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Dissection of Molecular Mechanisms by Which Human Host Factors Regulate JC Polyomavirus Internalization

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DISSECTION OF MOLECULAR MECHANISMS BY WHICH HUMAN HOST FACTORS REGULATE JC POLYOMAVIRUS INTERNALIZATION

By
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B.S. The University of Maine, 2015

A DISSERTATION
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Viruses require a host cell in order to replicate. Infection and the onset of disease result from direct virus-host cell interactions. My dissertation research is focused on understanding how a common human virus, JC polyomavirus (JCPyV), activates specific host cell factors to cause infection. When people are immunocompromised, JCPyV infection may exacerbate into the onset of progressive multifocal leukoencephalopathy (PML), a fatal neurodegenerative disease. Individuals with the greatest risk for the development of PML are those living with multiple sclerosis or infected with HIV. Unfortunately 50-80% of the population is infected by JCPyV, putting individuals at risk for developing PML. Limited therapies exist to treat PML, highlighting the need for effective therapeutics, candidates with the most promise target the initial interactions between JCPyV and host cell receptors, blocking their communication, thus preventing viral attachment, entry, and resultant infection. JCPyV recognizes and interacts with specific proteins, or receptors, on the host cell surface, resulting in the uptake of the virus into the cell. JCPyV requires the serotonin receptor subtype 2 (A, B, and C) family to internalize, however, the cellular proteins that mediate internalization and how they drive viral infection is poorly understood.

This dissertation research aimed to characterize the processes by which JCPyV usurps cellular endocytic machinery to internalize into host cells. The work described herein has demonstrated that proteins central to the clathrin-mediated endocytic pathway are crucial for JCPyV entry and infection,
including clathrin, AP2, β-arrestin, and dynamin. β-arrestin is capable of localizing to, and interacting with, serotonin receptors. Additionally, this work has further characterized the requirement of these contacts through modification of the β-arrestin binding domains within the receptor and through knockdown of the cellular protein, GRK2, responsible for initiating serotonin receptor-β-arrestin communications, both of which block JCPyV entry.

This research demonstrates that direct interaction between serotonin receptors and β-arrestin is a major determinant for JCPyV entry and is critical for driving the infectious process. Understanding the role of these proteins in virus internalization may serve as a platform for the development of novel treatments for PML. Together, this work defines how JCPyV internalization occurs within host cells.
DEDICATION

To my parents, my sister, and my brother. Thank you for your unwavering support.
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Throughout my graduate career I have had the privilege of working with individuals to whom I would like to extend my utmost gratitude. I would like to thank the other graduate members of the Maginnis lab, including Jeanne DuShane, Michael Wilczek, Kashif Mehmood, Mason Crocker, and Avery Bond, for providing a laboratory environment filled with humor and support. Truly, my success is linked to the supportive atmosphere and help that you all have provided for the last five years. I would also like to thank the past and present undergraduate students of the Maginnis lab, especially Conner Lajoie, Ashley Soucy, Sarah Nichols, and Tristan Fong, each of whom contributed to my publications. I would also like to extend my sincerest gratitude to every member of the Department of Molecular and Biomedical Sciences. In particular, every single professor, as well as, my fellow graduate students, from my undergraduate career to now completing my doctorate, your guidance and support has honed me into the scientist I am today, and for that, I am eternally grateful. I would also like to specifically thank each member of my committee; I appreciate your generosity with your time and all of the feedback and support you have provided throughout this process. I would like to thank the other laboratories with whom I have established collaborations over the course of my degree including the Atwood laboratory.

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LIST OF ABBREVIATIONS

5-Hydroxytryptamine (5-HT)

Acquired immunodeficiency syndrome (AIDS)

Adaptor protein 2 (AP2)

Antibody (Ab)

BK polyomavirus (BKVpV)

Central nervous system (CNS)

Cerebral spinal fluid (CSF)

Clathrin heavy chain (CHC)

Clathrin-mediated endocytosis (CME)

Deoxyribonucleic acid (DNA)

Dulbecco’s modified eagle medium (DMEM)

Endoplasmic reticulum (ER)

Epidermal growth factor receptor (EGFR)

Extracellular signal-regulated kinase (ERK)

Extracellular vesicle (EV)

Fetal bovine serum (FBS)

Fluorescent focus unit (FFU)

Glycosaminoglycans (GAGs)

Granule cell neuronopathy (GCN)

G protein coupled receptor (GPCR)
G protein receptor kinase (GRK)

Highly active antiretroviral therapy (HAART)

Human brain microvascular endothelial cell (HBMEC)

Human embryonic kidney cell (HEK)

Human immunodeficiency virus (HIV)

Immune reconstitution inflammatory syndrome (IRIS)

JC polyomavirus (JCPyV)

Lactoseries tetrasaccharide c (LSTc)

Magnetic resonance imaging (MRI)

Merkel cell polyomavirus (MCPyV)

Minimum essential medium (MEM)

Mitogen-activated protein kinase (MAPK)

Monoclonal antibody (mAb)

Mouse embryonic fibroblast (MEF)

Multiple sclerosis (MS)

Multiplicity of infection (MOI)

Murine polyomavirus (mPyV)

Non-coding control region (NCCR)

Nuclear pore complex (NPC)

Origin of replication (ORI)

Paraformaldehyde (PFA)

Phosphate buffered saline (PBS)
Polyomavirus (PyV)

Progressive multifocal leukoencephalopathy (PML)

Receptor tyrosine kinase (RTK)

Ribonucleic acid (RNA)

Selective serotonin reuptake inhibitor (SSRI)

Simian virus polyomavirus 40 (SV40)

Small-interfering RNA (siRNA)

Severe acute respiratory syndrome (SARS)

T antigen (T-Ag)

trans-Golgi network (TGN)

Tris buffered saline (TBS)

Viral protein 1, 2, 3 (VP1, VP2, VP3)

Western blot (WB)
CHAPTER 1
INTRODUCTION


1.1. Diversity of Pathogens and Capability of Causing Disease

The human body is an ecosystem; playing host to upwards of thousands of microbial species including bacteria, fungi, and viruses [1]. While the human body is made up of approximately $10^{13}$ cells, it is estimated that the number of microorganisms at any given time exceeds the number of human cells, with an estimated number of bacteria and fungi to be approximately $10^{14}$ [2]. These populations of microorganisms are further outnumbered by the number viruses, thought to exceed the amount of bacteria and fungi by tenfold [3]. While we play host to these microorganisms, they typically do not cause symptomology or disease.

Pathogens are distinct from the normal flora that inhabits the human body [2]. These microorganisms have developed the ability to overcome the cellular and biochemical barriers in order to cause disease in the human host. A successful infection by a pathogen involves the capacity of the pathogen to 1) establish an infection in a compartment of the host capable of supporting survival and replication of the pathogen, 2) avoid biological and immunological pressures that would otherwise serve to impair or eliminate infection, and 3) be able to replicate forming new infectious progeny that can then infect a new host [2]. In order to accomplish this, pathogens have evolved to exploit the host, and understanding the complex interplay between the host and the pathogen help us to understand the
biology of the resultant infectious disease. Thus, understanding the biology of a pathogen involves defining the contributions of both the host and the pathogen to the infectious process [2].

1.2. Viruses as Pathogens

Viruses are obligate intracellular parasites; they require a host cell in order to replicate because they lack the basic elements necessary for growth and replication [4, 5]. Many of the most lethal infectious agents are viruses, including smallpox, influenza, and HIV [6]. Moreover, viruses are capable of causing pandemics including the virus responsible for the most recent pandemic, SARS-CoV-2, the causative agent of COVID-19 [7]. Although viruses are simple in terms of structure and packaged materials, they are highly complex in their ability to overcome cellular defenses, resulting in viral propagation [6]. Furthermore, there is significant diversity among different species of viruses, each containing only the most critical elements necessary to cause an infection within a host [5, 6].

While viruses are capable of causing disease in humans, the mechanisms required for them to cause disease is very complex. There are significant barriers that a virus must overcome in order to deliver the enclosed genetic material into the host cell and ensure replicative success. In order for a virus to establish an infection the virus is dependent on the susceptibility of the target cell (capable of initiating infection, containing the proper signaling networks to allow for delivery of the genetic material to the appropriate compartment for replication), as well as the permissivity of the target cell (must contain the necessary resources to allow for viral replication and release of newly formed infectious particles) [5]. These critical elements typically define the tropism for a particular virus. However, not all infections result in the onset of virus-associated disease. The capacity for a virus to cause a disease within a host is dependent on many factors. There are different types of viral infections, lytic, non-lytic, and persistent infections; defining the ability for a virus to cause disease [5, 6]. A lytic infection is a consequence of active replication of the virus, generally resulting in lysis or breaking open of the virus-infected cell. This generally causes cell death, mediating the release of newly formed viral progeny. Non-
lytic infections are due to non-replicating viral infections. Persistent infections are the result of a virus infecting cells, though the viral infection is not cleared. In these cases the infection may be non-productive, result in integration of the viral genetic material into the host genome, or result in low-level viral replication [8]. The type of viral infection initiated is dependent on many factors. For example, in some cases the host immune system is able to prevent active viral replication resulting in a state of persistence [9]. Ultimately, the most advantageous strategy is for the virus to ensure productive infection, while also relying on the host environment. Polyomaviruses, which infect the majority of the population and cause a lifelong asymptomatic infection, are controlled by the host immune system [10].

1.3. **Polyomaviridae and JC Polyomavirus**

*Polyomaviridae* comprises a family of 14 human polyomaviruses [11] that are classified according to their morphological and genetic similarities. The *Polyomaviridae* family can be further sub-sectioned into three genera, *alpha-*, *beta-*, *delta-*, and *gammapolyomavirus* [12]. Collectively, these genera account for over 80 different viral species [12]. Polyomaviruses are dsDNA viruses containing genomes of roughly 5,000 base pairs. Their virions are approximately 40-45 nm in diameter with an icosahedral structure and lack a viral envelope [13-15]. The capsids of these viruses are comprised primarily of viral protein 1 (VP1), and secondarily of VP2 and VP3 [13, 16]. Due to their importance in human health, infection strategies of polyomaviruses BK (BKPyV), JC (JCPyV), and Merkel cell (MCPyV) are of significant interest. JC polyomavirus (JCPyV) was the first polyomavirus isolated from a human host [15]. However, advances in this field can be largely attributed to studies on nonhuman polyomaviruses, murine (mPyV) and SV40 [17]. While not discussed in detail in this work, other human polyomaviruses have been implicated with human disease including TSPyV, associated with the disease *Trichodysplasia spinulosa*, a rare skin condition [18]. Further, human polyomaviruses 6, 7, and 9 (HPyV6, HPyV7, HPyV9), originally isolated from the skin, have not been associated with the causation of any human pathology [19], though DNA from these polyomaviruses have been detected in cutaneous T-cell
lymphomas [20]. Additionally, New Jersey polyomavirus (NJPyV), though unconfirmed, may be associated with vasculitic myopathy, a condition affecting skeletal muscle [21]. BKPyV establishes an asymptomatic infection in the renourinary tract of healthy individuals. In spite of this, under conditions of immunosuppression, BKPyV may cause nephropathy and hemorrhagic cystitis; individuals at particular risk for BKPyV-associated disease are those who have received an organ transplant [22]. In comparison, JCPyV also establishes a lifelong infection within the kidney of healthy individuals [15]. In immunosuppressed individuals, JCPyV can cause progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease affecting the central nervous system (CNS) [15]. MCPyV, discovered in 2008, can cause Merkel Cell carcinoma, an aggressive, albeit rare, form of skin cancer [23]. While there are significant commonalities between these viruses, they differ in their utilization of cellular receptors and entry routes for initiating infection. However, one distinct overlap in infection is clear; all polyomaviruses studied to date traffic to the endoplasmic reticulum following attachment and entry [24-30]. Therefore, a deeper understanding of polyomavirus entry is necessary in order to define the similarities yet unique features that results in a broad range of diseases.

1.3.1. JC Polyomavirus Characteristics

JCPyV is categorized as a betapolyomavirus, along with other closely related polyomaviruses BKPyV and simian virus polyomavirus 40 (SV40) [12]. JCPyV VP1 mediates binding of JCPyV to target cells. The capsid of JCPyV contain 360 copies of VP1 organized as 72 pentamers on the capsid surface, with each pentamer forming a pore in the center. Each copy of VP1 is linked through its C-terminus to either a copy of VP2 or VP3, the minor capsid components, which are sequestered inside of the capsid. The overall ratio of VP1 to VP2 to VP3 in the capsid is 1:1:5. Further, the structure of the capsid is stabilized by interpentameric disulfide bonds, calcium ions, and the direct interactions that occur between VP1 and adjacent VP1 pentamers [13, 31, 32].
Enclosed within the capsid is the viral genome [33]. The genome of JCPyV can be divided into three sections, the non-coding control region (NCCR), which contains the origin of replication, the promoter, and enhancer elements, the early genes, which contain open reading frames (ORFs) for splice variants of T antigen (T-Ag), and the late genes, which contain ORFs for late gene products including the capsid components and agnoprotein (Figure 1.1.) [15]. The NCCR region is the most variable portion of the JCPyV genome, a single infected individual can harbor multiple strains of JCPyV due to the variability of this region [15]. Transcription of the viral genome begins with the early genes, the production of early gene products drives the transition from early gene transcription to transcription of the late genes and DNA replication [15]. As such, the JCPyV genome is described as bidirectional, with one strand of the genome allowing for transcription of early genes, including large T-Ag, small t antigen, and three additional T antigen splice variants [34]. Accumulation of T-Ag drives replication of the viral genome, through binding within the origin of replication (ORI), which occurs concurrently with cellular replication [35]. Then, the complementary strand is transcribed into late gene components including capsid proteins VP1, VP2, and VP3, as well as the agnoprotein, and two micro-RNAs which is thought to temper the production of T-Ag through a negative feedback loop [35].
Figure 1.1. The JCPyV genome

Early and late regions of the JCPyV genome are separated by the noncoding control region (NCCR). The NCCR contains the origin of replication (ORI) as well as enhancer and promoter elements (grey). The early segment of the genome contains open reading frames for T-Ag splice variants. The late region contains open reading frames for the Agno protein, and capsid components, VP1, VP2, and VP3. Modified from [15]. Figure used with permission from ([36]).

1.3.2. Transmission and Tropism of JC Polyomavirus

Due to variability in the NCCR, infected individuals are described to be either infected with archetype JCPyV or JCPyV with a rearranged NCCR region [15]. The archetype strain of JCPyV is thought to be the predominantly transmitted strain, as it is detected in the urine of infected individuals and in waste water [33]. Because of this, infection with JCPyV is thought to occur perorally, through ingestion of infectious JCPyV particles due to either poor sanitation practices or human-to-human contact.

Seroepidemiology studies have established that approximately 50-80% of the population is asymptomatically infected with JCPyV. These individuals are thought to become initially infected in tonsillar tissue followed by the establishment of a lifelong infection within the kidney while the host is healthy [37-39]. The kidney is thought to be a site of persistent infection rather than dormancy as infectious virions can be periodically shed in the urine throughout life [37]. Sequestration of archetype JCPyV within the kidney is believed to be due to immunocompetency. However, during prolonged severe immunosuppression JCPyV may traffic to the central nervous system (CNS) through the vascular system and establish a lytic infection of glial cells within the CNS, astrocytes and oligodendrocytes [40, 41]. While not well characterized, dissemination of JCPyV through the blood is thought to occur through JCPyV infection of B cells [42]. Spread of JCPyV across the blood brain barrier is poorly understood.

Under conditions of human immunodeficiency virus (HIV) infection/acquired immunodeficiency syndrome (AIDS) or through the use of immunomodulatory drugs, immune surveillance within the brain is reduced, particularly T cells. This reduced surveillance is thought to contribute to the spread of JCPyV
infection within the brain, virtually occurring unchecked. Within the CNS, the viral destruction of oligodendrocytes results in severe demyelination, rapidly progressing into multiple growing foci of cellular destruction, classified as progressive multifocal leukoencephalopathy (PML) (Figure 1.2.) [15, 43].

**Figure 1.2. Major sites of JCPyV infection.**

JCPyV asymptptomatically infects the kidney of healthy individuals, establishing a lifelong persistent infection. Cells commonly targeted by JCPyV in the kidney include renal proximal epithelial cells (approximate location indicated by black arrows). Upon severe immunosuppression JCPyV may traffic to the central nervous system (CNS), establishing a lytic infection within glial cells: astrocytes and oligodendrocytes (red arrow). Lytic infection of oligodendrocytes is thought to lead to the formation of multifocal regions within the brain (grey, black arrows), a hallmark of Progressive Multifocal Leukoencephalopathy (PML). Created using BioRender.
1.3.3. Neuroinvasion of JCPyV

Dissemination of JCPyV from primary sites of infection, the kidney and tonsillar tissue, to secondary sites, the CNS, is not well elucidated. However, multiple routes of spread have been proposed. One such method involves JCPyV infection of the bone marrow, primarily B cells, which then leads to spread of JCPyV infection to the CNS [42]. This route of transmission is supported by reports that glial cells, astrocytes and oligodendrocytes, and B cells, endogenously express similar transcription factors, components of the host cell critical for viral replication and production of infectious viral progeny [44, 45]. Alternatively, JCPyV escape into the CNS has also been described to occur through the choroid plexus. The choroid plexus lines the barrier between periphery blood and the cerebral spinal fluid (CSF) [46]. Detection of JCPyV DNA in the CSF is one of the criteria for diagnosis of PML in afflicted individuals [47, 48]. Importantly, cells of the choroid plexus express cellular receptors necessary for JCPyV attachment and entry, are susceptible to JCPyV infection in vitro, and tissue samples of the choroid plexus from infected patients demonstrate that JCPyV infection can occur within these cells in vivo [49-51]. In addition, some groups suggest that JCPyV may reside within the choroid plexus or brain parenchyma, only escaping into the CNS under conditions of decreased immune surveillance. This is supported by evidence that JCPyV DNA has been detected in the CNS of elderly individuals without conditions that would predispose them to PML [52]. However, the latter route of spread has the least amount of evidentiary support.

1.3.4. Utilization of Animal Models for Investigation of JC Polyomavirus Infection

Unfortunately, tractable animal models are not available for the investigation of JCPyV infection. One of the major difficulties in the establishment of an animal model for JCPyV infection occurs is the high reliance on human-specific transcription factors for replication of JCPyV [53-55]. Because of this, infection of JCPyV in animals does not reflect infection within the human host, either in the kidney or for the investigation of PML within the CNS. Rather than producing the infection seen in humans,
inoculation of some animal models results in tumorigenesis [56]. More recently, the establishment of a humanized mouse model was produced through the engraftment of human glial progenitor cells [57]. While a significant advancement towards the production of an animal model to study JCPyV-associated disease, a model that truly recapitulates the JCPyV infectious lifecycle within the human host remains enigmatic. However, much has been learned through evaluation of patient populations predisposed to PML. For example, careful monitoring of patients afflicted by multiple sclerosis (MS), taking the immunomodulatory drug natalizumab, or persons infected with HIV/AIDS, has been performed through the analysis of blood sampling, CSF, and magnetic resonance imaging (MRI). These tests, preformed with regularity, may indicate the onset of PML prior to severe disease, through identification of JCPyV in the blood (viremia) [58, 59].

1.4. Progressive Multifocal Leukoencephalopathy

1.4.1. Pathology of Disease

Progressive multifocal leukoencephalopathy (PML) was first described in a patient in 1958 as a unique disease affiliated with B-lymphocyte proliferative disorders [33, 60-62]. Initially PML was affiliated with multifocal lesions of the white matter in the CNS along with three distinct pathologies: demyelination, abnormal morphology of astrocytes, and oligodendrocyte nuclear inclusions [63]. The nuclear inclusions of oligodendrocytes pointed towards a disease of viral origin [63-65]. Early on, there were very few cases of PML until the 1980s when HIV had progressed to pandemic levels of infection [66]. It is estimated that 2-5% of the HIV+ population would develop PML, and is thus classified as an AIDS-defining illness [67-71]. However, the implementation of highly active antiretroviral therapy (HAART) significantly reduced the number of AIDS-associated PML cases, though introduction of HAART did not remove the risk of AIDS-associated PML [72]. For many, it is recommended to treat the underlying immunosuppression, resulting in restoration of immune function. While this has significantly reduced the mortality associated with PML, for many this results in the onset of induced immune
reconstitution syndrome (IRIS), associated with severe morbidity [73-76]. Although the HIV+ population
initially represented the largest risk group, due to introduction of HAART, the population of HIV+
individuals that progress to AIDS-associated PML is now relatively rare. However, the association of PML
with other risk groups are now recognized [77, 78].

1.4.2. Immunosuppressive Agent-associated PML

Although individuals infected with HIV and those with B-lymphoproliferative disorders were
originally thought to be the only populations at risk of developing PML, other predispositions have been
identified [77, 78]. The risk group now includes individuals taking immunomodulatory therapies like
natalizumab for treatment of MS [77, 79-81]. Natalizumab is an $\alpha_4$-integrin that prevents the
localization of leukocytes to sites of inflammation [79]. Moreover, PML has also been associated with
natalizumab treatment for Chrohn’s disease as well as other immunosuppressive drugs like adalimumab
treatment for rheumatoid arthritis [82-84]. Although the number of individuals taking
immunosuppressive agents is a fraction of those infected with HIV, the number of PML cases associated
with immunomodulatory therapies has significantly increased, select immunosuppressants provide the
highest risk for the development of PML, including natalizumab, rituximab, and dimethyl fumarate [78,
85].

1.4.3. Symptomology, Diagnosis, and Treatment of PML

PML causes severe symptomology. JCPyV infection of astrocytes and oligodendrocytes in the
CNS affects overall neuronal stability [47]. JCPyV infection of oligodendrocytes results in cell lysis, severe
demyelination, and ultimately death of the human host. Due to the location of demyelination, JCPyV-
induced PML may present as several other disorders including MS and stroke. Because of the sites of
demyelination, the most common symptoms associated with PML are semi paralysis, motor dysfunction,
speech impairments, and visual defects [33, 64, 65]. Unfortunately, PML diagnosis in the early stages of
the disease is uncommon, rather diagnosis occurs once severe symptomology presents itself [86, 87].
Diagnosis of PML occurs through classification of symptomology, MRI screening for presence of multifocal viral lesions, and detection of JCPyV DNA in the CSF by spinal tap [47, 48]. Unfortunately diagnosis of PML based on MRI screening alone is not recommended as lesions associated with JCPyV infection can be misidentified as lesions associated with other disorders including MS, as such, diagnosis prior to the onset of severe symptoms is uncommon [47, 88-90].

