors

Due to their remarkable ability to infect the CNS, attenuated EVs have been explored for use in gene delivery to cells of the CNS. Novel PV replicons that express green fluorescent protein (GFP) have been shown to deliver GFP to motor neurons without causing pathogenesis (Jackson *et al.*, 2001). Also, recombinant PVs designed by the Gromeier laboratory may be of clinical use for viral gene delivery into the CNS, or as oncolytic viruses to target glioblastoma cells and other tumorigenic cells (Goetz *et al.*, 2010). Attenuated CV may also represent an attractive option; our laboratory and others have shown the feasibility of generating relatively stable recombinant CVs expressing such gene products as GFP, dsRED, and renilla luciferase (Feuer *et al.*, 2005; Kim *et al.*, 2009). Also with their particular stem cell tropism in mind, attenuated recombinant CVB3 may be of value as a viral gene vector specifically for delivery of stem cell modulators, such as transcription factors inducing differentiation, into neural and embryonic stem cells. However, the high number of circulating antibodies in the population against EVs (Sawyer, 2002) may limit their use as vectors *in vivo*; In contrast, *in vitro* use may be of great benefit.

Conclusions and Future Perspectives

In summary, this review has attempted to cover many of the recent advances in EV research with regards to CNS infection and pathogenesis. EVs are among the most common and medically important human pathogens, are a frequent cause of CNS disease (Muir and van Loon, 1997), and may preferentially cause harm in the very young. Much remains to be learned regarding factors influencing EV tropism, activation of the immune response following infection, infiltration of immune cells into the CNS to combat infection, mechanism of pathogenesis, and antiviral/vaccine development (Nathanson, 2008). The large number of EV strains circulating in nature hinders the promise of vaccine design against these viruses. The potential cessation of the globally administered PV vaccine, either due to the successful eradication of poliomyelitis, or due to dwindling World Health Organization resources, may lead to emergence of uncharacterized CVs and other related non-polio EVs previously restrained by cross-reacting antibodies generated through yearly immunizations. These uncharacterized, yet circulating EV strains with potential CNS tropism might constitute a new impediment in our control over a devious viral pathogen. Finally, the ability of EVs to persist in the CNS and target neural stem cells might suggest lasting effects on brain function. Future studies might be directed at inspecting possible behavioral alterations and memory dysfunction following recovery from these challenging, yet successful neurotropic viruses.

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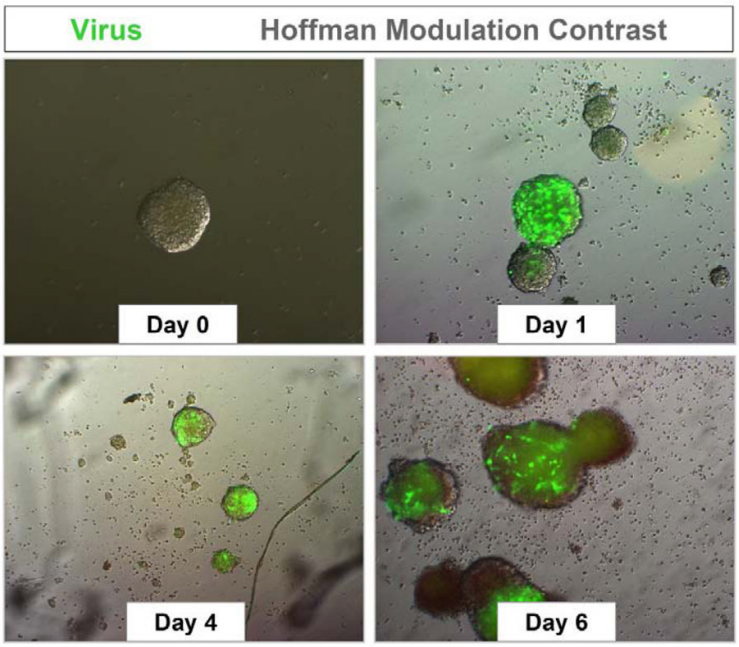

Figure 1. Neural progenitor and stem cells grown in culture are highly susceptible to coxsackievirus infection

NPSCs were isolated from the cortices of one day-old C57 BL/6 mice, cultured to form neurosphere aggregates, and infected with eGFP-CVB3 (moi = 0.1). Infected neurospheres were observed over time by fluorescence microscopy. Virus protein expression (green) was readily observed by day 1 PI. An increase in viral protein expression was seen until day 4 PI. By day 6 PI, virus protein levels were reduced, and signs of cytopathic effect (cpe) were readily observed at a higher magnification (not shown).