While there are diverse groups at risk for the development of PML, one theme is apparent among them, severe immunosuppression is a hallmark of PML [91]. Specifically, low levels of CD4+ T cells are associated with more severe cases of PML in those affiliated with AIDS and the use of immunomodulatory therapies [73, 92-95]. While CD4+ T cells are important for the prevention of PML, CD8+ T cells are important for recovery. Individuals successfully recovering from PML had increased levels of CD8+ T cells over time, and in individuals with severe PML, concentrations of CD8+ T cells are low [95, 96]. Collectively, this suggests that there may be a correlation between expression of CD8+ T cells and recovery rate [97]. Moreover, injection of JCPyV-antigen specific generated cytotoxic T cells in a combination therapy with a serotonin reuptake inhibitor resulted in clearance of JCPyV DNA from CSF and reduced symptomology in one patient [98].

While no specific antiviral therapy exists for PML, the introduction of antiretrovirals for the treatment of HIV has reduced the rate of AIDS-associated PML [33, 99]. Many drugs have been used to treat PML with mixed success in the clinic [100]. Currently, as no drug has effectively translated from protection of viral infection in vitro to in vivo, the recommended action is treatment of the underlying immunosuppression, often resulting in onset of immune reconstitution inflammatory syndrome (IRIS) [73-76]. Numerous strategies have been attempted to assist the host in clearance of viral infection, including the introduction of checkpoint inhibitors to help boost the immune response in the infected host [101, 102]. Recently, one target, programmed cell death 1 receptor (PD-L) and ligand PD-L1 have demonstrated promising results when used to treat patients suffering from PML. PD-L, a receptor found
on T cells, is associated with T cell exhaustion [101, 103]. These therapies, including pembrolizumab and nivolumab, have resulted in improved prognosis for patients suffering from PML [103-110]. However, one disadvantage of the utilization of checkpoint therapies is the risk of increased inflammation and onset of IRIS [101].

The utilization of T cell immunotherapy to treat PML has great potential. For some of these approaches, the genomic conservation between JCPyV and BKPyV, approximately 75%, could be used as a tactic. Reaction to one could result in reaction to the other. Using this tactic, bank-preserved T cells reactive to BKPyV were used as a therapy for treatment of PML, resulting in evidence of cross-reactivity and patient condition improvement, though in the majority of the patient pool, treatment also resulted in the onset of IRIS [111]. Aside from the onset of IRIS, the implementation of T cells specific to BKPyV may provide a more cost-effective method for the treatment of PML in comparison to checkpoint inhibitors. Moreover, due to the high prevalence of BKPyV infection in humans, the feasibility of collecting enough T cells to allow for treatment of those suffering from PML is enticing [111, 112].

1.5. JCPyV Association with Other Disorders

Although most often JCPyV-associated disease is attributed to PML, JCPyV has been described to cause other disorders including JCPyV granule cell neuronopathy (GCN), JCPyV-associated meningitis, JCPyV encephalitis, and JCPyV-associated nephropathy [113]. Similar to PML, GCN also results in JCPyV targeting of cells within the CNS. However, while PML is associated with JCPyV infection of glial cells, including astrocytes and oligodendrocytes, GCN is due to JCPyV infection of granule cells of the cerebellum [114]. Since GCN and PML both occur in the CNS, the likelihood that patients suffering from one of these disorders could also suffer from the other is most likely high [115]. JCPyV-associated meningitis and JCPyV encephalitis occur rarely, and are thought to be attributed to the immunosuppressed state of the patient [116, 117]. JCPyV-associated nephropathy, also a rare condition
which affects the kidney, is not though to produce severe symptomology, in contrast to nephropathy caused by other viruses, including BK polyomavirus [118].

1.6. Attachment of Polyomaviruses

Most often, the initiation of viral infection is mediated by interactions between viruses and specific cellular factors facilitating viral attachment [119]. Attachment can directly induce structural conformational changes or activate specific signaling pathways to allow for uptake of the virus within the cell [120, 121]. Because of this, more than one receptor may be necessary to allow for coordination of virus entry. Further, interactions with cellular receptors largely dictate the signaling networks critical for delivery of the virus to the appropriate cellular compartment, ensuring productive infection [14]. Many viruses utilize attachment factors such as sialic acid-containing receptors including glycoproteins or glycolipids, or glycosaminoglycans and integrins (Figure 1.3), and this conserved attachment strategy extends to polyomaviruses [14, 119].

The cell surface is decorated with glycoconjugates, carbohydrates covalently attached to biological molecules in the cell membrane, such as proteins or lipids [122]. The glycan chain of glycoproteins and glycolipids if often terminated by a sialic acid [123]. For example, glycolipids are composed of mono or oligosaccharides attached to lipid moiety embedded in lipid-rich areas of the cell membrane [124]. Common glycolipids include the glycosphingolipids, gangliosides, which are comprised of oligosaccharides with one or more sialic acids attached to a ceramide moiety anchored in the plasma membrane [125]. Gangliosides are a large group of glycosphingolipids, referred to as a, b, or c series gangliosides, based on their sialic acid branching patterns [126]. Sialic acids are neuraminic acid derivatives with either an N- or an O-substitution, and are comprised of a 9-carbon monosaccharide backbone [127]. Further, sialic acids may be additionally modified on their hydroxyl group through acetylation or methylation [128, 129]. The most common linkages are through either an $\alpha2,3$ or $\alpha2,6$ at
the 2-carbon position of the internal Gal or GalNAc [127]. Glycosaminoglycans (GAGs) are long polysaccharide chains with a repeating disaccharide containing an amino sugar [130]. Integrins are comprised of heterodimers of $\alpha$ and $\beta$ subunits. Though they primarily function as adhesion molecules in cell-cell interactions, integrins are recognized as attachment and entry factors for many viruses [14, 131]. Interactions between viruses and host cells are initiated through the aforementioned receptors; their distribution and diversity can have significant implications in tissue tropism and host range [132]. A deeper understanding of the attachment factors critical for infection by polyomaviruses will give increased insight into a virus family that in spite of having a narrow host range and tissue tropism, can cause a wide range of disease pathologies.

![Figure 1.3. Attachment factors commonly used by polyomaviruses.](image)

Sialic acids (SA) Glycosaminoglycans (GAGs) Integrins Gangliosides

- Sialic acid-containing receptors typically consist of glycoproteins or glycolipids (orange) with attached mono or oligosaccharide backbones (pink circles) and terminal sialic acid residues (pink diamonds). Glycosaminoglycans contain repeating disaccharides (green hexagons) and are attached to the polysaccharide sugar core (blue) by link proteins (light green). Integrins are heterodimeric proteins comprised of $\alpha$ and $\beta$ chains containing $\beta$ domains attached to thigh and calf domain regions. $\beta$ chains further contain epidermal growth factor (EGF) binding domains. Gangliosides are glycosphingolipids with a ceramide moiety embedded in the lipid bilayer and an oligosaccharide chain containing one or more sialic acids. Gangliosides may also contain a terminal sialic acid residue (pink diamond) [133].

Interestingly, all polyomaviruses studied to date use sialic acid-containing receptors for attachment to the host cell and/or entry [134, 135]. This binding has been demonstrated to occur with
at least one of the three known sialic acid linkages, $\alpha 2,3$, $\alpha 2,6$, or $\alpha 2,8$, with both mPyV and SV40 binding to $\alpha 2,3$-sialic acid containing receptors [136-147]. mPyV has more recently been identified to also bind to an $\alpha 2,8$-sialic acid containing receptor [148]. These initial interactions between the virus and host cell receptors occur through VP1, the major capsid component and the only protein expressed on the exterior surface [13, 149].

1.6.1. Cellular Attachment of JCPyV

JCPyV has been demonstrated to bind to GAGs [150] and either $\alpha 2,3$- or $\alpha 2,6$-terminal sialic acid receptors [140-145]. JCPyV was shown to utilize sialic-acid-containing gangliosides [142, 145], yet the virus does not bind to gangliosides with high affinity [151], instead preferring the $\alpha 2,6$-sialic acid-containing lactoseries tetrasaccharide c (LSTc) on glycoproteins or glycolipids [144]. Further, VP1 of JCPyV undergoes structural modifications for enhanced binding to LSTc, demonstrating further specificity for this receptor motif [144]. Interestingly, VP1 proteins of SV40, BKPyV, and JCPyV, have a greater than 74% sequence identity and engage their sialic acid-containing attachment factors through highly conserved interactions between the VP1 and the terminal sialic acid residue of their respective receptors [134, 152, 153]. Importantly, the interactions between these viruses and their unique receptors is dependent on interactions between VP1 and the terminally expressed Neu5Ac outside of the sialic acid binding pocket, conferring specificity for these receptors in attachment and thus, productive infection [144, 152, 154].

1.6.2. Cellular Attachment of Other Polyomaviruses

Visualization of murine polyomavirus (mPyV) VP1 in complex with receptor portions was instrumental in understanding the interactions between the viral capsid and cellular sugar residues [138, 155]. VP1 proteins are comprised of two anti-parallel $\beta$ sheets [138, 155]; the residues of the capsid implicated in binding with either $\alpha 2,3$- or $\alpha 2,6$-sialic acids were identified through at least three direct pocket interactions with VP1 [136-138, 155]. Mutagenesis of the internal residues of the binding pockets
results in loss of infectivity of mPyV [156, 157]. Some of the cellular receptors discussed in this work were identified through the implementation of binding and flotation assays, techniques that investigate the specific protein-protein interactions between viruses and sections of cellular membranes through fractionation [158]. Utilizing these techniques, interactions between mPyV and gangliosides GT1a, GD1a, or GT1b containing a terminal sialic acid residue were determined [159-161]. Each of these receptors was further characterized for their importance in mPyV infectivity in mouse embryo fibroblasts, as well as the identification of an additional α2,8-sialic acid containing receptor [148]. Membrane flotation and binding assays to identify protein-protein interactions were also used to demonstrate ganglioside GM1 containing an α2,3-sialic acid linkage is the cellular attachment factor for SV40 [160]. Alternative studies suggest that MHC I may also play a role in cellular attachment and/or entry [162-165]. Several studies have challenged the role of MHC I in promoting SV40 attachment and/or entry [146, 166], further supported by the demonstration that MHC I does not enter with SV40 [164]. In addition, the importance of GM1 as the primary receptor for SV40 was emphasized through the utilization of high-resolution crystallization [152]. Further, association of SV40 with GM1 is sufficient to induce membrane curvature resulting in the formation of deep invaginations at the cell surface [167].

Curiously, α4 integrins have also been identified as attachment factors for mPyV. While typically responsible for regulating cellular attachment to the extracellular matrix, cytoskeletal rearrangements, and proliferation, knockdown of α4 integrins significantly reduces mPyV infection [156, 168]. Further, VP1 of mPyV contains an α4 integrin-binding motif, and mutagenesis of this motif reduces viral infectivity [169]. Binding to both gangliosides and α4 integrins is critical for activation of phosphoinositide-3 kinase/mitogen-activated protein kinase (PI3K/MAPK) signaling networks, both necessary for viral infection; yet, α4 integrins may not come into play until after interaction with gangliosides has occurred [170].
BKPyV requires interactions with sialic acid residues for productive infection. Initial investigation into the cellular factors necessary for BKPyV attachment was employed through the use of sialidase S and neuraminidase, which removes $\alpha 2,3$ or $\alpha 2,3$ and $\alpha 2,6$ sialic acids, respectively, resulting in the inhibition of infection in Vero cells [171]. Additionally, binding assays using ganglioside-containing liposomes revealed that GD1b and GT1b support viral attachment through interactions between terminal $\alpha 2,8$-disialic acid motifs and VP1 [147]. Through a structure-function analysis, BKPyV was demonstrated to require the $\alpha 2,8$-disialic acid motifs for binding, and b-series gangliosides GD2, GD3, GD1b, and GT1b, containing this epitope can support infection. X-ray crystal structure analysis of BKPyV VP1 in complex with GD3 revealed that Lys68 makes specific contacts with two sialic acid moieties of GD3 [153]. The binding capacities of different strains of BKPyV may have altered attachment properties and virulence in vivo due to alterations in amino acid sequences in the binding pockets of VP1 [172]. Interestingly, through mutagenesis of a single residue in VP1 of BKPyV, Lys68, attachment preference can be switched from ganglioside GD3 to GM1, the primary attachment receptor for SV40. Likewise, mutagenesis of the Ser68 residue of SV40 can switch attachment receptor preference from GM1 to GD3, the primary attachment receptor for BKPyV [152, 153]. The capability of retargeting attachment and receptor switching of either SV40 or BKPyV through mutagenesis of a single capsid residue suggests a conservation of conformation and structure among VP1 capsid proteins among polyomaviruses, regardless of host range.

Cellular attachment of MCPyV relies on N-sulfated- or 6-O-sulfated heparin sulfate-containing GAGs in addition to ganglioside GT1b [173, 174]. Further, interaction with sialylated glycans occurs primarily between capsid protein VP1 and disaccharide Neu5Ac-$\alpha 2,3$-Gal in a conformation that differs from other polyomaviruses in complex with sialic acid-containing receptors [139]. Direct interaction with sialic acids does not interfere with MCPyV engagement of GAGs, suggesting that this complex occurs prior to interaction with sialic acids in a co-receptor attachment model [139].
Interestingly, HPyV6 and HPyV7, common viral inhabitants of human skin, have elongated loops in their VP1 protein that obstruct sialic acid binding sites commonly utilized by other polyomaviruses [175]. Because of this blockade, HPyV6 and HPyV7 engage nonsialylated glycans for cellular attachment [175]. However, HPyV12 and TSPyV both engage α2,3- and α2,6-sialylated glycans, while NJPyV binds sialylated glyans in a previously known sialic acid binding site on VP1 [176]. Further, B-lymphotrophic polyomavirus (LPyV), a simian polyomavirus, which has been isolated from both monkey and human cells [177], also engages sialylated glycans for cellular attachment [178]. Largely, aside from these viruses utilizing glycans for cellular attachment, mechanisms associated with their cellular entry downstream of attachment remain mostly uncharacterized. Collectively, six distinct binding conformations between VP1 of the aforementioned polyomaviruses and their respective sialic acid-containing cellular receptors have been classified according to their architectural similarities accounting not only for the location of interaction on the VP1 protein, but also for the conformation of the sialic-acid within the binding pocket: (1) for BKPyV, SV40, and JCPyV, (2) for mPyV, (3) for MCPyV, (4) for LPyV and HPyV9, (5) for TSPyV, and lastly, the most recently identified conformation, (6) for NJPyV [176]. Therefore, while human viruses within this family engage distinct receptors for attachment, they do so with a high degree of conservation and specificity.

1.7. Virus Entry

Host cell factors may serve as either virus attachment moieties or functional receptors that can coordinate internalization of the virus within the cell. However, in some instances, interactions between viruses and primary receptors is not sufficient to activate the internalization machinery or signaling networks necessary for viral entry. These primary receptors chiefly serve to temporally and spatially bring the viruses into close or direct contact with secondary receptors responsible for orchestrating virus internalization in a co-receptor system [14, 119]. While these primary receptors may not
functionally coordinate virus entry, they may additionally serve to activate downstream signaling cascades that play important roles in the propagation of infection.

Through inducing internalization, viruses are capable of causing an orchestration of cellular factors to further drive entry. Because of this, there are numerous pathways viruses can use to internalize into target cells including clathrin- and caveolin-mediated endocytosis, macropinocytosis, clathrin/caveolin-independent pathways, and also cholesterol dependent/independent routes [119]. Due to this stunning diversity in the ability of viruses to orchestrate internalization, virus entry is unsurprisingly complex to unravel.

1.7.1. JCPyV Entry by Clathrin-Mediated Endocytosis

Unlike other polyomaviruses studied, JCPyV internalization has been identified to occur through clathrin-mediated endocytosis (Figure 1.4) [179-181]. While this distinction occurs, it is unclear why JCPyV engagement of cellular receptors results in internalization by clathrin-mediated endocytosis (CME) in contrast to other polyomaviruses studied. CME serves as the most common entry pathway usurped by both enveloped and nonenveloped viruses [182, 183]. CME consists of the interplay of numerous cellular factors including, but not limited to, clathrin, AP2, and dynamin, predominantly isoforms I and II [184-191]. Through sucrose-rich flotation assays, JCPyV internalization has been demonstrated to rely on intracellular cholesterol, as well as actin [192, 193]. However, JCPyV entry is sensitive to chlorpromazine, an inhibitor of CME that also functions as a selective 5-hydroxytryptamine receptor (5-HTR) antagonist [179, 194, 195]. Importantly, this finding led to the hypothesis that JCPyV usurps the proteinaceous serotonin receptor family, subtype 2 (5-HT2R) for internalization, which was verified with inhibitors and antibodies to 5-HT2Rs as well as assays that specifically measure viral entry [181, 194, 196]. The 5-HT2Rs serve as entry receptors for JCPyV as overexpression of 5-HT2Rs does not enhance JCPyV binding [181, 194], but specifically enhances viral entry [194]. In addition, sites of JCPyV
infection are consistent with expression patterns of 5-HT₂Rs, including expression in kidney and glial cells [49, 197, 198]. In further support, recent literature has indicated an interaction between 5-HT₂Rs and JCPyV through the utilization of a PLA assay [199]. In this report interactions were found to occur as early as 5 min postinfection but were transient, dissipating at 15 min postinternalization [199]. It is thought that the difference in internalization strategy usurped by JCPyV in comparison to other polyomaviruses is due to JCPyV requiring the additional proteinaceous cellular receptor for mediating viral internalization, 5-HT₂Rs, though this remains unconfirmed though experimentation.

**Figure 1.4. Entry pathways usurped by polyomaviruses.** Polyomaviruses have been identified to hijack several of the currently known pathways to enter cells. JCPyV (green virion) is the only characterized polyomavirus to date that utilizes clathrin-mediated endocytosis, while SV40, BKPyV, mPyV, and MCPyV (red virion, collectively) usurp caveolin-mediated endocytosis and/or non-clathrin/non-caveolin lipid raft uptake mechanisms. JCPyV and BKPyV have also been affiliated with extracellular vesicles as a tactic for entry into target cells likely bypassing cellular attachment and entry factors (Adapted from [133]).

The determination that JCPyV likely uses CME for internalization was further supported by work outlined in Chapter 2, finding that JCPyV entry was sensitive to siRNA targeting the heavy chain of clathrin, and JCPyV localizes with clathrin at time points consistent with viral internalization [181].
Interestingly, JCPyV internalization also relies on the endocytic protein β-arrestin, the first association of this protein with the promotion of virus entry [181, 199]. β-arrestin binding domains within the 5-HT2R family, the Ala-Ser-Lys (ASK) motif as well as a proline, separated by six residues from a Asp-Arg-Tyr (DRY) motif on the third intracellular loop of 5-HT2Rs [200-203], have been demonstrated to be important for JCPyV internalization and infection [181, 199]. After recruitment and formation of a clathrin-coated pit, JCPyV is delivered to an early endosome for intracellular trafficking dependent on the GTPase activity of dynamin I [181, 185, 204].

1.7.1.1. Serotonin Receptor Endocytosis

Among many other GPCRs, 5-HT2R internalization can occur through CME requiring classic CME endocytic proteins: clathrin, AP2, dynamin, and β-arrestin [205]. Internalization of 5-HT2Rs by CME is used for receptor recycling, targeting of the receptor for degradation or activation of downstream signaling networks [205]. This mechanism relies heavily on β-arrestins, which are capable of binding to intracellular portions of the serotonin receptors [203, 206-209], interactions that promote 5-HT2R internalization by CME [210, 211]. The interaction between β-arrestin and 5-HT2Rs promotes the uncoupling of the G proteins resulting in the uptake of these receptors into a clathrin-coated pit [206, 212, 213]. One of these interactions occurs at a tri-peptide motif in 5-HT2Rs, conserved in either the third intracellular loop, or the C-terminus, depending on receptor subtype ([203], NCBI accession numbers 3356, 3357, 3358). This motif, the Ala-Ser-Lys (ASK) motif, is a primate specific motif [203], which is critical for β-arrestin dependent internalization of these receptors. The recruitment of β-arrestin to the 5-HT2AR following agonist mediated activation is reliant on G protein receptor kinases (GRKs), specifically GRK2 [203]. Interestingly, 5-HT2Rs of other species, including the rat 5-HT2AR, internalize independently of β-arrestin, with receptor turnover occurring more rapidly [203]. Moreover, mutagenesis of this motif from ASK to NCT, the amino acids of the rat 5-HT2AR in the same position, changes the properties of receptor internalization, negating the reliance of β-arrestin for entry, and
changing receptor signaling to that of the rat 5-HT$_2$R [203]. Furthermore, GRK2 is no longer necessary for entry of this mutated receptor [203]. Collectively this emphasizes the critical nature of the ASK motif and β-arrestin in internalization of 5-HT$_2$Rs in humans [203], the typical host of JCPyV. While the ASK motif is conserved within each of the 5-HT$_2$R subtypes (A, B, C), the necessity of GRKs for mediating β-arrestin interactions with the ASK motif, and thus, internalization, of 5-HT$_{2A}$R or 5-HT$_{2C}$R is uncharacterized. Furthermore, the impact of these motifs on receptor utilization by JCPyV is unclear.

In addition to binding to 5-HT$_2$Rs, β-arrestins also directly interacts with other endocytic proteins, promoting their activation during the internalization process. β-arrestin has been demonstrated to interact with clathrin [214, 215], and the β2 appendage of adaptor protein 2 (AP2) [216], resulting in changes to their activation and recruitment. Together, this highlights the critical contributions of β-arrestin to internalization, emphasizing its role as a central mediator of CME for endocytosis of 5-HT$_2$Rs.

1.7.1.2. Serotonin Receptors in JCPyV Internalization

JCPyV enters host cells by clathrin-mediated endocytosis as JCPyV infection is significantly reduced by chlorpromazine, an inhibitor of clathrin-mediated endocytosis [179, 194]. Further, expression of dominant-negative mutants of epidermal growth factor receptor kinase substrate clone 15 (eps15), a protein important for clathrin-mediated endocytic events, reduces infection [180]. Subsequently, the Atwood laboratory identified that 5-HT$_{2A}$R is required for JCPyV infection [196], based on the findings that infection of glial cells was sensitive to the use of 5-HT$_2$R-blocking antibodies specific to subtypes 2A and 2C, 5-HT$_{2A}$R expression rendered HeLa cells permissive for infection, and JCPyV colocalized with 5-HT$_{2A}$R-GFP at time points consistent with viral entry [196]. Furthermore, 5-HT$_{2A}$R antagonists including ritanserin, ketanserin, mianserin, and mirtazapine reduced JCPyV infection in
human glial cells [217]. Collectively, these data indicate that 5-HT2Rs are important for JCPyV infection and likely promote viral entry, most likely through clathrin-mediated endocytosis.

The 5-HT2Rs are seven transmembrane-spanning G protein coupled receptors widely expressed in the CNS and are commonly associated with physiologic and mood disorders [218]. Interestingly, 5-HT2Rs are expressed on a variety of cells in the CNS, including astrocytes and oligodendrocytes, and in the kidney, including the distal tubules and collecting ducts, all of which are sites of JCPyV infection [49, 197, 198]. Furthermore, 5-HT2Rs are expressed on neurons and subtype 2A is found abundantly in the cerebral cortex [219] where JCPyV has been identified in sites of significant demyelination in individuals with PML [114, 220]. Interestingly, JCPyV is not able to infect microglia, cells that express 5-HT2Rs but lack expression of the JCPyV attachment factor LSTc, further indicating that 5-HT2Rs are not the sole requirement for viral infection [49].

Given that treatment of cells with inhibitors and antibodies specific for serotonin subtypes 5-HT2A and 5-HT2C have shown diminished JCPyV infection, it was speculated that multiple 5-HTR subtypes may be capable of conferring infection or multiple subtypes may play functionally redundant roles in infection. Expression of 5-HT2A, 5-HT2B, and 5-HT2C receptors increased the susceptibility of poorly permissive human embryonic kidney (HEK) 293A cells, which express low levels of 5-HTRs, to JCPyV infection while other 5-HTR subtypes did not [194]. The presence of 5-HT2Rs in HEK293A cells did not impact viral attachment to the cell surface, but specifically enhanced viral entry that was blocked by chlorpromazine treatment [194]. These data demonstrate that the 5-HT2Rs subtypes are required for JCPyV entry [194], yet the direct entry mechanism remains unclear as chlorpromazine blocks clathrin-dependent endocytosis and is also a 5-HT2AR antagonist [195] (Figure 1.1). Interestingly, JCPyV was demonstrated to infect human brain microvascular endothelial cells (HBMECs), which lack 5-HT2ARs, indicating that infection could occur in the absence of 5-HT2AR [42]. However, these findings predate
evidence that JCPyV can also use 5-HTR subtypes 2B and 2C [194]. Furthermore, HBMECs are primary endothelial cells in contrast to the aforementioned HEKs, which are transformed epithelial cells. Therefore, entry strategies could vary in a tissue-specific manner or based on cellular transformation, and thus requires further investigation. Given the evidence that JCPyV entry and infection are dependent on 5-HT2Rs and the abundance of on-market therapies that target the 5-HT2Rs, such as selective serotonin reuptake inhibitors (SSRIs) for use in depression treatment [218], clinicians have prescribed 5-HT2R antagonists, including mirtazapine, in an off-label treatment for PML. Mirtazapine is a serotonergic antagonist that selectively inhibits 5-HT2 and 5-HT3 receptors [221]. Interestingly, case reports have indicated varying degrees of efficacy following mirtazapine treatments in PML patients. Several studies show that treatment with mirtazapine dramatically improves prognosis for individuals diagnosed with PML, including decreased neurological deterioration and undetectable viral loads [222, 223]. However, other reports have described instances where treatment with mirtazapine had little beneficiary effect, as patients rapidly deteriorated regardless of 5-HT2R antagonist treatment [224, 225]. As mirtazapine blocks 5-HT2Rs, it is tempting to speculate that treatment likely prevents JCPyV spread to other cells rather than treating established infection as MRI scans have remained unchanged in some cases [222, 224, 225]. Moreover, other reports indicate that mirtazapine, in combination with mefloquine, a treatment marketed for malaria, has been effective in treating some individuals with PML [226]. Treatment of cells with mefloquine decreased JCPyV infection by blocking viral replication, indicating that the combination treatment may be effective in treating PML [227]. While these reports are promising, conflicting evidence indicates that JCPyV-mediated infection by 5-HT2Rs requires further investigation as well as continued exploration of 5-HT2R antagonists as viable treatments for PML.

1.7.2. JCPyV Entry by Extracellular Vesicles

Recently, viruses have been identified to use extracellular vesicles (EVs) to infect cells [228]. EVs are cell membrane-derived vesicular structures that mediate transfer of material between cells [229].
Thus, as extracellular vehicles for transmitting material and mediating cellular communication, EVs can also transport viruses within the host. EVs have been associated with transporting both enveloped and nonenveloped viruses, including poliovirus, rhinovirus, hepatitis A and C viruses, coxsackievirus, dengue virus, rotavirus, and noroviruses [228]. These viruses have been identified to use EVs to transport either whole infectious virions or naked infectious genomes [228]. In transmitting virions, EVs can serve to enhance virulence of these viruses in multiple ways. EVs can increase the multiplicity of infection (numerous particles can be transmitted within a single EV), and EVs can protect naked infectious genomes and particles from neutralizing antibodies and from eliciting an immune response [228, 230, 231]. Further, within EVs, viruses are protected from harsh chemicals or environments that would otherwise serve to neutralize infection [228]. EVs may be the answer to key questions that remain in transmission of viruses within a host between target cells or, on a larger scale, sites of infection. While many RNA viruses have been associated with the transport within EVs, more recently, DNA viruses, including polyomaviruses, have also been identified to utilize EVs [232-234]. Of note, EVs may serve as enticing modes of transmission for viruses as a means of infecting cells that do not express the typically required cellular attachment and/or entry factors [235].

BKPyV has been associated with the use of EVs for transmission within an infected host. These EVs, isolated from infected Vero and renal proximal tubular epithelial cells, contained tens of BKPyV particles and were capable of infecting cells independent of sialylated glycan expression, suggesting that an alternative entry pathway is used to establish infection [234]. The identification that BKPyV may use EVs may indicate mechanisms of persistence and immune evasion of this virus in the asymptomatically infected host, prior to the onset of virus-associated disease.

A recent publication identified that JC polyomavirus-infected glial SVG-A cells released EVs into the medium. These EVs, approximately 100-200 nm in size, contained infectious virions, and appeared
to be derived from either multivesicular bodies or the plasma membrane. Importantly, JCPyV-containing EVs were capable of eliciting an infection in naïve cells independent of attachment factor LSTc and entry 5-HT\textsubscript{2} receptors [232]. This mode of transport of JCPyV is proposed as a means for transmission of the virus from sites of persistent infection, the kidney, to the CNS, for the development of PML. This is further supported by findings that oligodendrocytes and astrocytes, cell types targeted by JCPyV in the CNS [50], have been reported to lack expression of attachment factor LSTc, through the analysis of tissue sections [49], and that strains of JCPyV isolated from PML patient populations contain mutations within the LSTc binding site of VP1 [236]. However, incubation of JCPyV with LSTc pentasaccharide blocks attachment and infection in SVG-A cells, a mixed population of immortalized astrocytes and oligodendrocytes [151]. Together this emphasizes the importance of using various types of relevant cell models to study viral infection and highlights that there may be cell-type dependent differences and multiple entry strategies utilized simultaneously. Moreover, archetype JCPyV DNA was found in extracellular vesicles of human-derived plasma from both healthy and HIV+ individuals, those at significant risk for development of PML [15, 233]. The potential significance of these findings for JCPyV virulence and tropism cannot be understated.

1.7.3. Alternative Routes of Polyomavirus Endocytosis

1.7.3.1. Caveolin-/Lipid Raft-mediated Endocytosis

Most polyomaviruses have been identified, at least in part, to use caveolin-mediated endocytosis for entry, except for JCPyV [182, 237-241]. Characterized by the recruitment of caveolin, caveolin-mediated endocytosis involves a tight network of caveolins, primarily caveolin-1, an integral membrane protein. Upon recruitment, caveolins are capable of dimerization which can be seen by electron microscopy [242], and are maintained by the actin cytoskeleton [243]. Caveolins serve to assist in the formation and stabilization of caveolae, flask-shaped invaginations at the cell surface. These invaginations consisting of lipid-rafts rich in caveolin (Figure 2), can also be found in the \textit{trans}-Golgi
network (TGN), and among specialized intracellular vesicles [25, 244, 245]. Of note, upon changes in membrane cholesterol, caveolins may localize to endosomes, the Golgi, or the ER [246, 247], most of which are localization sites of polyomaviruses after entry [25, 29, 30, 248-251].

SV40 was one of the first viruses identified to use caveolin-mediated endocytosis as a cellular entry mechanism [163, 237, 252]. Since this discovery, caveolin-mediated internalization has been demonstrated for other polyomaviruses including BKPyV, mPyV, and MCPyV, despite the usage of different receptors [30, 238, 250, 253-255]. SV40 engages receptors GM1/MHC I and is then directed into clusters of caveolin, forming small, tight, flask-shaped invaginations [163]. The membrane invaginations are regulated by cavins, intracellular proteins that maintain caveolae morphology and dynamics through induction of membrane tubules [256]. After the recruitment of caveolin, dynamin II is recruited, acting as molecular scissors to pinch off the invagination through inherent GTPase function [248, 257, 258]. Following this, SV40 is sorted into early endosomes [259]. While SV40 was originally thought to be sorted into a ‘caveosome,’ the existence of such a vesicle has since been deemed to be an artifact of overexpression of caveolin-1 protein [260]. Following this, investigation of SV40 association with vesicles following endocytosis was revisited. It was determined that SV40 internalization by caveolin-mediated endocytosis results in depositing of the virions into early endosomes followed by association with late endosomes [259]. Interestingly, the late endosomes also contained caveolin-1, and upon overexpression caveolin-1 also accumulated in endolysosomes, which was deemed to likely be an artifact, that which had been previously associated with overexpression of caveolin-1 [259, 260].

SV40 internalization into caveolae triggers actin breakdown and recruitment, suggesting the involvement of receptor tyrosine kinases in SV40 entry [30, 258]. Receptor tyrosine kinases are often affiliated with ligand-mediated internalization of viruses by caveolin-/lipid-raft mediated endocytosis [261, 262]. While clathrin-mediated endocytosis was initially thought to occur independently of tyrosine
kinase activity, overlap between these two signaling pathways is becoming more appreciated, the importance of receptor tyrosine kinase signaling has been demonstrated for JCPyV infection [180]. Surprisingly, SV40 has also been demonstrated to enter into cells devoid of caveolin-1. In cells from a caveolin-1 knockout mouse SV40 internalization occurred more rapidly and is transported into pH neutral organelles [239].

Both caveolin-dependent and -independent internalization mechanisms have been identified for mPyV [238, 263]. mPyV is deposited into early endosomes after entry rather than caveolin-1+ vesicles [264, 265]. Like mPyV, BKPyV endocytosis also relies on interplay between the caveolar and endosomal endocytic systems [250, 253]. In contrast, in renal proximal tubule endothelial cells, endocytosis of BKPyV occurs independently of clathrin or caveolin [255]. Together this highlights the importance of investigation of cell type-specific differences during viral infection.

Similar to SV40, MCPyV entry was found to depend on intracellular cholesterol, which is critical for caveolar- and lipid-raft mediated endocytosis, and cellular phosphatases including protein phosphatase 1 (PP1), 2A (PP2A), and 2B (PP2B) [30]. Further, MCPyV internalization is dependent on actin dynamics as well as cellular GTPases, and Rho-like GTPase function [30]. The role of Rho-like GTPase in MCPyV entry is thought to occur through mediating actin-dependent endocytosis of MCPyV. Further, MCPyV entry was not perturbed upon inhibition of clathrin or through inhibition of macropinocytosis via the use of EIPA [30].

1.7.3.2. Non-clathrin/Non-caveolin Endocytosis

In recent years alternative endocytic pathways aside from the classic CME and caveolin-mediated endocytosis have also been described [266]. This pathway, deemed non-clathrin, non-caveolin endocytosis has been demonstrated to rely on tyrosine-kinase activity and cholesterol while, in contrast to clathrin and caveolin uptake routes, is independent of dynamin [239]. One of the first published
studies that began to describe this endocytic pathway was defined using SV40 in caveolin-1-devoid mouse embryonic fibroblast (MEFs) cells [239]. Upon uptake into these cells the viruses do not traffic by previously identified endocytic routes, and instead are delivered to the ER via cytosolic organelles that were nonendosomal in origin [239]. It is important to note that MEFs are not a commonly utilized cell model for studies of SV40 infection, which infects kidney cells of monkeys and humans [267]. Rather MEFs are utilized as an abortive model of infection, largely for studies of T-Ag transformation [268], and thus SV40 entry into these cells may differ from fully permissive cells.

It has been proposed that the non-clathrin non-caveolin uptake pathway is reliant on flotillins 1 and 2 [269, 270]. Assembly of these two proteins promotes the formation of microdomains that bud into the cell [271]. Further, GPI-linked proteins have been described to internalize into both flotillin-positive and caveolin-1+ vesicles suggesting that there may be overlap between these pathways and their induction by cellular receptors and/or ligands [271]. The activation of these two signaling pathways have since been linked to Src-family kinases and tyrosine-kinase activity, though much of their activation and crosstalk remains elusive [266, 271-273]. Further, the implication of this alternative route in uptake of polyomaviruses, aside from SV40, and its implications in viral pathogenesis remain to be elucidated.

1.8. Trafficking

One theme is apparent among polyomaviruses; while these viruses use distinct cellular attachment and entry factors and enter cells by differing mechanisms, all polyomaviruses are targeted to the ER [24-30]. It is postulated that direct interactions among polyomaviruses and their respective receptors largely dictate intracellular trafficking [14]. Regardless, targeting to the ER is critical for progression of the viral infectious cycle as disruption of ER trafficking halts the progression of infection [25, 29, 30]. To reach the ER, polyomaviruses have been attributed to following various trafficking routes (depicted in Figure 1.6).
Typical progression of the endosomal-lysosomal system begins at the early endosome. The early endosome is marked by the intracellular protein Rab5 [274]. Rab proteins serve to control the trafficking, fusion, sorting, and maturation of endosomes [274]. The early endosome serves as the first major sorting hub and from this hub, cargo proteins can be sent to recycling Rab11+ endosomes for recycling to the cell surface, by retrograde transport to late Rab7+ endosomes for targeting to the TGN or the ER, or to the lysosome for degradation [275].

1.8.1. Trafficking of JCPyV

After internalization, JCPyV colocalizes with Rab5+ endosomes as early as 15 minutes post infection [193]. Localization of JCPyV to early endosomes is critical for the progression of infection; expression of dominant-negative mutants of Rab5 inhibits infectivity [193]. Interestingly, after localization to early endosomes, JCPyV appears to exit the endosomal system and is transferred to a caveolin-1+ vesicle [193]. How JCPyV leaves the endosomal/lysosomal system remains unclear. Treatment of cells with either inhibitors or dominant negative constructs directed towards recycling (Rab11) or late (Rab7) endosomes do not impair infection [29, 192, 193]. Further, Retro-2, a compound the prevents ER transport from the endosome by an undefined mechanism, hinders JCPyV-ER localization [251], and cellular depletion of caveolin-1 or sequestration of intracellular cholesterol reduces JCPyV infection [193]. While originally thought to be distinct intracellular pathways, crossover between the endosomal and caveolar systems is becoming more appreciated [276, 277]; although JCPyV is internalized by CME, it still requires caveolin for transport to the ER, like other polyomaviruses studied [29].
Figure 1.5. Intracellular trafficking of polyomaviruses. After internalization of polyomaviruses into the cell, they are targeted to the endoplasmic reticulum (ER). Upon internalization of SV40, BKPyV, mPyV, or MCPyV (red virion, collectively) into a pH neutral early endosome, the virus is delivered to the ER via microtubule-mediated transport and the late endosome/lysosome. Meanwhile, upon internalization of JCPyV (green virion) into an early endosome, association with caveolin-1+ vesicles occurs prior to retrograde transport to the ER [133].

1.8.2. Trafficking of Other Polyomaviruses

Following entry of mPyV by caveolin-dependent/-independent endocytosis, mPyV is also deposited into an early endosome [264]. From the early endosome, mPyV is transported into a late endosome via microtubule-dependent transport [264]. Within the late endosome, low pH (5.5) induces conformational capsid changes that allow for enhanced disassembly within the ER [265]. The use of agents that prevent the pH drop in the endosome within these systems inhibits mPyV infection [264, 265]. From the endolysosomal system, mPyV is sorted to the ER for further disassembly [160, 265].

Similar to mPyV, BKPyV has also been attributed to require low pH prior to localization within the ER [253, 278]. Recently, the anti-diabetic drug, glibenclamide, which blocks the cystic fibrosis
transmembrane conductance regulator (CFTR), impeded BKPyV infection [279]. Importantly, the maximum inhibitory effect of this drug was found to be 4 hpi, suggesting that the hindrance in infection occurs during trafficking of the virus to the ER [279]. Collectively this highlights the importance of ion channel regulation during endosomal trafficking of BKPyV.

After sorting into a caveolin-1+ late endosome, SV40 is transported by microtubules to the ER [25]. Trafficking of SV40 through the caveolar/endosomal systems is reliant on acidification of the endosome as well as ionic balance of Ca$^{2+}$ [280]. Unsurprisingly, trafficking of MCPyV also involves the caveolar and endosomal systems, also relying on regulation of endosomal Ca$^{2+}$, however, transport of MCPyV also depends on the balance of K$^+$, of which is dispensable for SV40 infection [280]. This suggests that intracellular transport of polyomaviruses must be tightly regulated for infection to be successful, while also providing the appropriate tailored environment for individual polyomaviruses. After arrival into an early endosome by caveolin-mediated endocytosis, MCPyV traffics to late endosomes via microtubules. Interestingly, within the endosomal compartment, MCPyV has been described to acquire a lipid envelope [30]. This envelope is wrapped tightly around the virion and is posited to become acquired within the late endosomal system rather than in specialized compartments. The authors speculate that this envelope may be a new antiviral defense mechanism or a means of evading the low pH of the maturing/late endosomal compartments prior to arrival in the ER [30].

How polyomaviruses reach the ER from the endosomal/lysosomal system largely remains enigmatic. For mPyV, direct binding between mPyV and ganglioside GD1a results in targeting of mPyV from the late endosome to the ER [263]. After sorting into late endosomes, BKPyV colocalizes with Rab18, syntaxin 18, a member of the ER membrane fusion system, and NRZ, which form a complex. This complex serves to anchor BKPyV-containing vesicles at the ER. In the absence of Rab18, BKPyV becomes trapped in the late endosome and ER transport does not occur [281]. Given the conservation among
polyomavirus transport pathways, whether Rab18/syntaxin 18/NRZ complexes play a role in late sorting of other polyomaviruses to the ER warrants exploration.

1.9. Disassembly Within the Endoplasmic Reticulum

While it has been established for some time that polyomaviruses require transit through the ER for completion of the infectious cycle [25, 29, 30], discoveries made in recent years have just begun to unravel this complex process. Much of what is known about disassembly within the ER is largely due to advances for SV40. The capsid of polyomaviruses is stabilized by covalent bonds, which upon arrival in the ER, are reduced and isomerized by ER-resident redox proteins PDI, ERdj5, and ERp57 (Figure 4, step 1) [282]. Further, mPyV also requires a redox environment for capsid destabilization, though differing from SV40, ERp57 is dispensable for infection, instead relying on ERp29 for disassembly [283-285]. In this process, ERp29 acts as a chaperone to unravel the C-terminal linkages of VP1, the interactions that stabilize the overall architecture of the capsid; because of this, the hydrophobic capsid proteins, VP2 and VP3, become exposed [283, 285]. Similar to mPyV, JCPyV also usurps ERp29 for isomerization, as siRNA targeting ERp29, ERp57, and ERp72 all resulted in diminished infectivity [29]. It is important to note that ERp29 is not capable of disrupting the disulfide bonds of the viral capsid as it contains only one cysteine residue, highlighting the importance of isomerization for the progression of viral infection [286]. While MCPyV has recently been demonstrated to traffic to the ER, similar to other polyomaviruses [30], whether or not destabilization by ER-resident redox proteins is necessary for infection has yet to be determined.
Figure 1.6. Release of polyomaviruses from the ER to the cytosol. (1) Upon arrival of polyomaviruses JCPyV, BKPyV, SV40 (green virion, collectively), mPyV (orange virion), and MCPyV (purple virion) in the ER, the viral capsids are destabilized due to reduction and isomerization of disulfide bonds by ER redox proteins, PDI, ERdj5, ERp57, or ERp29. Destabilization of MCPyV remains elusive (indicated by ‘?’). (2) The now-hydrophobic particle embeds itself in the ER membrane and is stabilized by EMC1, preventing further disassembly of the viral capsid. As demonstrated by SV40 (potential pathway of PyV convergence indicated by blue virion), localization of the virus in the membrane of the ER induces the relocation of DNA J proteins (B12, B14, C18) and BAP31 to the site, forming a foci. (3) Direct interactions between DNA J proteins and cytosolic extraction machinery (Hsc70, HSP105, Bag2, and SGTA) form a complex, and with the assistance of derlin1, derlin2, or sel1, facilitate the extraction of the virus across the ER membrane and into the cytosol, primed for arrival at the nucleus [133].

As the disulfide bonds in the capsid of polyomaviruses are reduced and isomerized, the now-hydrophobic virion embeds itself in the membrane of the ER through the N-terminus of the exposed VP2, mimicking a misfolded protein [287]. In doing so, SV40 has been demonstrated to cause the
formation of a foci, or penetration site, in the ER membrane (Figure 4, step 2) [28, 287-290]. Virions devoid of VP2 arrive at the ER, but are incapable of release from the ER and the progression of infection [287]. The formation of foci in the ER leads to the recruitment of membrane-bound proteins to the site of penetration, including BAP31 and BiP, typically affiliated with ER-associated degradation (ERAD) of misfolded proteins [287]. Within the membrane, direct interactions between the membrane chaperone EMC1 and the virus occur, preventing further premature disassembly of the virion while in the ER [291]. Additional membrane-bound proteins are recruited to the foci, including DNA J proteins B12, B14, and C18 as well as cytosolic chaperones Hsc70, Hsc105, Bag2, and SGTA that function to extract SV40 through the ER membrane and into the cytosol (Figure 4, step 3) [288, 289, 292-294]. This extraction is further chaperoned by membrane proteins derlin1 and sel1 (SV40/BKPyV/JCPyV) or derlin2 (mPyV), or in the case of JCPyV, potentially through sel1, independent of derlin proteins [29, 249, 278, 282].

Importantly, the transmission of SV40 from the ER into the cytosol appears to be tightly regulated. Numerous publications highlight the critical nature of the foci for delivery of SV40 into the cytosol: (1) if the foci are not formed properly, infection does not progress [288, 290, 291], (2) the foci are formed prior to cytosolic delivery of the virions [288, 290, 291, 293, 294], (3) hydrophobic virions cluster to foci prior to release [28, 290, 295], and (4) disruption of cytosolic proteins that mediate extraction results in trapped virions within the ER [288, 293, 294]. It has been recently demonstrated that the formation of foci by SV40 involves the recruitment of cytosolic kinesin-1 to form the structure budding from the ER [295], though how SV40 recruits kinesin-1 while still residing within the ER is unclear. Once SV40 has been extracted from the ER into the cytosol, mechanical stress exerted by dynein-1 results in the further disassembly of the viral particle, priming the virus for nuclear localization [296], similar to that described for kinesin-1-mediated disassembly of adenovirus [297]. Moreover, the impact of Hsc70 cannot be discounted, as Hsc70-mediated cytosolic disassembly of polyomavirus capsids has been described for SV40 [288, 289, 295, 296] and for mPyV [298]. In fact, interactions
between hydrophobic polyomavirus capsid proteins and cytosolic chaperones may play a significant role in preventing the targeted degradation of the viruses, subverting the canonical fate of misfolded proteins [288, 299].