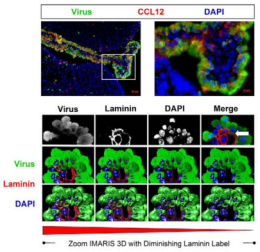

Figure 2. Induction of CCL12 and the progressive extravasation of infected myeloid cells through the basement membrane as determined by confocal microscopy and Imaris 3D analysis

Three day-old C57 BL/6 mice were intra-cranially infected with a recombinant coxsackievirus B3 expressing eGFP (eGFP-CVB3; 10^7 pfu). The brains were harvested 12 hours later and inspected by confocal fluorescence microscopy. Myeloid cells responding to CVB3 infection were observed entering through the tight junctions of the choroid plexus epithelium. As these myeloid cells entered the lateral ventricle, they expressed high levels of viral protein (green). Also, the chemokine CCL12 (red), a potential chemoattractant molecule for infiltrating myeloid cells, was expressed within the choroid plexus and surrounding subventricular zone. The kinetics of myeloid cell migration through the basement membrane (outlined by laminin staining in red) was observed by confocal microscopy and IMARIS 3D analysis. Immunofluorescence images showed infected myeloid cell migration (green) through the tight junctions of the choroid plexus epithelium. Grey scale images of all three colors (virus-green; laminin-red, DAPI-blue) at 12 hours PI revealed the intensity and organization of the myeloid cell infiltration in greater detail. In order to better visualize myeloid cell entry, IMARIS 3D with diminishing laminin label (diminishing red) was carried out, and the methodology revealed the extravasation of infected myeloid cells through the basement membrane (white arrow). Original images were obtained with a 63X Plan-Aprochromat objective at 0.3μm interval step slices.

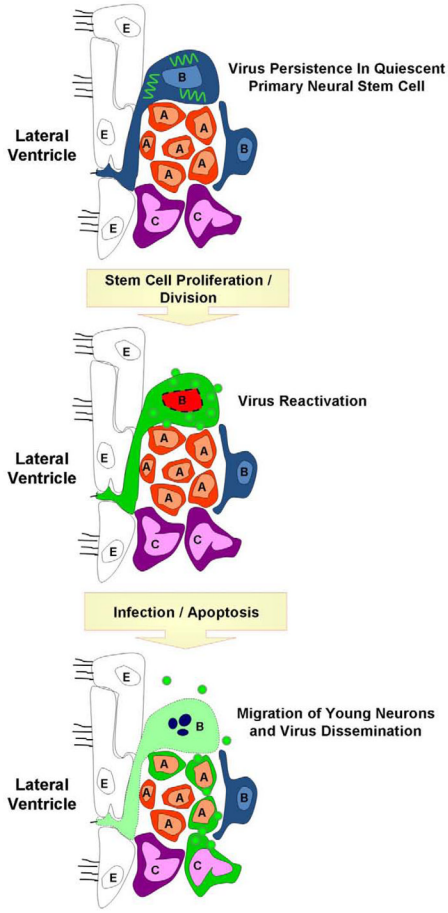

Figure 3. Potential reactivation of a persistent coxsackievirus infection within quiescent neural stem cell

Coxsackieviral RNA (squiggly green lines) may persist in a latent state within quiescent type B neural stem cells in the CNS. Following cellular division reactivation of viral RNA may lead to viral protein expression and sporadic infectious virus production. The production of infectious virions may infect nearby progenitor cells. Alternatively, asymmetric division of infected type B stem cells may generate downstream progenitor cells or immature neurons that remained infected and migrate to other regions of the CNS. The outcome over the long-term of continuous virus reactivation may be virus-mediated neuropathology and virus dissemination.