Until recently, a major question remained, how does a particle the size of a polyomavirus move through the ER membrane without disrupting the overall functionality of the organelle? Movement of large macromolecules likely induces significant mechanical stress on the ER, which may result in reduced structural integrity [300]. In moving through the membrane, SV40 has been demonstrated to co-opt ER-membrane-resident reticulon (RTN), responsible for maintaining the overall morphology of the ER membrane [300]. Under normal function, RTN maintains the structure of the ER membrane during movement of misfolded proteins from the ER into the cytosol, typically targeted for degradation. RTN aids in movement of SV40 by assisting in membrane curvature and flexibility during release [300], allowing for the membrane to undergo significantly more mechanical stress than what is normally tolerated.

Polyomavirus exploitation of the ERAD pathway is critical for partial disassembly of the viral capsid, highlighting the importance of ER arrival for all polyomaviruses studied to date. Partial disassembly of the viral capsid is essential for nuclear transport of polyomavirus virions as disassembly releases the internal genetic material. As dsDNA viruses, arrival in the nucleus is critical for transcription and replication. Disruption of any proteins utilized by these viruses between the plasma membrane and arrival of the viruses at the nucleus prevents transcription and thus replication, highlighting the vital regulation of these processes and their importance for the propagation of viral infection.

1.10. Nuclear Transport of Polyomaviruses

In contrast to small molecules (smaller than 9 nm), large macromolecules cannot transit through the nuclear pore complex (NPC) by passive diffusion. Transport of larger molecules across the nuclear
membrane (approximately 45 nm) occurs via karyopherin-β family proteins comprising importin transport factors for transit into the nucleus, and exportins for transit out of the nucleus [301]. Importantly, importins α and β heterodimerize, and this dimerization is critical for transport of the cargo to the nucleus [302]. However, importin β has also been associated with mediating transport out of the nucleus [301, 302]. Association of importins with cargo occurs in the cytoplasm where they then act as chaperones, facilitating the movement of the cargo across the nuclear membrane, through first docking with nucleoporins and then releasing the cargo on the opposite side of the membrane via GTPase hydrolysis [301]. Usurpation of these importins to guide nuclear transport of viruses have been well described [303]. In order to hijack the host’s nuclear transport machinery, a nuclear localization sequence (NLS) must become exposed, thereby acting as a ticket or voucher for nuclear transport. Importin α recognizes and binds to the NLS of the cargo in the cytoplasm while importin β directly interacts with the nucleoporins [304].

Exposure of polyomavirus NLS sequences occurs during uncoating within the ER. Direct interactions between the NLS of SV40s VP2 and VP3 with the importin complex are necessary for arrival of SV40 within the nucleus [305, 306]. While VP1 of SV40 also contains an NLS, interactions between this NLS and importins are not critical for infection [305]. In addition to SV40, disruption of the NLS of BKPyV VP2 and VP3 significantly impairs infection [307]. However, in contrast to BKPyV and SV40, interaction between the NLS of JCPyV VP1 and the importin complex is sufficient to support JCPyV infection, as demonstrated by VLPs comprised only of DNA and VP1 [308]. For mPyV, it was recently determined that the NLS of VP1 is sufficient to allow for nuclear transport, though the NLSs of VP2/3 may also contribute in a lesser capacity [309]. Interestingly, the authors noted that only a subset of the viral protein associated with importins is delivered into the nucleus [309]; this is in agreement with a separate report that SV40 association with importins α/β was an infrequently occurring event [306]. Several possibilities to explain this occurrence were rationalized by Soldatova et al.: (1) over the course of infection only a
subset of virions arrive in the cytoplasm for transport to the nucleus during entry, (2) upon arrival of the partially disassembled particles into the cytoplasm, a subset of these particles are likely targeted for degradation, and (3) transport of the particles into the nucleus must overcome barriers including detection by the innate immune system [309]. However, direct transport of viral particles may occur through the leaflets of the ER directly into the nucleus. Though not well characterized, it has been suggested that infection by SV40 may result in breakdown of the nuclear envelope, allowing for direct ER to nucleus transport [310]. This is conflicting with additional reports that SV40 requires transit through the cytoplasm prior to nuclear arrival for infection, identified through the implementation of anti-VP1 and -VP3 antibodies injected into the cytoplasm resulting in blocked infection [311]. This is further supported by the characterization of multiple cytosolic proteins in productive SV40 infection [288, 289, 292-294]. However, the implication of destabilization of the nuclear membrane during the promotion of viral infection cannot be discounted, as this may be necessary to allow for increased nuclear localization of SV40 [310, 312]. Aside from these data, there is limited understanding of the complex interactions that underscore the arrival of polyomavirus genetic material within the nucleus, highlighting a critical gap in our understanding of this event in the promotion of virus entry and thus infection.

1.11. Concluding Remarks

In the last few decades, numerous advances have been made to better define the infectious cycle of polyomaviruses from start to finish. Understanding the complex interactions that occur between a viral pathogen and target host cells are key to the development of improved therapies for the treatment of virus-associated diseases. Four polyomaviruses have been associated with human illness, BKPyV, JCPyV, TSPyV, and MCPyV. In usurping specific host cellular receptors and hijacking signaling networks, viruses co-opt intracellular endocytic and signaling pathways that orchestrate delivery of the virus to the appropriate cellular compartment for replication. Delivery of the virus to the right
compartment, at the right time, is tightly regulated, ensuring replicative success; failing to do so results in the disruption of viral infection either through nonproductive infection routes or through intracellular detection of the virus by the host immune system. Thus, in order to cause infection, polyomaviruses must either activate host cellular receptors and hijack signaling networks or usurp EVs to mediate their internalization within the cell.

JCPyV infection occurs in the majority of the population [37, 38] yet only results in PML in severely immunosuppressed individuals such as those with HIV-1 infection [100] or those under immunosuppressive therapies [313]. Viral attachment to host cells in culture is dependent on α2,6-sialic acid on LSTc via interactions with specific residues on VP1 [144]. Following attachment, JCPyV enters cells by 5-HT2Rs [194, 196], and although antagonists to 5-HT2Rs have shown some clinical promise [222, 223], there are other instances in which no clinical improvement has been observed [224, 225]. A deeper understanding of how JCPyV utilizes 5-HT2Rs will undoubtedly result in improved antiviral therapies. Together, viral attachment, entry, and trafficking represent novel targets for anti-viral therapeutics, presenting an opportunity for the continued development of improved PML treatments.

The goal of this dissertation research was to define the mechanisms of JCPyV entry into host cells by investigating the role of various endocytic proteins and protein-interaction domains of the serotonin receptor. In doing so we have characterized the necessity of numerous host factors involved in the uptake of JCPyV within cells, and worked to define the mechanism by how viral entry occurs. Collectively, this work largely advances our understanding of JCPyV utilization of 5-HT2Rs and how this receptor promotes virus internalization. Proteins and receptor domains characterized within this work for internalization of JCPyV could serve as targets for the development of improved or novel antiviral therapeutics.
CHAPTER 2

JC POLYMOMAVIRUS ENTRY BY CLATHRIN-MEDIATED ENDOCYTOSIS IS DRIVEN BY β-ARRESTIN


2.1 Chapter Summary

JC polyomavirus (JCPyV) establishes a persistent, lifelong, asymptomatic infection within the kidney of the majority of the human population. Under conditions of severe immunosuppression or immune modulation, JCPyV can reactivate in the central nervous system (CNS) and cause progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease. Initiation of infection is mediated through viral attachment to α2,6-sialic acid containing lactoseries tetrasaccharide c (LSTc) on the surface of host cells. JCPyV internalization is dependent on serotonin 5-hydroxytryptamine (5-HT)2 receptors, and entry is thought to occur by clathrin-mediated endocytosis (CME). However, the JCPyV entry process and the cellular factors involved in viral internalization remain poorly understood. Treatment of cells with small molecule chemical inhibitors and RNA interference of 5-HT2R endocytic machinery, including β-arrestin, clathrin, AP2, and dynamin, significantly reduced JCPyV infection. However, infectivity of polyomavirus simian virus 40 (SV40) was not affected by CME-specific treatments. Inhibition of clathrin or β-arrestin specifically reduced JCPyV internalization but did not affect viral attachment. Furthermore, mutagenesis of a β-arrestin binding domain (Ala-Ser-Lys) within the intracellular C-terminus of 5-HT2AR, severely diminished internalization and infection, suggesting that β-arrestin interactions with 5-HT2AR are critical for JCPyV infection and entry. These conclusions illuminate key host factors that regulate clathrin-mediated endocytosis of JCPyV, which is necessary for viral internalization and productive infection.
2.2. Introduction

Viruses use multiple strategies to internalize into host cells including clathrin- and caveolin-mediated endocytosis, macropinocytosis, non-clathrin, and non-caveolin mechanisms [119, 314]. Of these, clathrin-mediated endocytosis (CME) is the most common viral entry mechanism, serving as an enticing pathway for both enveloped and nonenveloped viruses to gain entry into host cells [182, 183]. While JCPyV has been suggested to utilize CME, other polyomaviruses including SV40 and BKPyV, those most closely-related to JCPyV, enter cells via caveolae- or non-clathrin-, non-caveolae-mediated uptake mechanisms [163, 237, 239, 253, 255]. SV40 enters cells in a non-clathrin dependent mechanism [239], including caveolae-dependent and independent-lipid-mediated endocytic mechanisms [163, 237]. BKPyV entry also requires caveolae, and entry is independent of clathrin [253, 254, 315]. Regardless of the mechanism utilized for internalization, following entry events, all polyomaviruses ultimately traffic to the endoplasmic reticulum (ER) for partial uncoating prior to retrotranslocation to the nucleus [24-29].

CME involves direct interactions between clathrin and other scaffolding proteins, like AP2, to coordinate internalization [186, 191]. AP2 is comprised of four subunits (α2, β2, σ1, and µ1), and serves multiple roles in CME, including assisting in the assembly of clathrin-coated pits, providing a proteinaceous link between internalizing cargo and clathrin, and coordinating interactions between clathrin and other CME proteins [184, 187-190]. Following the activation and interaction between these proteins, a clathrin-coated pit is formed, which becomes pinched off through GTPase activity of dynamin into an endocytic vesicle [185, 204]. The endocytic vesicle is then free to traffic cargo, including viruses, to the appropriate intracellular compartment [316].

GPCRs, including 5-HT2Rs, can be internalized through clathrin-mediated endocytosis in a process involving specific scaffolding proteins, leading to receptor recycling, receptor degradation, or
activation of downstream signaling cascades [205]. 5-HT2R-mediated CME requires β-arrestins, which bind to cytoplasmic domains within the receptor [206-209] and drive receptor internalization [210, 211]. β-arrestin binding to intracellular domains prevents G-protein coupling and directs the receptor to the clathrin-coated pit with the assistance of endocytic proteins including clathrin and AP2 [206, 212, 213]. 5-HT2Rs have a conserved tripeptide motif, Ala-Ser-Lys (ASK), that serves as a β-arrestin-binding domain in the C-terminus, which has been demonstrated to modulate internalization of 5-HT2AR [203]. In addition to receptor interactions, β-arrestins can bind to endocytic scaffolding proteins including clathrin [214, 215] and the β2 subunit of AP2 [216], and modulate their activity. Therefore, β-arrestins are critical for mediating GPCR signaling events and determine the outcome of agonist-mediated receptor endocytosis [205].

The focus of this study was to characterize the host cell proteins required for JCPyV internalization and to further define the role of 5-HT2Rs in JCPyV internalization and infection. We have further defined the cell entry pathway by identifying specific endocytic proteins required for JCPyV entry and infection. Collectively, these results elucidate the internalization strategy usurped by JCPyV to infect host cells and highlight the role of 5-HT2R-scaffolding proteins in viral internalization strategies.

2.3. Materials and Methods

2.3.1 Cells, Viruses, Antibodies, Plasmids, and Reagents

SVG-A cells [317] were maintained in complete minimum essential medium (MEM) (Corning) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (Mediatech, Inc.) and 0.2% Plasmocin prophylactic (Invivogen) (complete). HEK293A cells were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM) (Corning) supplemented with 10% FBS, 1% P/S, and 0.2% Plasmocin prophylactic (complete). HEK293A cells stably expressing YFP fusions of 5-HT2R subtypes 2A, 2B, and 2C [194], were maintained in complete DMEM additionally supplemented with 1% G418 (Corning). All cell
types were stored in a humidified incubator at 37°C and 5% CO₂. Cell lines were generously provided by the Atwood Laboratory (Brown University) and were verified by ATCC.

Generation and proliferation of JCPyV strain Mad-1/SVEΔ and SV40 strain 777 (provided by the Atwood Laboratory (Brown University)) was described previously [251, 318]. Alexa Fluor-488 and -647 labelled JCPyV were prepared as previously described [151]. Titering of JCPyV and SV40 was performed in SVG-A cells by fluorescent focus unit (FFU) infectivity assay. Clathrin inhibitor Pitstop® 2 (Abcam, ab120687) and dynamin inhibitor Dynole® series kit (Abcam, ab120474) were reconstituted according to manufacturer’s instructions in DMSO and used at indicated concentrations at time points specified. JCPyV neutralizing antisera was used to neutralize noninternalized virus (1:10,000, generously provided by Dr. Walter Atwood). Antibodies used for FFU infectivity assays include PAB597 (1:10), a supernatant containing a mAb against JCPyV VP1 derived from a hybridoma (generously provided by Ed Harlow), PAB962 (neat or 1:2), a mAb for large T-antigen specific to JCPyV and does not react to SV40 large T-antigen (generously provided by the Tevethia Laboratory (Penn State University)) [319], Ab-2 (1:50), a PAb for that detects large T-antigen of SV40 and JCPyV (Calbiochem, DP02), and secondary polyclonal goat anti-mouse Alexa Fluor 488 or 594 antibodies (Thermo Fisher). Antibodies used for western blot analysis include anti-β-arrestin 1/2 monoclonal Ab (generously provided by Dr. Cheryl Craft, Institute for Genetic Medicine, described in [320], used for detection in SVG-A cells) or β-arrestin 1/2 monoclonal Ab (1:500, Cell Signaling Technology, 4674S, used for detection in HEK293A cells), polyclonal anti-clathrin heavy chain (1:250, Abcam, ab21679), anti-dynamin 1 polyclonal (1:250) and anti-dynamin 2 polyclonal (1:250) (Thermo Fisher, PA1-660, PA1-661), monoclonal anti-AP2 μ2 (1:50, BD Biosciences, 611350), monoclonal anti-AP50 β-adaptin (1:250, BD Biosciences, 610381), monoclonal anti-GAPDH mouse-specific (1:2000, Abcam, ab8245) or anti-GAPDH rabbit-specific (1:2000, Abcam, ab8245) antibodies corresponding infrared 680 goat anti-mouse and 800 goat anti-rabbit secondary antibodies (1:15,000, LI-COR).
2.3.2. siRNA Treatment

SVG-A or HEK293A cells were seeded in 12 well plates to ~50% confluence. siRNA transfections were performed using RNAiMax (Thermo Fisher) according to manufacturer’s instructions as indicated in Table 2.1. Following addition of siRNA, cells were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 h. Transfection efficiency was monitored through the use of Block-iT Alexa Flour Red control oligo (Life Technologies) at 48 h post-transfection. Following siRNA treatments, cells were processed for western blot analysis or infected with JCPyV or SV40 (MOIs indicated in figure legends). siRNA-induced toxicity was measured by propidium iodide flow-cytometry based viability assay (Thermo Fisher).

<table>
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<th>Target</th>
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<th>Time of siRNA addition (h)</th>
<th>Supplier, sequence targeted, and/or catalog number</th>
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<tr>
<td>Block-iT Alexa Flour Red control oligo</td>
<td>Control used at same concentration as target</td>
<td>Control added at same time as target</td>
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</tbody>
</table>

Table 2.1. Conditions for siRNA of CME-affiliated Proteins.

2.3.3. siRNA Western Blot Analysis

To determine efficacy of siRNA knockdown, following siRNA treatment, SVG-A or HEK293A cells were washed in PBS, removed from wells with a cell scraper, washed in PBS, and then pelleted by
centrifugation at 414 x g at 4°C for 5 min. Cell pellets were resuspended in 50μL of Tris-HCL lysis buffer (1mM EDTA, 50mM Tris-HCL, 120mM NaCl) or RIPA, containing protease (1:10, Sigma-Aldrich) and phosphatase inhibitors (1:100, Sigma-Aldrich) and incubated on ice for 10 min or 20 min respectively. Cellular insoluble material was pelleted at 21,130 x g, at 4°C for 10 min. For CHC siRNA-treated samples, cellular pellets were sonicated on ice at 35% amplitude for 10 s, three times, prior to centrifugation. Lysates containing protein were mixed 1:1 with Laemmli sample buffer (BioRad), boiled at 95°C for 5 min, and resolved by SDS-PAGE through a 10% TGX-mini gel (BioRad). Proteins were transferred to nitrocellulose membranes (BioRad) using a Semi-Dry Transblot System (BioRad) at 2.5A (25V) for 3 min. Membranes were equilibrated in 1X Tris buffered saline (TBS) for 10 min before blocking in 5% non-fat milk/TBS-T (1XTBS-Tween 20 (0.1%)) or 5% BSA/TBS-T at RT for 1.5 h before 3 washes with TBS-T at RT. Membranes were then incubated with primary antibody indicated and a GAPDH (housekeeping) antibody in 5% BSA/TBS-T or 5% nonfat milk/TBS-T at 4°C overnight (O/N), rocking. Membranes were washed extensively in TBS-T at RT before incubation with secondary antibodies (LI-COR) in 5% non-fat milk/TBS-T at RT for 1 h, rocking. Membranes were washed before imaging on a LI-COR Odyssey CLx.

Reduction in protein expression was calculated by defining the relative fluorescence of each target protein band and loading control in each lane utilizing LI-COR ImageStudio western blot analysis software (version 5.2). Target protein bands were normalized to the loading control and the protein reduction was calculated in comparison to the irrelevant EGFR siRNA control. Knockdown by siRNA was optimized for reductions of at least 70%.

2.3.4. JCPyV and SV40 FFU Infectivity Assay

Following chemical treatments, siRNA, or transfections, cells were infected with JCPyV or SV40 at MOIs indicated in figure legends. Virus was diluted in infection inoculum, either MEM or DMEM, containing 2% FBS and 1% P/S, at a total volume of 200 μL per well (24 well plates) or 300μL (12 well plates). Cells were incubated in viral inoculum at 37°C for 1 h, cell-type specific complete medium was
added and cells were incubated at 37°C for 48 h (T-antigen) or 72 h (VP1). Following infection, cells were washed in 1X PBS then fixed in ice cold MeOH at -20°C for 20 mins or 4% PFA at RT for 10 min before staining by indirect immunofluorescence. Following fixation cells were washed extensively with 1X PBS, and permeabilized with 1% TritonX-100-PBS (Thermo Fisher) at RT for 15 mins. Cells were incubated with the mAb for T-antigen PAB962 or VP1 antibody PAB597 to detect newly synthesized T-antigen or VP1 protein in SVG-A cells at 48 and 72 hpi, respectively [319] or pAB AB-2 to detect T-antigen for SV40 in HEK293As in 1X PBS at 37°C for 1 h, washed 3 times, and incubated at 37°C for 1 h with a secondary polyclonal goat anti-mouse Alexa Fluor 488 or 594 antibodies. Infection was quantified by determining the number of VP1- or T-antigen-positive nuclei divided by the total number of DAPI-positive cells per 10X visual field (% infection). The number of DAPI positive cells per visual field was determined through use of a binary screen described previously [321] utilizing Nikon NIS-Elements Basic Research software (version 4.50.00, 64-bit). Average percent infection was then normalized to the control indicated.

2.3.5. JCPyV and SV40 Infection in Presence of Inhibitors

SVG-A cells were seeded to ~70% confluence in a 24 well plate before pretreatment with clathrin inhibitor Pitstop 2 at indicated concentrations in 2% FBS-containing MEM at 37°C for 15 min. Cells were infected with JCPyV in 200 µL of MEM (2% FBS) at MOIs indicated in figure legends, in the presence of chemical inhibitors, at 37°C for 1h. Cells were fed with 1mL complete medium containing JCPyV antisera (1:10,000) and incubated at 37°C for 72 h. For Pitstop 2 addback experiments cells were treated with Pitstop 2 at time points indicated, either prior to, at the time of, or 4 h following infection by JCPyV. After infection had progressed for 4 h, cells were fed with 1mL of complete medium containing JCPyV antisera (1:10,000). For infectivity in the presence of Dynole, SVG-A cells were seeded to ~70% confluence in a 24 well plate before pretreatment with dynamin 1/2 inhibitor Dynole 34-2 or negative control Dynole 31-2 at indicated concentrations in 2% FBS-containing MEM at 37°C for 30 min. Cells were infected with JCPyV in 200 µL of MEM (2% FBS) at MOIs indicated in figure legends, in the
presence of chemical inhibitors, at 37°C for 1 h. Cells were fed with 1mL complete medium and incubated at 37°C for 4 h. At 4 hpi viral inoculum was removed and replaced with 1mL complete medium. Following infection cells were fixed and stained by indirect immunofluorescence for nuclear expression of VP1. Inhibitor-induced toxicity was measured by MTS viability assay (Promega) to define optimal working concentrations of inhibitors.

2.3.6. Site-directed Mutagenesis of 5-HT₂ Receptors

5-HT₂AR-YFP fusion construct plasmid generation was described previously [144]. Potential β-arrestin binding sites were altered by site-directed mutagenesis with the QuickChange II site-directed mutagenesis kit (Agilent). Primers for each mutation were designed through use of Agilent QuickChange Primer Design software and were manufactured and HPLC purified by Integrated DNA Technologies (IDT). Primers used are provided in Table 2.2 below. Following mutagenesis, plasmids were transformed into DH5α competent cells (Invitrogen). Mutated sequences were verified by DNA sequencing (Genewiz (Plainfield, NJ)) or University of Maine DNA Sequencing Facility), analysis via MacVector, and subsequently transiently expressed in HEK293A cells. Accession numbers are provided in Table 2.2.

<table>
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<th>Mutation</th>
<th>Forward primer (5’-3’)</th>
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</table>

Table 2.2. Primers and Accession Numbers for 5-HT₂A Mutated Receptors.

2.3.7. Transfection of mutated receptor plasmids in HEK293A cells

HEK293A cells were seeded to ~80% confluence in 24 well plates in 500 µL or 96 well #1.5 glass-bottomed plates (CellVis) in 100 µL of DMEM containing 10% FBS without antibiotics. Transfection
complexes containing 1 µg of DNA:1 µL of Lipofectamine 2000 (Invitrogen) in incomplete medium (lacking FBS and antibiotics) at a total concentration of 1 µg/well (24 well plate) and 0.16 µg/well (96 well plate) were incubated at RT for 20 min. Following incubation, complexes were added to the cells and incubated at 37°C for 4 h. Transfection medium was removed and complete medium containing antibiotics was added, and plates were then incubated at 37°C for an additional 20 h. Transfection efficiency was determined through visualization of YFP expression by fluorescence microscopy, and cells were fixed or infected as indicated.

2.3.8. Surface Expression of Mutated Receptor Constructs in HEK293A Cells

Following transfection of wild type and mutated 5-HT2A-YFP plasmids in HEK293A cells, cells were washed with 1X PBS then fixed in 4% PFA for 10 min followed by 3 washes with 1X PBS. Cells were incubated in blocking reagent (1X PBS containing 2% goat serum, 0.2% TritonX-100, and 0.1% BSA) at RT for 1 h then stained with a pan Cadherin-specific antibody (Abcam, ab6528, 1:200) in blocking reagent at 4°C O/N. Cells were extensively washed with 1X PBS and incubated in secondary goat anti-mouse Alexa Fluor 594 antibody (1:1000) in blocking reagent at RT for 1 h. Cells were washed with 1X PBS, and DAPI was added to stain for the nucleus (1:1000) for 10 min at RT prior to final 1X PBS washes and visualization by confocal microscopy. Fields of view were collected using an Olympus laser scanning confocal microscope (model IX81, Olympus America Inc.) under 60X magnification (oil immersion) using Fluoview software (Version 04.01.01.05). Fields of view were defined using DAPI visualization, then cells were viewed via Z sectioning, and fluorescence images were collected using 405/635 and 543/488 multi line argon lasers. At least 10 fields of view were captured for each sample measured. Cell-surface expression of mutant receptors was determined by ImageJ. Percent surface expression was determined by the Mander’s overlap coefficient utilizing colocalization threshold analysis (ImageJ [322]). Each field of view in comparable Z planes were analyzed representing the percentage of overlap between the pan Cadherin cell-surface marker and YFP expression for transfected receptors. Generation of box and
whisker plots was performed using MATLAB and Statistics Toolbox (release R2018a, The MathWorks Inc., Natick, Massachusetts).

2.3.9. Colocalization Analysis of JCPyV in SVG-A cells

SVG-A cells were seeded to ~80% confluence in 96 well #1.5 glass-bottomed plates in 100 µL of MEM containing 10% FBS without antibiotics. Transfection complexes of either emerald-clathrin (em-clath) or emerald-caveolin (em-cav) (gifts from Michael Davidson, described in [323] and [324], Addgene #54040 and #54026, respectively), containing 1 µg of DNA:1 µL of Lipofectamine 2000 in incomplete medium (lacking FBS and antibiotics) at a total concentration of 0.16 µg/well were incubated at RT for 20 min. Following incubation, complexes were added to each well and incubated at 37°C for 4 h. Transfection medium was removed and complete medium containing antibiotics was added, and plates were incubated an additional 20 h at 37°C. Cells were then prechilled on ice at 4°C for 45 min prior to addition of 10% phenol-free medium containing JCPyV-647 (MOI indicated in figure legend). Cells were then incubated on ice at 4°C for 1 h allowing for synchronized viral attachment, washed with 1X PBS, fixed with 4% PFA, or incubated at 37°C for 5 min, washed with 1X PBS, and fixed. Cells were incubated with DAPI nuclear stain (1:1000) and imaging by confocal microscopy. Fields of view were defined using DAPI visualization, then cells were viewed via Z sectioning, at least 10 fields of view were collected for each sample. Colocalization between JCPyV-647 and em-cav or em-clath was determined for each field of view in comparable Z planes by the Mander’s overlap coefficient using colocalization threshold software in ImageJ for each field of view (% colocalization). Generation of box and whisker plots was performed using MATLAB and Statistics Toolbox.

2.3.10. Viral Attachment by Flow Cytometry Following siRNA

Following siRNA treatment or transfection of wild-type or mutated receptors, SVG-A or HEK293A cells were removed from plates using CellStripper (Corning) and pelleted by centrifugation at
414 x g at 4°C for 5 min. Cells were washed with 1X PBS, pelleted, resuspended in 10% complete phenol-
free MEM (Corning), and incubated on ice at 4°C for 45 min. Cells were pelleted and resuspended in 200
µL of 10% complete phenol-free MEM containing Alexa Fluor488 labeled-JCPyV (siRNA SVG-A treated
samples) or Alexa Fluor 647-labeled JCPyV (transfected HEK293A samples) and incubated on ice at 4°C
for 1 h to allow for viral attachment. Cells were then pelleted by centrifugation, washed with 1X PBS,
and fixed in 4% PFA for 10 min, and subsequently resuspended in 300 µL 1X PBS. Fixed cells were then
analyzed for viral attachment by flow cytometry using a BD FACSCanto (BD Biosciences) equipped with a
488 and 633 AP-C laser line (Benton, Dickinson, and Company) for 10,000 events before analysis with BD
FacsDIVA (Benton, Dickinson, and Company) and FlowJo software (Tree Star, Inc.). Samples were gated
to exclude complex and dead cells utilizing FlowJo software.

2.3.11. Viral Entry by Confocal Microscopy

Cells were seeded in a 96-well #1.5 glass-bottomed plate (CellVis) to ~50% confluence before
treatment with siRNA or ~80% confluence for transfection with wild-type or mutated receptors.
Following siRNA treatment or transfection, cells were chilled on ice at 4°C for 45 min before addition of
Alexa Fluor 488-labeled JCPyV (SVG-A cells) or Alexa Fluor 647-labeled JCPyV (HEK293A cells) in 2% FBS
MEM containing 1% P/S and incubated on ice at 4°C for 1 h for viral attachment. Cells were then
incubated at 37°C for 1.5 h, allowing for viral entry, washed in 1X PBS, and fixed in 4% PFA for 10 min.
Cells were stained with DAPI and imaged by confocal microscopy. Fields of view were defined using DAPI
visualization, then cells were viewed via Z sectioning utilizing a 60X objective (oil immersion), and
fluorescent and DIC images for each field of view were collected. Viral entry was quantified using
Fluoview Software single measurement analysis by outlining individual regions of interest (ROI) utilizing
the corresponding DIC image to define the plasma membrane of the cell. ROIs were drawn to exclude
the plasma membrane to measure internalized virus by relative fluorescence units per cell for
background-corrected samples within an applied threshold intensity. Cross sections were quantified for
> 30 individual cells for control and target siRNA-treated samples (SVG-A cells) or cells expressing wild type and mutated receptors (HEK293A cells) for at least three independent experiments.

2.4. Results

2.4.1. Inhibition of Clathrin Decreases JCPyV Infection

To define whether clathrin is required for JCPyV infection, SVG-A cells, a model human glial cell line, were treated with the clathrin inhibitor Pitstop 2, which specifically inhibits the clathrin terminal domain [325]. Cells were pretreated and infected with JCPyV in the presence of Pitstop 2 and fed with medium containing JCPyV-specific antisera 1 h postinfection (hpi) to neutralize non-internalized virus. Infectivity was scored by a fluorescence focus assay (FFA) at 72 hpi to measure newly synthesized VP1 following a single replication cycle. Pitstop 2 treatment resulted in a dose-dependent decrease in JCPyV infection (Figure 2.1A). Additionally, to determine whether Pitstop 2 was impacting JCPyV infection at a time consistent with JCPyV entry, Pitstop 2 was added at specific times based on the reported kinetics of JCPyV internalization [29, 194]. SVG-A cells were either pretreated for 15 min with Pitstop 2, treated at the time of infection, or incubated in Pitstop 2 at 4 hpi, when the majority of virus should be internalized [29, 194] (Figure 2.1B). Additionally, JCPyV-specific antisera was added to the medium at 4 hpi. Pitstop 2 reduced infection when cells were pretreated or when the inhibitor was included in the viral inoculum (Figure 2.1B). There was no impact on JCPyV infection when Pitstop 2 was added 4 hpi, suggesting that clathrin inhibition specifically reduces JCPyV infection at times consistent with viral attachment and entry. To further confirm the role of clathrin in JCPyV infection, SVG-A cells were treated with a siRNA targeting clathrin heavy chain (CHC), and knock down was measured by western blot (WB) analysis (Figure 2.1D). Additionally, CHC siRNA-treated cells were infected with either JCPyV or SV40, at MOIs resulting in comparable levels of infectivity [179, 321, 326] (indicated in figure legends). Viral infectivity was scored by indirect immunofluorescence 72 hpi, following a single round of infection, for newly synthesized VP1. CHC knockdown resulted in a significant reduction in JCPyV infection in comparison to
EGFR and scrambled control siRNAs (Figure 2.1C). Conversely, infectivity of SV40, which utilizes a clathrin-independent internalization pathway [163, 237, 239], was not impacted by CHC knockdown (Figure 2.1C).

**Figure 2.1. Clathrin inhibition decreases JCPyV infection.**

SVG-A cells were pretreated with clathrin inhibitor Pitstop 2 or a DMSO control (A) at indicated concentrations at 37°C for 15 min prior to infection with JCPyV, and at 1 hpi cells were fed with media containing JCPyV-specific neutralizing antibody (JC-specific Ab). (B) 20 µM Pitstop 2 or DMSO control was added at times indicated prior to or following infection with JCPyV, and at 4 hpi cells were fed with media containing JC-specific Ab. (C) SVG-A cells were transfected with siRNA targeting clathrin heavy chain (CHC), EGFR control, or a non-targeting scrambled (Scr) control. (A-C) Cells were infected with JCPyV (MOI = 0.5 FFU/cell) or (C) SV40 (MOI = 0.001 FFU/cell). (D) Cellular lysates from (C) were processed for western blot to confirm protein knockdown of CHC. (A-C) Infected cells were fixed 72 hpi and analyzed by indirect immunofluorescence utilizing an Ab that detects both JCPyV and SV40 VP1. Data represent the percentage of JCPyV- or SV40-infected VP1+ cells/visual field normalized to the number of DAPI+ cells/field for five 10x fields of view for triplicate samples (all samples normalized to EGFR control siRNA-treated cells (100%)) for at least three independent experiments. Error bars = SD. *P<0.05.

To determine if JCPyV colocalizes with clathrin at times consistent with viral internalization, SVG-A cells were transfected with plasmids to express either emerald-clathrin (em-clath) or emerald-caveolin (em-cav) and infected with JCPyV labeled with Alexa Fluor-647 (JCPyV-647) on ice for 1 h to allow for synchronized viral attachment. Cells were then fixed or shifted to 37°C for 5 min for viral internalization prior to fixation (Figure 2.2A). Samples were imaged by confocal microscopy (Figure 2.2A) and fields of view were analyzed for colocalization between em-clath or em-cav and JCPyV-647. Virus
particles colocalized with em-clath at 5 min post internalization yet did not demonstrate significant colocalization with em-cav (Figure 2.2B). These data suggest that clathrin is critical for JCPyV infection and that JCPyV colocalizes with clathrin at times consistent with viral internalization.

Figure 2.2. JCPyV colocalizes with clathrin during entry.

(A-B) SVG-A cells were transfected with either emerald-clathrin (em-clath) or emerald-caveolin (em-cav) (green), prechilled, then incubated with JCPyV-647 (MOI = 5 FFU/cell) (pseudocolored magenta) on ice for 1 h (viral attachment) followed by incubation at 37°C for viral entry. (A) Cells were fixed at times indicated, nuclei stained with DAPI (blue), and 10 fields of view per sample were measured by confocal microscopy at 60X magnification. (B) Colocalization was analyzed using ImageJ software by measuring the percent correlation of em-clath or em-cav and JCPyV-647 using Mander’s Coefficient. Data represent the percentage of em-clath and em-cav colocalized with JCPyV/visual field for at least 10 fields of view for triplicate samples. Data is depicted as a box and whisker plot denoting the median and the distribution of % colocalization for both samples and is representative of at least 3 independent experiments. Upper and lower whiskers = 1.5 times the interquartile range. Data points in gray (+) = outliers. Scale bars = 5 µm. Arrows = sites of colocalization. P*, <0.05.

2.4.2. JCPyV Infection Requires Adaptor Protein AP2 Subunits β2 and μ2

AP2 is a key regulator in clathrin-coated pit assembly and clathrin-mediated endocytosis [188-190] and is required for internalization of 5-HT$_2$Rs [327]. The AP2 subunit μ2 interacts with the internalizing receptor and cargo [328, 329] while subunit β2 interacts with other endocytic proteins, such as clathrin and β-arrestin [330-333]. To determine if AP2 is required for JCPyV infection, SVG-A cells were treated with siRNA targeting subunits AP2 μ2 and β2, and knock down was measured by WB analysis (Figure 2.2B). Following siRNA treatment, cells were infected with JCPyV (Figure 2.2A).
Knockdown of \( \mu 2 \) resulted in a slight, though significant reduction in JCPyV infection while knockdown of \( \beta 2 \) significantly reduced infection in comparison to EGFR and scrambled control siRNAs (Figure 2.2A). Collectively, these data demonstrate a requirement for AP2 subunits \( \mu 2 \) and predominantly, \( \beta 2 \), for JCPyV infection.

2.4.3. Dynamin is Required for JCPyV Infection

Clathrin-coated pits are pinched off into vesicles by the molecular GTPase dynamin [185, 204]. To test the role of dynamins in JCPyV infection, SVG-A cells were pretreated for 30 min with increasing concentrations of Dynole 34-2, an inhibitor of dynamin isoforms 1 and 2 [334], which are expressed within the brain and function in CME [335]. Cells were subsequently infected with JCPyV in the presence of Dynole, resulting in a dose-dependent decrease in JCPyV infection (Figure 2.3A). To further explore the role of dynamin in infection, dynamin isoforms 1 and 2 were individually knocked down in SVG-A
cells by siRNA interference (Figure 2.3C). Following siRNA treatment, cells were infected with JCPyV and infectivity was measured (Figure 2.3B). Knockdown of dynamin 1 resulted in a significant decrease in infection, while knockdown of dynamin 2 lead to a lesser, though significant reduction in infectivity (Figure 2.3B). Collectively, these data demonstrate the requirement of dynamins, mainly dynamin 1, for JCPyV infection.

![Figure 2.4. Dynamin is required for JCPyV infection.](image)

(A) SVG-A cells were pretreated with dynamin 1/2 inhibitor Dynole at indicated concentrations or the Dynole-specific negative control at 37°C for 30 min before infection with JCPyV (MOI = 0.5 FFU/cell) in the presence of Dynole. (B) SVG-A cells transfected with siRNA targeting EGFR control, non-targeting scrambled (Scr) control, dynamin 1 (Dyn1), or dynamin 2 (Dyn2) siRNAs were infected with JCPyV (MOI = 0.5 FFU/cell). Infected cells were fixed at 72 hpi, stained using a VP1-specific antibody, and analyzed by indirect immunofluorescence. (C) Protein knockdown of Dyn1 and Dyn2 was confirmed by western blot. Data represent the percentage of VP1+ cells/visual field normalized to the number of DAPI+ cells/field for five 10x fields of view for triplicate samples (all samples normalized to EGFR control siRNA-treated cells (100%)) for at least three independent experiments. Error bars = SD. $P^*$, <0.05.

2.4.4 Knockdown of β-arrestin Significantly Reduces JCPyV Infection in Glial Cells

β-arrestin acts as an adaptor protein and assists in directing internalizing GPCRs into clathrin-coated pits through direct interactions with clathrin and AP2 [212, 213]. While there are reported
species-specific and cell type- and ligand-specific differences in β-arrestin-mediated internalization [336-338], human 5-HT2R has been reported to be β-arrestin-dependent [203], suggesting that β-arrestin may play a role in JCPyV infection. SVG-A cells were transfected with siRNA targeting β-arrestin (Figure 2.4B), infected with JCPyV or SV40, and infectivity was measured. β-arrestin knockdown resulted in a significant reduction in JCPyV infection while not impacting SV40 infection (Figure 2.4A). These data suggest that JCPyV infection requires the activity of the 5-HT2R scaffolding protein β-arrestin.

Figure 2.5. JCPyV infection requires β-arrestin.

SVG-A cells transfected with siRNA targeting β-arrestin (β-arr), EGFR control, or non-targeting scrambled (Scr) control (A) were infected with JCPyV (MOI = 0.5 FFU/cell) or SV40 (MOI = 0.001 FFU/cell) or (B) processed for western blot to confirm β-arr knockdown. Infected cells were fixed 72 hpi, stained using a VP1-specific antibody, and analyzed by indirect immunofluorescence. Data represent the percentage of VP1+ cells/visual field normalized to the number of DAPI+ cells/field for five 10x fields of view for triplicate samples (all samples normalized to EGFR control siRNA-treated cells (100%)) for at least three independent experiments. Error bars = SD. *P < 0.05.

2.4.5. Reduction of Clathrin Heavy Chain and β-arrestin Do Not Affect JCPyV Attachment but Specifically Reduce Internalization

Reduction of clathrin heavy chain (CHC) and β-arrestin, key proteins in the CME pathway, significantly reduced JCPyV infection (Figures 2.1 and 2.4) and JCPyV was found to colocalize with clathrin during internalization (Figure 2.2). To determine whether knockdown of CHC and β-arrestin
specifically reduces JCPyV internalization, viral attachment and entry were measured following siRNA treatment (Figure 2.6 and 2.7). SVG-A cells were treated with siRNAs targeting CHC, β-arrestin, or a non-relevant control EGFR, and subsequently incubated with Alexa Fluor-488 labeled JCPyV (JCPyV-488) virions on ice to allow for viral attachment. Attachment of JCPyV-488 to siRNA-treated cells was measured by flow cytometry (Figures 2.6A and 2.6B). Analysis of mean fluorescence intensities indicate that binding of JCPyV to SVG-A cells is not reduced by siRNA silencing of CHC (Figure 2.6A) or β-arrestin (Figure 2.6B).

Figure 2.6. Reduction of clathrin heavy chain or β-arrestin does not affect attachment of JCPyV.

SVG-A cells were transfected with EGFR control siRNA or siRNA targeting (A) clathrin heavy chain (CHC) or (B) β-arrestin (β-arr). Cells were stripped from plates, pre-chilled at 4°C for 45 min, incubated with JCPyV-488 (MOI = 5 FFU/cell) on ice for 1 h, and analyzed by flow cytometry. Histograms represent the mean fluorescence intensity for cells transfected with CHC or β-arr siRNA (green), EGFR siRNA (blue), or cells alone (gray). Data are representative of 10,000 events for triplicate samples for at least 3 independent experiments.

To measure JCPyV entry, SVG-A cells were treated with CHC, β-arrestin, or epidermal growth factor receptor (EGFR) siRNAs and incubated on ice at 4°C with JCPyV-488 for synchronized viral attachment. Cells were then incubated at 37°C for 1.5 h to allow for viral entry [29]. Following fixation, JCPyV internalization was measured by confocal microscopy (Figures 2.7A and 2.7B insets) by quantifying the relative fluorescence intensity for internalized JCPyV-488 within individual cells, excluding the plasma membrane. Treatment of cells with either CHC (Figure 2.7A) or β-arrestin (Figure 2.7B) siRNAs resulted in a significant reduction in JCPyV internalization in comparison to EGFR control.
siRNA-treated samples. Taken together, these results suggest that reduction of β-arrestin or CHC does not impact viral attachment to cells but specifically reduces JCPyV internalization.

Figure 2.7. Reduction of clathrin heavy chain or β-arrestin prevents JCPyV entry. SVG-A cells were transfected with siRNAs for (C) CHC or (D) β-arr or EGFR control and incubated at 37°C for 72 h, prechilled, then incubated with JCPyV-488 (MOI = 5 FFU/cell) (green) on ice for 1 h (viral attachment) followed by incubation at 37°C for 1.5 h (viral entry). Cells were fixed, nuclei were stained with DAPI (blue), and viral entry was analyzed by confocal microscopy at 60X magnification (A, B inset). Utilizing DIC overlay, JCPyV internalization was quantified as the relative fluorescence per cell for at least 30 cells for CHC-, β-arr-, and EGFR-siRNA treated samples using Olympus software Fluoview10-ASW. Data is depicted as a box and whisker plot denoting median and the distribution of % internalization for both samples and is representative of at least three independent experiments. All samples normalized to EGFR-siRNA treated cells (100%). Upper and lower whiskers = 1.5 times the interquartile range. Scale bars = 5 µm. P*, <0.0001.
2.4.6. Clathrin, AP2, and β-arrestin Are Required for JCPyV Infection of Kidney Cells

JCPyV infects both kidney and glial cells, yet demonstrates poor permissivity in established kidney cell lines [194]. Human embryonic kidney (HEK293A) cells express the JCPyV attachment factor LSTc, but lack sufficient expression of 5-HT2Rs to support JCPyV infection and are poorly permissive for infection unless 5-HT2Rs are expressed [194]. HEK293A cells stably-expressing 5-HT2A, 5-HT2B, and 5-HT2C receptors support JCPyV infection and demonstrate a specific increase in viral entry in comparison to control HEK293A cells [194]. To determine whether JCPyV infection of kidney cells is dependent upon clathrin-mediated endocytosis, 5-HT2A, 5-HT2B, and 5-HT2C-R-expressing HEK293A cells [194] were treated with siRNA targeting clathrin heavy chain (CHC). Protein knockdown was determined by WB analysis (Figure 2.6A). Following siRNA treatment, cells were infected with JCPyV and infectivity was measured 48 hpi for newly synthesized T-antigen (T-Ag). Reduction of CHC in 5-HT2A, 5-HT2B, and 5-HT2C-R-expressing HEK293A cells resulted in significantly reduced JCPyV infection (Figure 2.8A). To further determine the CME components necessary for JCPyV infectivity of kidney cells, 5-HT2A, 5-HT2B, and 5-HT2C-R-expressing HEK293A cells were treated with siRNA targeting AP2 subunit β2 and β-arrestin. Protein reduction was determined by WB analysis (Figures 2.8B and 2.8C). Following siRNA treatments, cells were infected with JCPyV and infectivity was measured 48 hpi. Reduction of AP2 β2 in 5-HT2A, 5-HT2B, and 5-HT2C-R-expressing HEK293A cells significantly reduced JCPyV infectivity (Figure 2.8B). Moreover, treatment with a β-arrestin siRNA resulted in significant reductions in JCPyV infection (Figure 2.8C), while not impacting infection by SV40 (Figure 2.6C). These data suggest that clathrin, AP2, and β-arrestin are required for JCPyV infection of 5-HT2R-expressing-HEK293A cells (Figure 2.8).
Figure 2.8. Reduction of clathrin, AP2 β2, and β-arrestin in HEK-5-HT2A, 2B, and 2CR-YFP cells significantly reduces infection.