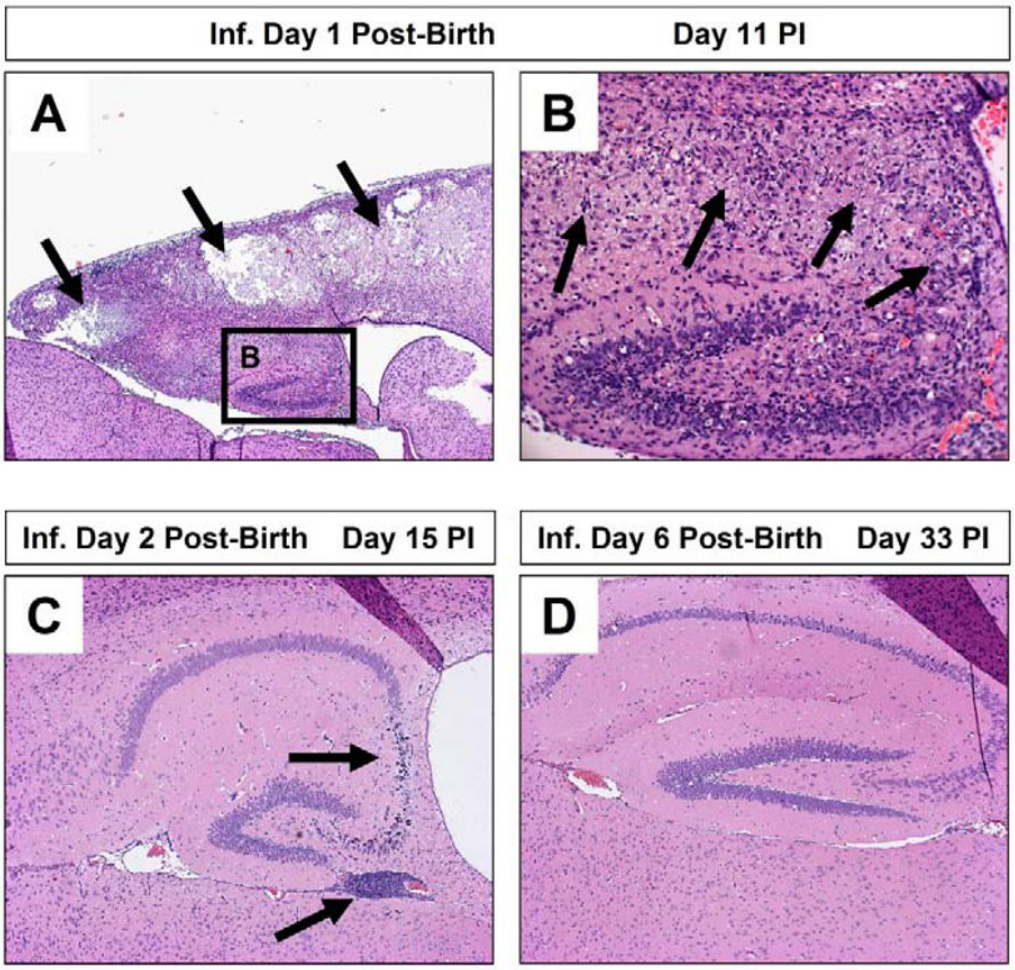

Figure 4. Increased susceptibility of young mice to coxsackievirus infection and subsequent CNS pathology

Neonatal SJL mice (day 1, 2, or 6 post-birth) were intra-cranially infected with 100 pfu CVB3. The brains of infected mice were harvested on day 11, 15 or 33 post-infection, respectively. De-paraffinized sections of the brains were stained with hematoxylin and eosin and inspected by microscopy for lesions and inflammatory cells. ***(A)*** One day-old mice suffered the greatest level of neuropathology following infection. Lesions were observed within the cortex and hippocampus (black arrows). ***(B)*** A higher magnification of ***(A)*** revealed the loss of pyramidal neurons in the hippocampus and the presence of inflammatory cells (black arrows). ***(C)*** In contrast, two day-old mice infected with an identical amount of CVB3 showed reduce signs of neuronal loss restricted to the CA3 and CA4 field of the hippocampus (black arrow). The presence of immune cell enriched perivascular cuffs were observed near the hippocampus (black arrow). ***(D)*** Six day-old mice infected with CVB3 showed little or no signs of CNS disease.