HEK293A cells stably expressing 5-HT2A, 2B, and 2CR-YFP were transfected with siRNA targeting (A) clathrin heavy chain (CHC), (B) AP2 β2, or (C) β-arrestin (β-arr), and EGFR control, and non-targeting scrambled (Scr) control. Cells were infected with JCPyV (MOI = 0.5 FFU/cell) or (C) SV40 (MOI = 0.001 FFU/cell) or (A-C insets) processed for western blot to confirm CHC, AP2 β2, and β-arr knockdown. Infected cells were fixed at 48 hpi and stained using a JCPyV or SV40 T-antigen (T-Ag)-specific antibody, and analyzed by indirect immunofluorescence. Data represent the percentage of JCPyV- or SV40-infected TAg+ cells/visual field normalized to the number of DAPI+ cells/field for five 10x fields of view for triplicate samples (all samples normalized to EGFR control siRNA-treated cells for each receptor subtype cell line (100%)) for at least three independent experiments. Error bars = SD. *P < 0.05.
2.4.7. Site-directed Mutagenesis of a Conserved β-arrestin Binding Domain Within 5-HT2AR Reduces JCPyV Infection

The human 5-HT2R subtypes contain conserved domains within the intracellular loops that serve as docking sites for endocytic scaffolding machinery that can promote receptor internalization [202, 203]. The ASK motif, conserved in human 5-HT2Rs, is a reported direct interaction site for β-arrestin [203]. The role of this protein-binding domain has been extensively characterized for internalization and trafficking of 5-HT2AR; expression of the ASK motif confers β-arrestin-mediated 5-HT2AR internalization [203] (depicted in Figure 2.9A). Furthermore, β-arrestin scaffolds 5-HT2AR to clathrin and AP2 to mediate CME [339]. To determine the role of the ASK motif in JCPyV infection, single and double amino acid point mutations were engineered in the 5-HT2A-YFP receptor, in which the serine and lysine at amino acid positions 457 and 458 were substituted with alanine residues (Figure 2.9A). To ensure accessibility of the mutated 5-HT2ARs for viral utilization, cell-surface expression of wild type and mutated receptors was measured by confocal microscopy. The cell-surface was defined by staining the plasma membrane with an antibody specific for pan Cadherin, and colocalization of wild type and mutated 5-HT2ARs with pan Cadherin was measured. Mutagenesis of 5-HT2A-YFP receptors did not reduce the surface expression of the receptor in comparison to wild type cell-surface expression (Figure 2.9B). Wild-type and mutated 5-HT2A-YFP receptors were expressed in HEK293A cells and infected with JCPyV or SV40, and viral infectivity was measured by expression of newly synthesized viral protein T-Ag by indirect immunofluorescence 48 hpi (Figure 2.9C). Cells expressing 5-HT2AR-S457A (AAK) and 5-HT2AR-SK457-58AA (AAA) demonstrated significant reductions in JCPyV infection in comparison to wild-type 5-HT2AR-YFP (Figure 2.9C). However, 5-HT2AR-K458A (ASA) supported JCPyV infection to the level of wild-type 5-HT2AR-YFP (Figure 2.9C). Furthermore, mutation of residues in the 5-HT2AR ASK motif had no significant effect on SV40 infection of HEK293A cells (Figure 2.9D). Collectively these data suggest that the β-
The α2A adrenergic receptor (α2A-AR) binds to β-arrestins, which are crucial for the regulation of receptor signaling. Our experiments revealed that the arrestin-binding domain in the 5-HT2AR is essential for JCPyV infection, and specifically, the serine residue within the ASK motif is critical for JCPyV infection in HEK293A cells.

**Figure 2.9. β-arrestin-binding motif in 5-HT2AR is required for JCPyV infection of HEK293A cells.**

(A) Schematic demonstrating 5-HT2AR ASK motif. (A-D) HEK293A cells were transfected with wild type unmutated 5-HT2AR-YFP (WT), 5-HT2AR-YFP receptors containing point mutations in ASK motif, or empty vector YFP. (B) Cell-surface expression of WT and mutated receptors was measured by confocal microscopy at 60X magnification (inset). Cell-surface expression was analyzed using ImageJ software by measuring the percent correlation of receptor expression (green) and a pan Cadherin cell-surface marker (red) using Mander’s Coefficient. Data represent the percentage of receptor that is cell-surface expressed/visual field normalized to the WT for at least 10 fields of view for triplicate samples. (C) Following transfection, cells were infected with JCPyV (MOI = 0.5 FFU/cell) or (D) SV40 (MOI = 0.001 FFU/cell), fixed at 48 hpi, stained with a JCPyV or SV40 T-antigen (T-Ag)-specific antibody, and analyzed by indirect immunofluorescence. Data represent the percentage of JCPyV-infected T-Ag+ cells/visual field normalized to the number of DAPI+ cells/field for five 10x fields of view for triplicate samples for at least three independent experiments. Scale bars = 5 µm. Error bars = SD. P*, <0.05.
2.4.8. Mutagenesis of the ASK Motif in 5-HT2AR Reduces Internalization of JCPyV

To determine whether the ASK motif is required for JCPyV internalization, viral attachment and entry were measured. HEK293A cells transiently expressing wild-type and mutated 5-HT2A-YFP receptors were removed from plates and incubated with Alexa Fluor 647 labelled JCPyV (JCPyV-647) virions on ice. Attachment of JCPyV-647 to cells expressing wild-type and mutated 5-HT2A-Rs was measured by flow cytometry (Figure 2.8A). Mean fluorescence intensities of JCPyV-647 bound virus was equivalent in cells expressing wild-type and mutated 5-HT2A-YFP receptors suggesting that mutagenesis of the ASK motif does not reduce JCPyV binding to cells (Figure 2.8A). Furthermore, expression of WT 5-HT2A-R did not enhance JCPyV binding in comparison to untransfected cells, in correlation with previously published results [194]. To measure viral entry, HEK293A cells expressing wild-type and mutated 5-HT2A-YFP receptors were incubated on ice with JCPyV-647 for synchronized viral attachment, followed by incubation at 37°C for 1.5 h for viral entry. Following fixation, viral internalization was measured by confocal microscopy by quantifying the relative fluorescence intensity for internalized JCPyV-647 within individual cells, excluding the plasma membrane (Figure 2.8C). Cells expressing 5-HT2A-R-S457A (AAK) and 5-HT2A-R-SK457-58AA (AAA) demonstrated a significant reduction in JCPyV internalization while 5-HT2A-R-K458A (ASA) supported JCPyV internalization comparable to the wild-type 5-HT2A-YFP receptor (Figure 2.8B). Collectively, these data demonstrate that the serine residue within the ASK motif is critical for JCPyV entry within host cells.
Figure 2.10. Mutagenesis of β-arrestin-binding motif in 5-HT2AR decreases JCPyV internalization in HEK293A cells.

HEK293A cells were transfected with wild type unmutated 5-HT2AR-YFP (WT), mutated receptors 5-HT2AR-S457A-R-YFP (AAK), 5-HT2AR-SK457-58AA-R-YFP (AAA), or 5-HT2AR-K458A-R-YFP (ASA), or empty YFP vector. (A) Cells were stripped from plates, prechilled at 4°C, followed by incubation with JCPyV-647 (MOI = 5 FFU/cell) on ice for 1 h for viral attachment then analyzed by flow cytometry. Histograms represent the mean fluorescence intensity for cells (no virus, gray), YFP (no virus), or samples treated with JCPyV-647. Data are representative of 10,000 events for triplicate samples for at least 3 independent experiments. For viral entry, (B, C) HEK293A cells were transfected with WT, AAK, AAA, or ASA. Cells were chilled to 4°C, incubated with JCPyV-647 (MOI = 3 FFU/cell) (pseudocolored magenta) on ice for 1 h (viral attachment) followed by incubation at 37°C for 1.5 h (viral entry). Cells were fixed, nuclei were stained with DAPI (blue), and entry was analyzed by confocal microscopy at 60X magnification. (B) Utilizing DIC overlay, JCPyV internalization was quantified as the relative fluorescence within individual cells for at least 30 cells for 5-HT2AR-WT-, AAK-, AAA-, and ASA-transfected samples using Olympus software Fluoview10-ASW. Data is depicted as a box and whisker plot denoting the median and the distribution of % internalization across samples measured and is representative of at least three independent experiments. All samples normalized to WT transfected cells (100%). Upper and lower whiskers = 1.5 times the interquartile range. Data points in gray (+) = outliers. Scale bars = 5 µm. P*, <0.0001.
2.5. Discussion

Viral entry is a complex process, requiring numerous host-cell factors, including cell-surface receptors and intracellular proteins, to facilitate internalization [119]. In this study we have further characterized the endocytic proteins in the clathrin-mediated pathway that facilitate JCPyV internalization. Disruption of clathrin (Figure 2.1 and 2.2) and CME-accessory proteins β-arrestin (Figure 2.5), AP2 (Figure 2.3), and dynamin (Figure 2.4), all significantly reduced JCPyV infection. Furthermore, disruption of CHC and β-arrestin specifically reduced JCPyV entry into host cells (Figure 2.7) while not impacting viral attachment (Figure 2.6). Interestingly, chemical inhibitors and siRNAs utilized did not impact infection by SV40, which is known to enter by clathrin-independent pathways [163, 237, 239]. Additionally, mutagenesis of the ASK motif, a conserved β-arrestin interaction motif within the C-terminus of human 5-HT2AR, reduced JCPyV infection (Figure 2.9) and entry (Figure 2.10) indicating that the β-arrestin-binding domain of 5-HT2AR is critical for JCPyV infection and internalization. These results demonstrate that JCPyV utilizes CME to infect multiple host cell types and characterizes the key host-cell proteins involved, further defining the role of 5-HT2Rs in JCPyV internalization strategies (Figure 2.11).

Viruses usurp many endocytic pathways to enter host cells [119] including clathrin-mediated endocytosis, macropinocytosis, caveolae, clathrin- and caveolae-independent mechanisms, and cholesterol-mediated or -independent pathways [314]. Interestingly, polyomaviruses have been reported to enter cells by a number of the aforementioned routes [239, 250, 253], yet, one distinct feature is clear, following internalization all polyomaviruses reach the endoplasmic reticulum (ER) for uncoating [24-29]. Internalization strategies and targeting of cargo to the proper cellular compartment for viral uncoating events is largely reliant on receptors utilized for viral attachment and entry [119].
Figure 2.11. Model of JCPyV entry.

JCPyV initially interacts with LSTc via direct interactions with VP1. Internalization of JCPyV is facilitated by 5-HT₂Rs. 5-HT₂R-mediated entry likely first activates β-arrestin to the site of infection facilitating an interaction with the receptor through the conserved 5-HT₂R ASK motif. Clathrin and AP2 subunits β2 and μ2 are recruited, likely forming a clathrin-coated pit around the viral cargo. Internalization is completed by the GTPase scission mechanism of dynamin 1 and 2 allowing for JCPyV to traffic through the endocytic compartment in a clathrin-coated vesicle.

Following internalization, SV40 is deposited into a caveolin-positive vesicle, then traffics to the ER for partial uncoating [340]. Like other polyomaviruses, localization of SV40 to the ER is a direct result of binding to and internalization by specific ganglioside receptors [17, 25, 167, 265, 341]. Although JCPyV binds to gangliosides with low affinity, it does not utilize gangliosides as functional viral receptors [151]. Instead, JCPyV infection is dependent on attachment to the sialic acid containing LSTc receptor motif [144] and requires 5-HT₂Rs [196] for internalization by CME [194]. This dichotomy in polyomavirus entry pathways suggests that JCPyV may utilize proteinaceous receptors, 5-HT₂Rs, in order to facilitate
clathrin-mediated endocytosis, rather than the ganglioside-, non-clathrin-dependent pathways utilized by other polyomaviruses [163, 237] for arrival to the ER.

Upon activation, GPCRs, including 5-HT$_2$Rs, are regulated by two distinct mechanisms, G-protein- and β-arrestin-dependent signaling [212, 342]. The activation of these pathways is based on the ligand that induces either G-protein and/or β-arrestin coupling to the receptor [206, 327, 336, 343-345]. Upon recruitment, β-arrestins interact with the intracellular loops of 5-HT$_2$Rs, leading to further recruitment of endocytic proteins [198, 200, 336, 338, 346-348] and activation of signaling events [338, 343, 345, 349, 350]. In addition to regulation of 5-HT$_2$R internalization and recruitment of β-arrestin, internalization of GPCRs into clathrin-coated vesicles also implicates the potentiation of downstream signaling cascades, including the mitogen-activated protein kinase (MAPK) pathway [338, 351, 352]. Interestingly, the MAPK extracellular signal-regulated kinase (ERK) is activated at time points coinciding with JCPyV entry [253, 321], and is required for viral infection [321]. We have demonstrated that β-arrestin, a known activator of MAPK and ERK1/2 signaling, is essential for JCPyV infection and internalization. However, the influence of β-arrestin-mediated internalization of 5-HT$_2$Rs on ERK signaling in the context of JCPyV pathogenesis, remains to be explored.

Conserved intracellular motifs of 5-HT$_2$Rs have been implicated to serve as points of interaction between endocytic scaffolding machinery and the receptors [202, 203]. Among those motifs are domains associated with interactions for β-arrestins including the Asp-Arg-Tyr (DRY), Asn-Pro-X-X-Tyr (NPXXY), and ASK motifs in human 5-HT$_2$Rs [353-355]. The ASK motif is required for β-arrestin-induced internalization, and disruption of this domain has been demonstrated to impede normal trafficking of 5-HT$_2\alpha$R [203]. Interestingly, expression patterns of these motifs are not conserved across all 5-HT receptor subtypes, and only 5-HT$_2$Rs of human origin express the ASK motif (determined by MacVector amino acid alignment). Our findings demonstrate that the ASK motif within 5-HT$_2\alpha$R is essential for JCPyV entry.
(Figure 2.10) and infection (Figure 2.9), suggesting that the presence of the ASK motif within 5-HT₂Rs may confer specificity for JCPyV utilization of this receptor family. However, the presence of multiple β-arrestin interacting domains suggests that more than one interaction may be necessary to facilitate ligand-mediated internalization of these receptors and warrants further exploration.

Within this study we have characterized the key proteins required for JCPyV internalization, including β-arrestin, clathrin, AP2, and dynamin. Furthermore, we have identified a β-arrestin interaction motif within 5-HT₂A that is required for JCPyV infection, suggesting that it may contribute to JCPyV internalization strategies utilizing 5-HT₂Rs. Collectively, these findings contribute to our understanding of JCPyV internalization and infection, identifying new targets for the development of antiviral therapies to treat JCPyV infection and prevent PML pathogenesis.
CHAPTER 3
GRK2 MEDIATES $\beta$-ARRESTIN INTERACTIONS WITH 5-HT$_2$ RECEPTORS FOR JC POLYOMAVIRUS ENDOCYTOSIS

The work presented in this chapter has been submitted for consideration for publication in the *Journal of Virology* on 03NOV2020

3.1. Chapter Summary

JC polyomavirus (JCPyV) infects the majority of the population, establishing a lifelong, asymptomatic infection in the kidney of healthy individuals. People that become severely immunocompromised may experience JCPyV reactivation, which can cause progressive multifocal leukoencephalopathy (PML), a neurodegenerative disease. Due to a lack of therapeutic options, PML results in fatality or significant debilitation among affected individuals. Cellular internalization of JCPyV is mediated by serotonin 5-hydroxytryptamine subfamily 2 receptors (5-HT$_2$Rs) via clathrin-mediated endocytosis. The JCPyV entry process requires the clathrin-scaffolding proteins $\beta$-arrestin, adaptor protein 2 (AP2), and dynamin. Further, a $\beta$-arrestin interacting domain, the Ala-Ser-Lys (ASK) motif, within the C-terminus of 5-HT$_{2a}$R is important for JCPyV internalization and infection. Interestingly, 5-HT$_2$R subtypes A, B, and C equally support JCPyV entry and infection, and all subtypes contain an ASK motif, suggesting a conserved mechanism for viral entry. However, the role of the 5-HT$_2$R ASK motifs and the activation of $\beta$-arrestin-associated proteins during internalization has not been fully elucidated. Through mutagenesis, the ASK motifs within 5-HT$_{2a}$R and 5-HT$_{2c}$R were identified as critical for JCPyV internalization and infectivity. Further, utilizing biochemical pulldown techniques, mutagenesis of the ASK motifs in 5-HT$_{2a}$R and 5-HT$_{2c}$R resulted in reduced $\beta$-arrestin binding. Utilizing small-molecule chemical inhibitors and RNA interference, G protein receptor kinase 2 (GRK2) was determined to be required for JCPyV internalization and infection by mediating interactions between $\beta$-arrestin and the
ASK motif of 5-HT$_2$Rs. These findings demonstrate that GRK2 and β-arrestin interactions with 5-HT$_2$Rs are critical for JCPyV entry by clathrin-mediated endocytosis and productive infection.

3.2. Introduction

5-HT$_2$Rs are G-protein coupled receptors (GPCRs) that can be activated by G protein-dependent or β-arrestin-mediated signaling pathways, resulting in differing signaling outcomes [212, 342]. 5-HT$_2$Rs can be internalized by CME in an agonist- and cell-type specific-fashion [203, 205, 206, 209] through the recruitment of scaffolding proteins, including clathrin, β-arrestin, and adaptor protein 2 (AP2) [203, 205, 206]. The activation of these proteins ultimately dictates the signaling outcomes of the receptor and associated cargo [14, 205, 212, 342], facilitating the delivery of 5-HT$_2$Rs to endocytic vesicles resulting in recycling, trafficking, degradation of the receptor, or activation of specific signaling cascades [205]. We have previously determined that JCPyV usurps the CME proteins β-arrestin, AP2, and clathrin, to facilitate a productive infection, yet had no impact on SV40 infection [181]. While these proteins were demonstrated to be important for viral infection, β-arrestin and clathrin were specifically identified to facilitate viral entry [181]. Expression of 5-HT receptors 2A, 2B, or 2C can confer infection in poorly permissive cell types, suggesting a reliance on conserved signaling inherent to 5-HT$_2$Rs [194]. Further, HEK293A cells stably expressing 5-HT$_{2a}R$, 5-HT$_{2b}R$, or 5-HT$_{2c}R$ can promote infection; however, when β-arrestin is knocked down, these cells become resistant to JCPyV infection [181, 199]. This highlights the importance of β-arrestin in the viral usurpation of 5-HT$_2$R signaling capabilities, likely occurring in a conserved manner.

The C-terminus of 5-HT$_{2a}R$ contains a conserved, tripeptide motif, the ASK (Ala-Ser-Lys) motif, a β-arrestin binding domain; this motif has been identified to be critical for endocytosis and trafficking of 5-HT$_{2a}R$ within cells [203]. Mutagenesis of this motif alters the signaling capability of 5-HT$_{2a}R$ and negates receptor reliance on β-arrestin for intracellular trafficking and recycling [203]. Upon β-arrestin
interactions with the receptor, further endocytic scaffolding machinery, including AP2 and additional β-arrestin, are recruited to the site of the activated receptor and can directly interact with clathrin, assisting in the formation of the clathrin-coated pit [206, 212, 213]. Through these direct interactions β-arrestin can promote downstream signaling in the cell, including the activation of the mitogen activated protein kinase (MAPK) cascade [338, 351, 352], which has been demonstrated to drive JCPyV infection [180, 321, 356, 357]. Mutagenesis of the ASK motif in 5-HT₂AR drastically reduces JCPyV internalization and infection [181], suggesting a dependence on this domain for β-arrestin-induced signaling events critical for viral infection. Interestingly, in addition to 5-HT₂AR, the ASK motif is also conserved in related 5-HT₂R subtypes 2B and 2C (NCBI accession numbers 3356, 3357, 3358, respectively). However, the importance of the conserved ASK motif for canonical function of the 5-HT₂AR and 5-HT₂CR or a reliance on β-arrestin for their intracellular trafficking is not well characterized.

β-arrestin-mediated signaling is facilitated by G protein receptor kinases (GRKs), which promote uncoupling of G proteins through specific phosphorylation events [203, 209]. GRKs, a family of seven kinases, are recruited to GPCRs and recognize specific amino acid sequences, or ‘bar codes,’ on the receptor, resulting in phosphorylation of mainly Ser or Thr residues within the signal sequence [358]. This phosphorylation event prevents the activation of G proteins and promotes β-arrestin recruitment and binding to the receptor, resulting in rapid desensitization, or internalization, of 5-HT₂Rs by CME. Furthermore, the β-arrestin binding domain of 5-HT₂AR, the ASK motif, is phosphorylated by GRK2 [203]. This phosphorylation promotes the recruitment of β-arrestin which engages the phosphate residue-tagged Ser within the ASK motif [203]. The direct interaction between β-arrestin and 5-HT₂AR then promotes internalization of the receptor by CME. Trafficking of the receptor within the cell is reliant on β-arrestin engagement of the ASK motif as mutagenesis of the Ser within the motif results in β-arrestin-independent internalization of the receptor with drastically different signaling capabilities [203]. Interestingly, mutagenesis of the Ser residue within the ASK motif of 5-HT₂AR significantly reduces JCPyV infection [181].
entry within cells [181]. Therefore, the presence of the ASK motif within 5-HT₂Rs is critical for canonical signaling functionality of the receptor, and as such, β-arrestin is a regulator of receptor trafficking and signaling outcomes [181, 203, 205].

The focus of this study was to determine the signaling networks responsible for induction of JCPyV internalization through 5-HT₂Rs and β-arrestin. We have identified a cellular kinase and β-arrestin interacting domains within the 5-HT₂Rs responsible for promoting JCPyV internalization and infection. Collectively, the results presented herein further define JCPyV entry strategies, as well as, provide improved insight into JCPyV utilization of 5-HT₂Rs as functional entry receptors for viral infection and pathogenesis.

3.3. Materials and Methods

3.3.1. Cells, Viruses, Antibodies, Plasmids, Reagents, and siRNAs

SVG-A cells [317] were cultured in complete minimum essential medium (MEM) (Corning) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) (Mediatech, Inc.) and 0.2% Plasmocin prophylactic (Invivogen). HEK293A cells were maintained using Dulbecco’s modified Eagle medium (DMEM) (Corning) containing 10% FBS, 1% P/S, and 0.2% Plasmocin prophylactic. HEK293A cells stably expressing 5-HT₂R subtypes 2A, 2B, and 2C [194] (NCBI accession numbers 3356, 3357, 3358, respectively) in fusion with YFP were cultured in complete DMEM additionally containing 1% G418 (Corning). All cell types were grown in a humidified incubator at 37°C with 5% CO₂. Cell lines used for this study were generously provided by the Atwood Laboratory (Brown University), all of which were verified by ATCC.

JCPyV strain Mad1-SVEΔ was generated and produced (provided by the Atwood Laboratory (Brown University)) as described previously [251, 318]. Labeling of JCPyV with Alexa Fluor 647 was previously described [151], and determination of JCPyV titer was performed in SVG-A cells by
fluorescent focus unit (FFU) assay for infectivity. Noninternalized virus was neutralized using JCPyV-neutralizing antiserum (1:10,000; provided by Atwood Laboratory). Antibodies used to detect infectivity by FFU assay include PAB597 (1:10), an anti-JCPyV VP1 monoclonal antibody (mAb) grown from a hybridoma (generously provided by Ed Harlow); PAB962 (1:3), a mAb grown from a hybridoma for specific detection of JCPyV large T antigen (T-Ag, generously provided by the Tevethia Laboratory, Penn State University) [319]; and secondary polyclonal goat anti-mouse and anti-rabbit Alexa Fluor 594 or 647 antibodies (1:1000, Thermo Fisher). Antibodies used for western blot analysis include rabbit GRK5 polyclonal (1:500, Abcam, 64943), mouse GRK2 mAb (1:1000, ThermoFisher, MA5-15840), β-arrestin 1/2 rabbit mAb (1:500, Cell Signaling Technologies, 4674S), GFP rabbit mAb (1:1000, CST, 2956), and mouse and rabbit GAPDH (1:2000, Abcam, 9484, and 9485, respectively). Plasmids used in this study include WT GRK2 (a gift from Robert Lefkowitz, Addgene; RRID:Addgene_14691, [359], and kinase defective GRK2-K220R (a gift from Robert Lefkowitz, Addgene; RRID:Addgene_35403, [360]). Plasmids for mutated 5-HT2B and 5-HT2CRs, as well as accession numbers, are included in Table 3.2. 5-HT2AR plasmids used in this study have been previously described [181]. Plasmids used for transfection of the infectious clones include JCPyV-Puc19, JCPyV strain JC12 DNA, a subclone of Mad1-SVEΔ, subcloned into Puc19 at a BamHI site [361], and SV40-Puc19, SV40 strain 776 DNA subcloned into Puc19 at a EcoRI site (generously provided by the Atwood Laboratory, Brown University). siRNAs used in this study targeted EGFR (irrelevant control, Cell Signaling, 6482), GRK2 (ThermoFisher, AM51331), and GRK5 (ThermoFisher, AM16708). Concentrations of siRNAs used in this study are as described in ‘siRNA treatment’ Materials and Methods section. siRNAs were screened for toxicity through a propidium iodide flow cytometry assay as in [181]. Average knockdown is reported in Table 3.1.
### Site-directed Mutagenesis of 5-HT2 Receptors

Generation of 5-HT2A, 2B, 2C-R-YFP fusion construct plasmids was previously described [194, 319]. Individual amino acids within the ASK motif of 5-HT2A and 5-HT2C were altered by site-directed mutagenesis. 5-HT2A ASK motif mutagenesis was previously described [181]. Primers for each desired mutation were designed using Agilent QuickChange Primer Design software and were HPLC purified by Integrated DNA Technologies (IDT) (Table 1). Individual mutagenesis reactions were designed containing wildtype DNA template (5 ng/reaction), forward and reverse primers (175 ng/reaction), Phusion High-Fidelity DNA Polymerase (2 units/reaction, New England Bio Labs), and 5X Phusion HF Buffer (New England BioLabs), as described (Table 1). All sample mutagenesis reactions were conducted in a BioRad thermocycler (c1000) as follows: samples were denatured at 95°C for 30 sec, followed by 30 cycles of 95°C for 30 sec, annealing for 1 min (varying temperatures listed in Table 1), 72°C for 5 min, and a final extension at 72°C for 7 min. Following mutagenesis, template DNA was digested by incubation with 10 U of Dpn1 enzyme (New England BioLabs) in a 37°C water bath for 1 h. Plasmids were then transformed in DH5α-competent cells (Invitrogen) and sequences were verified by The University of Maine Sequencing Facility, analyzed by MacVector (version 15.5), and purified by Plasmid DNA Maxiprep (Qiagen).

Genbank accession numbers are provided in Table 3.2.

<table>
<thead>
<tr>
<th>Cell type</th>
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<th>Average knockdown</th>
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<tr>
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<td>GRK5</td>
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<td>SVG-A</td>
<td>GRK2/5</td>
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<td>HEK293A-5-HT2A</td>
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<tr>
<td>HEK293A-5-HT2B</td>
<td>GRK2</td>
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<tr>
<td>HEK293A-5-HT2B</td>
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<td>HEK293A-5-HT2C</td>
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<tr>
<td>HEK293A-5-HT2C</td>
<td>GRK5</td>
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</table>

Table 3.1. Average Knockdown of Cellular Proteins Following siRNA.
Table 3.2. Conditions for Site-directed Mutagenesis Used in This Study.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers for mutagenesis of 5-HT₂₅R-YFP</th>
<th>Annealing temp</th>
<th>GenBank Accession Numbers</th>
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<td></td>
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3.3.3. Transfection of Plasmids in HEK293A and SVG-A Cells

For transfection of control GFP, GRK2 wildtype, or GRK2 K220R mutant plasmids, SVG-A cells were plated to 80% confluence in a 24-well plate in 500 µL in MEM only supplemented with 10% FBS (lacking antibiotics). Transfection complexes containing 1 µg DNA:1 µL of Lipofectamine 2000 transfection reagent (Invitrogen)/well for were incubated at RT for 20 min prior to addition to cells. Cells transfected with wildtype GRK2 or GRK2 K220R plasmids were co-transfected with GFP to allow for determination of transfection efficiency (0.5 µg of wildtype GRK2 or GRK2 K220R DNA and 0.5 µg of GFP DNA/well). Cells treated with transfection complexes were incubated at 37°C for 4 h. Transfection media was removed and cells were fed with complete media and incubated at 37°C for 20 h. Transfection efficiency was determined through GFP expression by fluorescence microscopy, determined to be at
least 70%, and cells were infected as indicated. For expression of 5-HT2A, 2B, 2CR-YFP wildtype and mutated plasmids, HEK293A cells were seeded to 80% confluence in 500 µL/well (24-well plate) or in 100 µL/well (96-well #1.5 glass-bottom plates (CellVis)). All cells were plated in DMEM supplemented with 10% FBS (lacking antibiotics). Transfection complexes were performed as described above with a final concentration of 1 µg DNA/well (24-well plate) and 0.16 µg DNA/well (96-well plate). Transfection efficiency was determined by fluorescence microscopy for expression of YFP, determined to be at least 70%, and cells were fixed or infected as indicated.

3.3.4. Cell-surface Expression of 5-HT2B and 5-HT2C Wildtype and Mutated Receptors

Cell-surface expression was performed as previously described [181]. Briefly, following transfection of wildtype and mutated 5-HT2BR- and 5-HT2CR-YFP plasmids in HEK293A cells in #1.5 96-well glass bottom plates, cells were incubated for 24 h and fixed in 4% PFA at RT for 10 min. After fixation, cells were washed with 1X PBS 3 times and incubated in blocking reagent (1X PBS containing 2% goat serum, 0.2% Triton X-100, and 0.1% BSA) at RT for 1 h. Cells were then stained with an anti-pan-Cadherin antibody (1:200, Abcam, ab6528) in blocking reagent at 4°C rocking O/N. Cells were washed with 1X PBS and subsequently stained with secondary Alexa Fluor 647 antibody (1:1,000) in blocking reagent at RT for 1 h. Cells were washed with 1X PBS and nuclei were stained with DAPI (1:1,000) at RT for 10 min prior to imaging by confocal microscopy. Samples were imaged utilizing an Olympus laser scanning confocal microscope (model IX81) at 60X magnification (oil immersion) and Flouview software (version 04.01.01.05). Fields of view were defined using DAPI staining, and then viewed via z sectioning for background-corrected samples within an applied threshold, as previously described [181]. Images were acquired using 405/635- and 543/488-nm argon laser lines; at least 10 fields of view were acquired for each sample. Cell-surface expression of mutant receptors was quantified using ImageJ by defining the percentage of overlap between the pan-Cadherin cell-surface marker and YFP expression of transfected receptors. Colocalization analysis in ImageJ was used to determine the Mander’s overlap
3.3.5. JCPyV and SV40 Infection

Following siRNA treatment, chemical inhibitor treatment, or transfections, SVG-A or HEK293A-5-HT2R cells were infected with JCPyV or SV40 at the MOIs indicated in figure legends. Virus inoculum was prepared using either MEM (SVG-A) or DMEM (HEK293A), containing 2% FBS and 1% P/S, and 200 µL (24-well plates) or 300 µL (12-well plates) was added per well. Cells were incubated in a 37°C humidified incubator containing 5% CO2 for 1 h, complete medium was added to each well, and cells were further incubated for 48 h (T Ag, HEK293A-5-HT2R cells) or 72 h (VP1, SVG-A cells). Cells were washed with 1X PBS and fixed with 4% paraformaldehyde (PFA) at RT for 10 min followed by staining for indirect immunofluorescence. For infectivity staining, cells were washed with 1X PBS and permeabilized with 1% Triton X-100-PBS (ThermoFisher) at RT for 15 min. Cells were incubated with mAb PAB962 (T Ag) or PAB597 (VP1) to detect T Ag or VP1 protein in SVG-A or HEK293A-5-HT2R cells at 48 or 72 hpi, respectively [362], at 37°C for 1 h. Following incubation, cells were washed with 1X PBS, three times, followed by incubation with polyclonal secondary antibody goat anti-mouse Alexa Fluor 488 or 594 antibodies. Infection was determined through quantifying the number of VP1-positive (VP1+) or T Ag-positive (T-Ag+) cells per 10X visual field divided by number of DAPI-positive cells within the field (percent infection). The number of DAPI-positive cells per field was determined using a binary code as previously described [181, 321], using Nikon NIS-Elements Basic Research software (version 4.50.00, 64 bit). The average percent infection was then normalized to that of the control as indicated.
3.3.6. Viral Attachment by Flow Cytometry

Following siRNA treatment or transfection of wildtype or mutated receptors, SVG-A or HEK293A cells were removed from plates using CellStripper (Corning) and centrifuged at 414 x g at 4°C for 5 min. Cells were then washed with 1X PBS, centrifuged, and resuspended in cell-type specific phenol-free MEM or DMEM (Corning) containing 10% FBS. Cells were prechilled on ice for 45 min. Chilled cells were pelleted by centrifugation and resuspended in 500 µL of either complete phenol-free MEM or DMEM containing Alexa Fluor 647 labelled JCPyV (JCPyV-647; MOI indicated in figure legend) and incubated on ice for 1 h for viral attachment. Cells were centrifuged, washed with 1X PBS, and fixed in 4% PFA for 10 min prior to resuspension in 300 µL of 1X PBS. Cells were then analyzed by flow cytometry for viral attachment using a BD FACSCanto system (BD Biosciences), equipped with 488 and 633 AP-C laser lines (Becton, Dickinson, and Company) for at least 20,000 events followed by analysis with BD FACSDiva (Becton, Dickinson, and Company) and FlowJo (TreeStar, Inc.) software. Gating was performed to exclude complex and dead cells using FlowJo software.

3.3.7. Viral Entry by Confocal Microscopy

Cells were plated to 50% confluence in #1.5 96-well glass bottom plate (CellVis) prior to siRNA treatment or transfection with wildtype or mutated plasmids. After transfection, cells were prechilled on ice for 45 min then incubated with JCPyV-647 (MOIs indicated in figure legends) on ice for 1 h for viral attachment, plates were then shifted to a 37°C humidified incubator with 5% CO₂ for 2 h for viral entry. Following entry cells were washed with 1X PBS and fixed in 4% PFA for 10 min at RT. Cells were washed with 1X PBS, then subsequently incubated in blocking reagent (1X PBS containing 2% goat serum, 0.2% Triton X-100, and 0.1% BSA) at RT for 1 h while rocking. Cells were then incubated with a primary anti-pan-Cadherin antibody (1:200) in blocking reagent, at 4°C overnight. Cells were washed with 1X PBS and incubated with secondary Alexa Fluor 488 or 647 antibody (1:1,000) in blocking reagent at RT for 1 h. Cells were washed with 1X PBS then incubated with DAPI (1:1,000) for 10 min in 1X PBS for
nuclear visualization. Samples were visualized by confocal microscopy utilizing an Olympus laser scanning confocal microscope (model IX81) at 60X magnification (oil immersion) and Fluoview software (version 04.01.01.05). Fields of view were defined using DAPI staining, and Fluoview software single-measurement analysis was used to define regions of interest (ROI) using z-sectioning excluding the plasma membrane (using either pan-Cadherin or DIC overlay) to measure relative internalized virus by relative fluorescence units per cell for background-corrected samples within an applied threshold, as previously described [181]. Images were acquired using 405/635- and 543/488-nm argon laser lines. Cross sections of individual cells were analyzed (at least 30 cells per sample) for siRNA treated samples (SVG-A cells) or cells expressing wildtype or mutated receptors (HEK293A cells). Microscopy experiments were performed independently three times in triplicate, containing at least 30 cells per sample per replicate; graphs are comprised of three independent replicates (N = 90).

3.3.8. siRNA Treatment

SVG-A or 5-HT₂R stably-expressing HEK293A cells were seeded to 50% confluence in 12-well plates. siRNA transfections targeting either EGFR (irrelevant control), GRK2, or GRK5 were performed using RNAiMax (ThermoFisher) per the manufacturer’s instructions (7.5 pmol/well). Following siRNA transfection, cells were incubated in a humidified incubator at 37°C with 5% CO₂ for 72 h. Efficiency of siRNA transfection was determined using Block-iT Alexa Fluor red oligonucleotide control (Life Technologies) at 48 h following transfection. Following incubation with siRNAs cells were either processed for western blot analysis, infected with JCPyV or SV40, (MOIs indicated in figure legends), or were transfected with the infectious clones of JCPyV or SV40.

3.3.9. siRNA Western Blot Analysis

To determine the efficiency of knockdown by siRNA, western blot analysis was performed as described in [181]. In brief, SVG-A or HEK293A-5-HT₂R cells were washed with 1X PBS, and subsequently
removed from wells with a cell scraper. Cells were pelleted by centrifugation at 414 × g at 4°C for 5 min and pellets were resuspended in Tris-HCl lysis buffer (1mM EDTA, 50mM Tris-HCl, 120mM NaCl) containing protease inhibitors (1:10; Sigma-Aldrich) and phosphatase inhibitors (1:100; Sigma-Aldrich), and incubated on ice for 10 min. Cellular pellets were then sonicated at 35% amplitude, on ice, for 10 s, repeated three times. Insoluble cellular material was then pelleted at 21,130 × g at 4°C for 10 min. Cellular protein-containing lysates were mixed 1:1 with Laemmli sample buffer (Bio-Rad), boiled at 95°C for 5 min, and processed by SDS-PAGE with a 10% TGX minigel (Bio-Rad). Resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad) using a semidry transblot apparatus (Bio-Rad) at 2.5 amps (25 V) for 3 min. Membranes were equilibrated in 1X TBS for 5 min, blocked with Odyssey blocking buffer (LI-COR) at RT for 1.5 h, then washed with 1X TBS-T for 5 min, three times. Membranes were incubated with primary antibodies targeting either GRK2 (1:1000) or GRK5 (1:500) and GAPDH (housekeeping; 1:2000) in Odyssey blocking buffer at 4°C overnight, rocking. Following extensive washing with 1X TBS-T, membranes were incubated with secondary antibodies (LI-COR) in Odyssey blocking buffer at RT, for 1 h while rocking, then washed. Imaging was performed using a LI-COR Odyssey CLx system. siRNA-induced knockdown of protein expression was determined by defining the relative fluorescence for each housekeeping control protein band and target protein band using LI-COR ImageStudio software (version 5.2). Target protein bands were normalized to the housekeeping control, and reduction in protein expression was determined in comparison to the control-siRNA control treated samples. Knockdown by siRNA was determined to be at least 70% for all experiments reported or shown in Table 3.2.

3.3.10. Transfection of JCPyV and SV40 Infectious Clones

SVG-A cells were plated in 24-well plates to 50% confluence followed by transfection of either EGFR (CTL), GRK2, or GRK5 siRNA using RNAiMax (ThermoFisher) per the manufacturer’s instructions (3.75 pmol/well). Following siRNA transfection, cells were incubated in a humidified incubator at 37°C
with 5% CO₂ for 72 h. Linearization of viral plasmids was accomplished through digestion with BamHI (JCPyV-Puc19, Promega) or EcoRI (SV40-Puc19, Promega) at 37°C for 2 h, successful cut of plasmids was determined by agarose gel electrophoresis. Cells were then transfected with 1 µg of DNA containing the linearized plasmids of either JCPyV-Puc19 or SV40-Puc19 DNA using Fugene (Roche) at a ratio of 1.5 µL Fugene:1 µg of DNA prior to incubation at 37°C. 24 h posttransfection cells were fed with 500 µL of complete medium containing antibiotics, additionally supplemented with 1% amphotericin B (Corning). Cells were fixed and stained for newly synthesized VP1 at either 4 or 7 days posttransfection.

3.3.11. 5-HT₂R Pulldown in HEK293A-5-HT₂R Cells

5-HT₂R pulldown assays were adapted from [203]. Briefly, HEK293A or HEK293A-5-HT₂R cells were plated to 90% confluence in 6-well plates in DMEM containing 10% FBS (no antibiotics). Cells were then either transfected with siRNAs for 72 h (HEK239A-5-HT₂R) or WT or mutated 5-HT₂AR, 5-HT₂BR, or 5-HT₂CR plasmids (HEK293A, 10 µg DNA/well/sample) at 37°C for 4 h. At 4 h posttransfection media was replaced with incomplete DMEM (no FBS or antibiotics) for serum starvation O/N (transfected samples) or for 4 h (siRNA-treated samples). Cells were then prechilled at 4°C for 45 min followed by incubation with either 5-HT (200 µM/well) or JCPyV (MOI indicated in figure legend) at 4°C for 1 h for attachment. Cells were spiked with warm DMEM (10% FBS) and shifted to 37°C incubator for 15 min prior to removal from plates by scraping. Cells were pelleted by centrifugation at 414 x g at 4°C for 5 min and resuspended in 200 µL lysis buffer (10mM Tris HCL/150mM NaCl/0.5mM EDTA/0.5% Triton X-100, pH 7.5) additionally supplemented with phosphatase (1:100, Sigma-Aldrich) and protease (1:10, Sigma-Aldrich) inhibitors for 30 min with vigorous pipetting every 10 min. Samples were pelleted by centrifugation at 21,130 x g at 4°C for 10 min, and 50 µL of supernatant was mixed 1:1 with Laemmli sample buffer (Bio-Rad) and boiled at 95°C for 5 min (input). GFP-Trap magnetic agarose beads (25 µL/sample) were washed 3 times with wash buffer (10mM Tris HCL/150mM NaCl/0.5mM EDTA, pH 7.5) and separated using a magnetic tube rack until clear per manufacturer instructions (CST). Remaining
supernatant was mixed with beads and incubated at RT for 2 h rotating. Beads were pelleted using a magnetic tube rack and 75 µL of supernatant was mixed 1:1 with Laemmli sample buffer (Bio-Rad) and boiled at 95°C for 5 min (non-bound). Beads were washed three times with wash buffer, transferred to a new sterile microcentrifuge tube in 50 µL of wash buffer, mixed 1:1 with Laemmli sample buffer (Bio-Rad) and boiled at 95°C for 10 min. After magnetic separation, supernatant was transferred to a new microcentrifuge tube (bound). Negative control beads (magnetic agarose beads lacking a pre-conjugated antibody) were also used to detect non-specific bead interactions (not shown). Half of the bound samples were processed by SDS-PAGE using a 10% TGX mini gel (Bio-Rad). Resolved proteins were transferred to a nitrocellulose membrane (Bio-Rad) using a semidry transblot apparatus (Bio-Rad) at 2.5 amps (25 V) for 3 min. Membranes were then equilibrated in 1X TBS at RT for 5 min and blocked with Odyssey blocking buffer (LI-COR) at RT for 1.5 h. Membranes were washed with 1X TBS-T three times for 5 min and incubated with a primary monoclonal antibody targeting β-arrestin 1/2 (1:500, CST, 4674), diluted in blocking buffer, at 4°C O/N, rocking. Membranes were washed and imaged on a LI-COR Odyssey CLx system, stripped using Restore™ western blot stripping buffer according to manufacturer’s instructions (Bio-Rad), and re-probed with a primary monoclonal anti-GFP antibody (1:1000, CST, 2956) for 5-HT2R detection. The percentage of bound β-arrestin was determined by defining the relative fluorescence for each β-arrestin protein band and corresponding receptor protein band using LI-COR ImageStudio software (version 5.2). β-arrestin bands were normalized to the receptor expression within each sample, and the percentage of bound β-arrestin was determined in comparison to the WT- or CTL-siRNA transfected samples treated with 5-HT.

3.3.12. Statistical Analysis

All experiments were performed in triplicate with three independent replicates. Microscopy experiments were performed independently three times, in triplicate, containing at least 30 cells per sample per replicate; graphs are comprised of three independent replicates (N = 90). A two-sample
student’s T test was used to determine statistical significance assuming unequal variance, comparing the mean values of triplicate samples collected, performed in Microsoft Excel. Additionally, the normality of distribution of data was determined using the Shapiro-Wilk’s normality test and a Q-Q plot (quantile-quantile) in RStudio (version 1.2.1335, 2019, Integrated development of RStudio, Inc.). A pairwise Wilcoxon Rank Sum Test was used for comparing data populations that were not normally distributed (microscopy experiments containing samples of least 90 cells). For these data, statistical analysis was performed in RStudio, and plots were generated using ggplot2, plotting the raw values that had been imported from Excel into RStudio using library XLConnect. Significance was determined by a $P$ value < 0.05.

3.4. Results

3.4.1. The ASK Motifs of 5-HT$_{2B}$R and 5-HT$_{2C}$R are Required for JCPyV Infection

β-arrestin and the Ser residue of the β-arrestin-binding ASK motif within 5-HT$_{2A}$R are necessary for JCPyV entry and infection [181]. 5-HT$_{2B}$R and 5-HT$_{2C}$R also support JCPyV entry and infection, and the β-arrestin-binding ASK motif is conserved in 5-HT$_{2}$Rs [181]. Interactions between β-arrestin and the Ser residue within the motif of 5-HT$_{2A}$R ultimately regulate receptor internalization and subsequent trafficking [203]. Thus, investigation of the ASK motif in the 5-HT$_{2B}$R and 5-HT$_{2C}$R is necessary to define the mechanism of 5-HT$_{3}$R-mediated entry of JCPyV. To determine whether the ASK motif is required for JCPyV utilization of 5-HT$_{2B}$R and 5-HT$_{2C}$R for infection, site directed mutagenesis was employed, engineering single or double amino acid mutations within the ASK motif of either receptor in fusion with YFP. Through mutagenesis, the Ser and Lys residues of 5-HT$_{2B}$R and 5-HT$_{2C}$R were replaced with Ala at residue positions 323 and 324 (5-HT$_{2B}$R) and positions 310 and 311 (5-HT$_{2C}$R) (depicted in Fig. 3.1).
Figure 3.1. Schematic of conserved ASK motifs in 5-HT₃Rs.

Schematic demonstrating the conserved β-arrestin-binding Ala-Ser-Lys (ASK) motif positions within 5-HT₃Rs. The ASK motif of 5-HT₃A is located within the C-terminus of the GPCR, at amino acid positions 456-458. The ASK motifs of 5-HT₂BR and 5-HT₂CR are within the third intracellular loop of the GPCR, at amino acid positions 322-324, and 309-311, respectively.