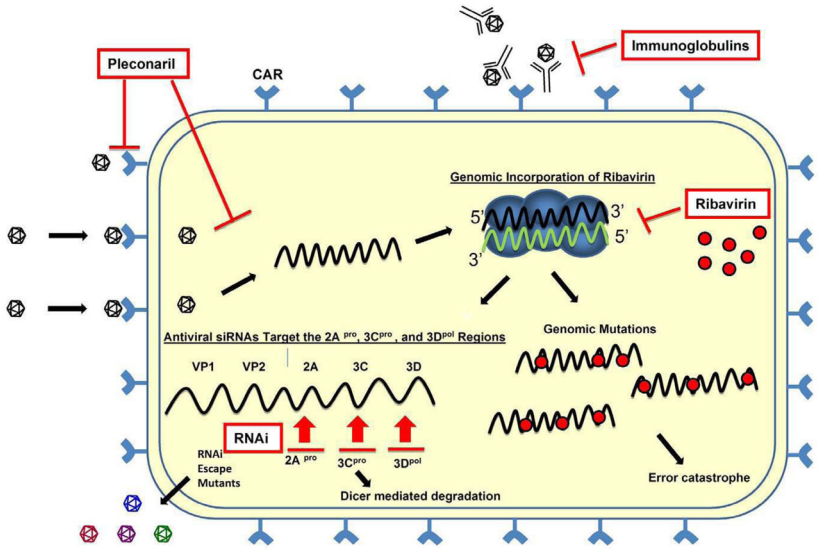

Figure 5. Antivirals Against Enteroviruses: Mechanisms of Action

Pleconaril, ribavirin, intravenous immunoglobulins (IVIg) and RNAi each inhibit the production of infectious virions at different steps. Pleconaril induces a conformational change in the viral VP1 capsid protein that inhibits both genome uncoating and the binding of the virus to its cognate receptor, CAR. Ribavirin, a nucleoside analog, is incorporated into the viral genome. This incorporation results in viral genomic mutations that lead to “error catastrophe”. Virus-specific IVIg may bind to virions extracellularly and inhibit the virus from entering the cell. RNAi molecules target the positive sense viral RNA strand leading to Dicer-mediated degradation of the viral genome. However, escape mutants may form that avoid the effects of RNAi. Positive-sense strand viral RNA is shown in black; negative sense strand viral RNA is shown in green. Antiviral compounds are shown in red. The replication complex is shown in blue.

Table 1

Enterovirus tropism in the CNS

Virus	CNS Localization	Cell Types Infected - Acute	Cell Types Infected - Persistence
Poliovirus	entire CNS	neurons, astrocytes, oligodendrocytes	neurons
Enterovirus-71	CNS - but not dorsal root ganglia or trigemina	neurons, astrocytes	persistence documented; site unknown
Coxsackievirus	choroid plexus, neurogenic regions (SVZ, SGZ), hippocampus, cortex	nestin^{+} myeloid cells, NPSCs, neurons	NPSCs (in culture)
TMEV	grey matter (acute) white matter (persistence)	neurons, astrocytes, oligodendrocytes	macrophages, microglia, oligodendrocytes

Table 2

Methods used to identify and classify enterovirus CNS infections

Technique	Method of Identification	Host Isolate	Tools	Specificity
Tissue Culture (Leland, 2008)	Presence of Cellular Cytopathic Effect	Cerebral Spinal Fluid	Selective Cell Lines	Genus
Reverse-Transcription Polymerase Chain Reaction + Genomic Sequence (Romero, 1999)	Genomic Amplification	Cerebral Spinal Fluid	Enterovirus Specifc Primers – Majority Amplify 5′ UTR (Rotbart, 1990)	Serotype – Nucleotide Mutations (Mirand, 2008)
Quantitative Real Time PCR (Dierssen, 2008)	Genomic Amplification	Cerebral Spinal Fluid	Enterovirus Specifc Primers – Majority Amplify 5′ UTR	Genus
Immunohistochemistry	Visualization Using Virally Specific Antibodies	Central Nervous System Tissue	Visualization/Microscopy	Cellular Localization
In situ Hybridization	Visualization using Virally Specific Probes	Central Nervous System Tissue	Visualization/Microscopy	Cellular Localization

Table 3

Compounds used to treat enterovirus infections

Compound	Target	Stage of Development	BBB Penetration	Resistance	Mechanism
Ribavirin (Li, 2008)	Viral Genome	Patient Prescription	Conflicting Reports (Hosoya 2001 + Honda 1994)	Yes (Poliovirus Isolates) (Vignuzzi, 2005 + Pfeiffer 2003)	Nucleoside Analog - Genomic Incorporation- Error Catastrophe
Pleconaril (Pevear, 1999)	VP1 Protein of Nucleocapsid	Clinical-Phase III-IV (Webster 2005, Desmond 2006)	Yes (Schmidtke 2009)	Yes (CVB3 Nancy Strain) (Schmidtke 2005)	Conformational Change of VP1 Protein - Receptor Attachment - Genomic Uncoating (Chen 2008)
RNAi (Multiple Formulations)	Viral Genome – Majority Target Protease 2A + 3C or Polymerase 3D (Yuan, 2005) (Tan 2010) (Tan 2007)	In Vitro/In Vivo (Li, 2008)	Undocumented	Yes (Coxsackie + Poliovirus Isolates) (Merl 2007) (Gitlin 2002)	Viral RNA Degradation via Dicer Recognition (Aliyari 2009)