To determine the expression of the mutated receptors at the cell surface in comparison to wildtype (WT), cell-surface expression was measured by confocal microscopy. The surface of the cell was defined by staining with an antibody (Ab) that detects cell-surface proteins cadherins [181]. Individual fields of view were measured and the percentage of cell-surface expression was determined for mutated 5-HT₂BR and 5-HT₂CR in comparison to their respective WT, unmutated, receptors (Fig. 3.2A, 3.2B). Mutagenesis of the Ser or Lys residues in either receptor did not significantly alter cell-surface expression in comparison to the WT receptors (Fig. 3.2A, 3.2B).
Figure 3.2. Cell surface expression of mutated 5-HT$_{2A}$R and 5-HT$_{2C}$R.

HEK293A cells were transfected with plasmids containing wildtype 5-HT$_{2A}$R-YFP or 5-HT$_{2C}$R-YFP (WT), or 5-HT$_{2A}$R-YFP or 5-HT$_{2C}$R-YFP with amino acid point mutations within the ASK motif, or empty vector YFP. (A, B) Receptor cell-surface expression of WT or mutated receptors was measured by confocal microscopy at 60X magnification (top). Cell surface expression was determined using ImageJ software for colocalization between expressed receptor in YFP (green) and a cell surface marker (pan-Cadherin-647, pseudocolored orange) using Mander’s coefficient. Data are represented as a box-and-whisker plot denoting the percentage of receptor that is cell-surface expressed/visual field for 10 fields of view (bottom). Outliers are indicated in grey. Samples were measured in triplicate. Upper and lower whiskers represent 1.5 times the inter-quartile range. NS = no significance. *, P < 0.05.

WT and mutated receptors were expressed in HEK293A cells and subsequently infected with JCPyV, and viral infectivity was determined through the quantitation of nuclear T antigen (T-Ag) expression by indirect immunofluorescence (Fig. 3.3A). 5-HT$_{2A}$R-S323A (AAK) and 5-HT$_{2A}$R-SK323-24A (AAA) demonstrated approximately 80% reductions in JCPyV infectivity in comparison to 5-HT$_{2A}$R-K324A (ASA), which demonstrated infectivity levels comparable to 5-HT$_{2A}$R (Fig. 3.3A). Interestingly, 5-HT$_{2C}$R-S310A (AAK) and 5-HT$_{2C}$R-SK310-11AA (AAA) also demonstrated significant reductions of nearly 80% in infection in comparison to 5-HT$_{2C}$R-K311A (ASA) and 5-HT$_{2C}$R (Fig. 3.3B). Collectively, these results
suggest that the β-arrestin-binding ASK motif, conserved in all three subtypes of 5-HT$_2$Rs, is necessary for JCPyV infectivity, and that specifically, the Ser residue within the ASK motif is important for infection across the 5-HT$_2$R subtypes.

Figure 3.3. β-arrestin binding ASK motif within 5-HT$_2$BR and 5-HT$_2$CR is required for JCPyV infection.

(A, B) Transiently transfected cells were infected with JCPyV (MOI = 1 FFU/cell) and fixed at 48 hpi. Cells were stained using a JCPyV-specific T-Ag antibody and infectivity was measured by indirect immunofluorescence. Data represent the percentage of infected cells/visual field normalized to the total number of DAPI+ cells/visual field for five 10X fields of view for triplicate samples and were independently repeated three times. Samples were normalized to the WT receptor within each receptor subtype (100%). A two-sample student’s T test was used to determine statistical significance. NS = no significance. Error bars = SD. *, $P < 0.05$.

3.4.2. The Serine Residue of the 5-HT$_2$AR and 5-HT$_2$CR ASK Motif is Critical for JCPyV Internalization

JCPyV requires the Ser residue within the ASK motif of 5-HT$_2$AR for internalization and productive infection [181], and the ASK motifs of 5-HT$_2$BR and 5-HT$_2$CR are critical for JCPyV infection (Fig. 3.3). Thus, the impact of mutagenesis of the ASK motifs within 5-HT$_2$BR and 5-HT$_2$CR during JCPyV attachment and entry was explored. HEK293A cells were transfected with WT and mutated 5-HT$_2$BR and 5-HT$_2$CRs, each in fusion with YFP. The transiently transfected cells were incubated with JCPyV labeled with Alexa Fluor 647 (JCPyV-647) on ice for viral attachment. The cells were fixed and viral attachment was measured by flow cytometry. The mean fluorescence intensity was comparable in cells expressing the mutated ASK
motifs in comparison to the unmutated 5-HT$_{2a}$R or 5-HT$_{2c}$R, suggesting that mutagenesis of the Ser or Lys residues did not impact viral attachment (Fig. 3.4A, 3.4B).

**Figure 3.4. Attachment of JCPyV in kidney cells expressing mutated 5-HT$_{2a}$R or 5-HT$_{2c}$R.**

**(A, B)** HEK293A cells were transfected with plasmids containing wildtype 5-HT$_{2a}$R-YFP or 5-HT$_{2c}$R-YFP (WT), or 5-HT$_{2a}$R-YFP or 5-HT$_{2c}$R-YFP with amino acid point mutations within the ASK motif, or empty vector YFP. Cells were removed from plates, prechilled on ice, and subsequently incubated with JCPyV-647 (MOI = 5 FFU/cell) on ice for 1 h for viral attachment. Cells were analyzed by flow cytometry. Histograms represent the mean fluorescence intensities for cells without treatment (grey), empty YFP vector (no virus, black), or cells treated with JCPyV-647. Data are representative of 20,000 events for three independent experiments and were performed in triplicate.

To measure viral entry, HEK293A cells expressing WT and mutated 5-HT$_{2}$Rs were incubated with JCPyV-647 on ice for synchronized viral attachment, then cells were shifted to a 37°C incubator for 2 h for viral internalization prior to fixation. Viral entry was measured by confocal microscopy and single cell quantitative analysis (Fig. 3.5A, 3.5B) [181]. The relative fluorescence intensity for internalized JCPyV-647 was measured by drawing individual regions of interest excluding the plasma membrane, defined by DIC overlay, utilizing Olympus Fluoview software [181]. Mutagenesis of either 5-HT$_{2a}$R or 5-HT$_{2c}$R with an altered Ser residue (AAK, AAA) resulted in reductions in JCPyV internalization by approximately 75% while mutation of the Lys residue alone (ASA) was not significantly different in comparison to WT receptors (Fig. 3.5A, 3.5B). Together, this highlights the importance of the conserved 5-HT$_{2}$R ASK motif and specifically the Ser residue within the motif for internalization of JCPyV, as disruption of this residue significantly diminishes viral entry.
Figure 3.5. Mutagenesis of 5-HT₂aR or 5-HT₂cR ASK motif reduces JCPyV entry.

(A, B) To measure JCPyV entry, cells were prechilled, and then incubated with JCPyV-647 (MOI = 5 FFU/cell, pseudocolored magenta) on ice for 1 h for viral attachment. Cells were then shifted to 37°C for 2 h for viral entry prior to fixation. Cell nuclei were stained with DAPI (blue). Viral entry was analyzed by confocal microscopy at 60X magnification (top). Fluoview software single measurement analysis was used to define regions of interest (ROI) using z-sectioning excluding the plasma membrane to measure internalized virus per cell. JCPyV internalization was defined as the relative fluorescence per cell for at least 30 cells in triplicate per sample for (WT), 5-HT₂aR-YFP, or 5-HT₂cR-YFP with amino acid point mutations within the ASK motif (green) using Olympus Fluoview 10-ASW software. Data are depicted as a raincloud plot where the height and width of each raincloud represents the distribution of each sample (bottom). Individual rain points (black) denote the percentage of virus internalized for each cell measured. Samples were normalized to the average of WT (100%). Microscopy experiments were performed independently three times, and at least 30 cells in triplicate samples per replicate were analyzed; graphs are comprised of three independent replicates (n = 90). Statistical significance was determined using a pairwise Wilcoxon Rank Sum Test. Scale bars = 20 µm. NS = no significance. *, P < 0.05.
3.4.3. Mutagenesis of the ASK Motif in 5-HT2Rs Reduces β-arrestin-receptor Interactions

The Ser residue of the conserved ASK motifs of 5-HT2Rs are required for JCPyV infection and entry (Fig. 3.1 and 3.2, [181]). Further, the Ser residue of the ASK motif of 5-HT2A receptors has been implicated as a direct β-arrestin-binding domain when 5-HT induces canonical signaling events or pathways [203]. To determine if JCPyV induces β-arrestin interactions with 5-HT2Rs during entry and whether mutagenesis of the ASK motif reduces this interaction, biochemical pulldown assays were employed. HEK293A cells were transfected with WT or mutated receptors, 5-HT2A-R-YFP, 5-HT2B-R-YFP, or 5-HT2C-R-YFP, in which the ASK motif was mutated to AAK or ASA, as this mutation did not alter JCPyV entry or infection (Fig. 3.3, 3.5). Cells were incubated with either 5-HT, JCPyV, or were not treated at 4°C for 1 h for attachment and then shifted to 37°C for 15 min for entry, subsequently harvested, and lysed. Magnetic agarose beads pre-conjugated to a GFP antibody for detection of YFP were incubated with the cellular lysates for immunoprecipitation of 5-HT2R-YFPs and direct-interacting proteins. Samples containing the bound fraction of proteins were resolved by SDS-PAGE and analyzed through western blotting using anti-GFP and anti-β-arrestin antibodies. Quantitation of the percentage of bound β-arrestin relative to 5-HT2R-YFP was determined through LI-COR ImageStudio analysis. Briefly, the borders of each protein band were defined by ROI, resulting in determination of the intensity of the band, β-arrestin band intensity was then internally normalized to the 5-HT2R band intensity to normalize for protein loaded/well. (Fig. 3.6A). Incubation of WT-expressing cells with either 5-HT or JCPyV resulted in increased β-arrestin binding, in comparison to cells that did not receive agonist treatment (Fig. 3.6B). Further, AAK-expressing HEK293A cells treated with either 5-HT or JCPyV demonstrated reduced β-arrestin binding at 15 min posttreatment compared to WT controls, and β-arrestin binding in ASA-expressing cells was similar to WT (Fig. 3.6B). Mutagenesis of the Ser residue in the ASK motif of the 5-HT2Rs (AAK) reduced β-arrestin binding for JCPyV-treated samples by 60% (5-HT2A-R), 60% (5-HT2B-R), and 70% (5-HT2C-R) in comparison to the WT receptors (Fig. 3.6B). Together these results suggest that upon
stimulation of 5-HT$_2$Rs by either 5-HT or JCPyV, $\beta$-arrestin localizes with the receptor, resulting in direct interactions between $\beta$-arrestin and the receptor. Further, these findings also demonstrate that the Ser residue of the ASK motif is critical for conferring $\beta$-arrestin interactions with 5-HT$_2$Rs when receptors are activated by either 5-HT or JCPyV.

Figure 3.6. Mutagenesis of ASK motif of 5-HT$_2$R reduces $\beta$-arrestin-receptor interactions during JCPyV internalization.
Figure 3.6. (Continued)

HEK293A cells were transfected with unmutated 5-HT2A-R-YFP, or 5-HT2B-R-YFP, or 5-HT2C-R-YFP (WT), or receptors containing a point mutation within the ASK motif (AAK, ASA). (A) Cells were incubated with JCPyV (MOI = 12 FFU/cell), or serotonin (5-HT, 200 mM/well), or received no treatment (NT), at 4°C for 1 h (attachment) and then shifted to 37°C for 15 min prior to lysis (whole cell lysate, WCL). Lysed cells were incubated with magnetic agarose beads conjugated to a GFP antibody for immunoprecipitation (IP) of YFP-conjugated 5-HT2Rs. Bound samples for WT, AAK, and ASA receptors were processed by SDS-PAGE and immunoblotted (IB) for β-arrestin and GFP (corresponding 5-HT2R). WCL samples were stained for total protein. (B) The percentage of β-arrestin bound to 5-HT2R was determined using LI-COR ImageStudio software. Data represent the quantitation of the relative amounts of β-arrestin that are bound to 5-HT2Rs at 15 min post-internalization, normalized to the 5-HT WT (100%), and contain data from individual replicates performed in three independent experiments. Error bars = SDs. A two-sample student’s T test was used to determine statistical significance.*, P < 0.05.

3.4.4. GRKs are Required for JCPyV Infection

Prior to recruitment of β-arrestin for endocytosis, 5-HT2A-R is phosphorylated by GRK2 [203, 209]. Further, 5-HT2A-R internalization by β-arrestin has been reported to be both GRK2-dependent and GRK2/GRK5-independent [203, 206, 327]. The involvement of either GRK2 or GRK5 is dependent on cell type and agonist [206], and ultimately determines receptor internalization and downstream signaling cascades [203, 209]. To determine whether GRK2 and/or GRK5 are required for JCPyV infection of glial cells, SVG-A cells were transfected with siRNAs targeting an irrelevant control (CTL) [181], GRK2, or GRK5. Cells were then infected with either JCPyV or SV40 and scored for infectivity by indirect immunofluorescence of newly synthesized VP1 or processed for western blot analysis to determine efficiency of protein knockdown (Fig. 3.7). SV40 infection of SVG-A cells occurs independently of cellular proteins involved in clathrin-mediated endocytosis, including β-arrestin [181, 239]. Further, mutation of the ASK motif in the 5-HT2A-R had no impact on SV40 infection [181]; however, the necessity of GRKs has not been studied for this closely related polyomavirus. Interestingly, treatment of SVG-A cells with siRNA targeting GRK2 or GRK5 reduced JCPyV infection, by approximately 70% and 40%, respectively. (Fig. 3.7). In contrast, infection of SVG-A cells by SV40 was not hindered by knockdown of GRK2 while siRNA
targeting GRK5 resulted in reduced SV40 infection, by approximately 45% (Fig. 3.7). Combinatory siRNA knockdown of both GRK2 and GRK5 further reduced JCPyV infectivity beyond that seen for GRK2 alone, by approximately 7%, while SV40 infection was not further reduced (Fig. 3.7).

Figure 3.7. GRKs 2 and 5 are necessary for JCPyV infection of glial cells, while SV40 only requires GRK5.

SVG-A cells were transfected with siRNA targeting an irrelevant control (CTL), GRK2, or GRK5, and were either processed for protein knockdown by western blot analysis (below) or infected with either JCPyV (MOI = 1 FFU/cell) or SV40 (MOI = 0.001 FFU/cell) and fixed at 72 hpi. Noninternalized virus was neutralized using JCPyV-neutralizing antiserum (1:10,000). Cells were stained with an antibody specific for VP1 and infectivity was measured by indirect immunofluorescence. Data represent the percentage of infected cells/visual field normalized to the number of DAPI+ cells/visual field for 5, 10X fields of view for triplicate samples and were independently repeated three times. Samples were normalized to the control siRNA for each cell type (100%). Error bars = SD. *, P < 0.05.

To determine whether the reliance on GRKs is required for infection of kidney cells and attributed to specific 5-HT2Rs, HEK293A cells stably expressing 5-HT2A, 2B, or 2C Rs were similarly treated with siRNA targeting an irrelevant control (CTL), GRK2, or GRK5 prior to JCPyV challenge. JCPyV infectivity was quantified by T-Ag expression at 48 h postinfection by indirect immunofluorescence. JCPyV infection of HEK293A cells expressing 5-HT2Rs, regardless of subtype, exhibited similar reductions
(~70-80%) in JCPyV infection when GRK2 was knocked down. Infection following GRK5 knockdown was reduced by approximately 40% (Fig. 3.4B).

Figure 3.8. GRK2 and GRK5 are required for JCPyV infection of kidney cells.

HEK293A cells stably expressing the 5-HT₂R subtypes, were transfected with siRNA targeting an irrelevant control (CTL), GRK2, or GRK5, and were either processed for protein knockdown by western blot analysis (right) or infected with JCPyV (MOI = 1 FFU/cell) and fixed at 48 hpi. Noninternalized virus was neutralized using JCPyV-neutralizing antiserum (1:10,000). Cells were stained with an antibody specific for T-Ag and infectivity was measured by indirect immunofluorescence. Data represent the percentage of infected cells/visual field normalized to the number of DAPI+ cells/visual field for 5, 10X fields of view for triplicate samples and were independently repeated three times. Samples were normalized to the control siRNA for each cell type (100%). Error bars = SD. *, P < 0.05.

In addition, to determine if a functioning GRK2 kinase domain is important for either JCPyV or SV40 infection, SVG-A cells were transiently transfected with GFP (CTL), wildtype GRK2 and GFP (WT), or kinase deficient GRK2 (K220R) and GFP [203] prior to viral challenge. Expression of kinase deficient K220R significantly hindered JCPyV infection in comparison to control GFP and the overexpressed WT GRK2 by nearly 50% (Fig. 3.9), while SV40 infection was not reduced (Fig. 3.9). Interestingly, overexpression of WT GRK2 did not enhance JCPyV infection beyond that of the GFP control (Fig. 3.9). Collectively, these results suggest that JCPyV requires the kinase activity of GRK2 for infection of either glial or kidney cell types, regardless of 5-HT₂R subtype used. Further, these results suggest that both
JCPyV and SV40 infection of glial cells requires GRK5, and due to the disparate mechanisms in JCPyV and SV40 entry, this is perhaps through a conserved post-entry polyomavirus effect.

Figure 3.9. Kinase function of GRK2 is required for JCPyV infection.

SVG-A cells were transfected with plasmids for GFP (CTL), or wildtype GRK2 and GFP (WT), or a kinase deficient GRK2 containing a K220R point mutation and GFP (K220R). Cells were infected with either JCPyV (MOI = 1 FFU/cell) or SV40 (MOI = 0.001 FFU/cell) and fixed at 72 hpi. Cells were stained with an antibody specific for VP1 and infectivity was measured by indirect immunofluorescence. Data represent the percentage of infected cells/visual field normalized to the number of DAPI+ cells/visual field for 5, 10X fields of view for triplicate samples and were independently repeated three times. Samples were normalized to the CTL sample (100%). A two-sample student’s T test was used to determine statistical significance. NS = no significance. Error bars = SD. *, P < 0.05.

3.4.5. GRK2, but not GRK5, is Required for JCPyV Internalization

Internalization of 5-HT2A R involves the recruitment of GRKs resulting in phosphorylation of 5-HT2AR and leads to the recruitment of β-arrestin and subsequent internalization of the receptor [203]. Inhibition of GRK2 and GRK5 reduced JCPyV infectivity (Fig. 3.7, 3.8, and 3.9), thus necessity of these kinases in promoting either JCPyV attachment or internalization warranted further characterization. To determine whether reduction of GRK2 or GRK5 impacted JCPyV attachment or entry, SVG-A cells were transfected with siRNA targeting an irrelevant control (CTL), GRK2, or GRK5 and then incubated with JCPyV-647 on ice, fixed, and viral attachment was measured by flow cytometry. The mean fluorescence
intensity of virus attached to cells treated with GRK2 or GRK5 siRNA was equivalent to that of the CTL-siRNA samples (Fig. 3.10).

**Figure 3.10. Knockdown of either GRK2 or GRK5 does not impact JCPyV attachment.**

SVG-A cells were transfected with siRNA targeting either an irrelevant control (CTL), GRK2, or GRK5. Cells were stripped from plates, prechilled on ice and then incubated with JCPyV-647 (MOI = 5 FFU/cell) on ice for 1 h, and then analyzed by flow cytometry for viral attachment. Histograms represent the mean fluorescence intensities for cells transfected with either CTL siRNA (blue), GRK2 or GRK5 siRNA (green), or cells alone (no siRNA or virus, shaded grey). Data are representative of 20,000 events for at least three independent experiments.

To determine whether GRK2 or GRK5 are required for JCPyV internalization, SVG-A cells treated with siRNAs were incubated with JCPyV-647 for viral synchronized attachment on ice, then shifted to a 37°C incubator for 2 h for internalization. Cells were fixed and viral entry was measured by confocal microscopy and single cell quantitative analysis. The relative fluorescence intensity for internalized JCPyV-647 was measured by drawing individual regions of interest excluding the plasma membrane, defined by a pan-Cadherin stain, and analyzed using Olympus Fluoview software (Fig. 3.11). In comparison to irrelevant control siRNA-treated samples, JCPyV internalization in cells treated with GRK2 siRNA was reduced by approximately 80% (Fig. 3.11). Interestingly, GRK5 knockdown in SVG-A cells did not impact JCPyV entry (Fig. 3.11). Collectively, these results suggest a requirement for GRK2 in facilitating JCPyV internalization, for which, GRK5 is dispensable.
SVG-A cells were transfected with siRNA targeting either an irrelevant control (CTL), GRK2, or GRK5. To measure JCPyV entry, cells were prechilled, and then incubated with JCPyV-647 (MOI = 5 FFU/cell, pseudocolored magenta) on ice for 1 h for viral attachment. Cells were then shifted to 37°C for 2 h for viral entry prior to fixation. Cell nuclei were stained with DAPI (blue) and the plasma membrane was stained with an anti-pan-Cadherin antibody (pseudocolored green). Viral entry was analyzed by confocal microscopy at 60X magnification (top). Fluoview software single measurement analysis was used to define regions of interest (ROI) using z-sectioning excluding the plasma membrane to measure internalized virus per cell. JCPyV internalization was defined as the relative fluorescence per cell for at least 30 cells per sample for CTL, GRK2, and GRK5 siRNA-treated samples using Olympus Fluoview 10-ASW software. Data are depicted as a raincloud plot where the height and width of each raincloud represents the distribution of each sample (bottom). Individual rain points (black) denote the percentage of virus internalized for each cell measured. Samples were normalized to the average of CTL-treated samples (100%). Microscopy experiments were performed independently three times in triplicate, containing at least 30 cells per sample per replicate; graphs are comprised of three independent replicates (n = 90). Statistical significance was determined using a pairwise Wilcoxon Rank Sum Test. Scale bars = 20 μm. NS = no significance. *, P < 0.05.

3.4.6. GRK5 is Required for JCPyV or SV40 in a Post-trafficking Step

While GRK2 is required for JCPyV infectivity (Fig. 3.7, 3.8, and 3.9) and internalization (Fig. 3.11), GRK5 knockdown reduced infectivity of both JCPyV and SV40 (Fig. 3.7), yet siRNA knockdown of GRK5 did not hinder JCPyV internalization (Fig. 3.11). These data suggest that GRK5 may function to facilitate a step in polyomavirus infection following entry. Thus, the impact of GRKs for the promotion of either JCPyV or SV40 infection was further explored. To determine if either GRK2 or GRK5 are required for
infection postinternalization, SVG-A cells were transfected with siRNA targeting the irrelevant control (CTL), GRK2, or GRK5, and incubated for 72 h. Cells were then transfected with the infectious clone of either JCPyV or SV40, bypassing viral attachment, entry, and trafficking. Cells were fixed at either 4 or 7 days post infection and viral infectivity was determined by indirect immunofluorescence of newly synthesized VP1 [321]. siRNA knockdown of GRK2 did not impact JCPyV infection while knockdown of GRK5 reduced infection by approximately 40% and 55% at days 4 or 7 postinfection, respectively (Fig. 3.12A). Meanwhile, SV40 infection was also not impacted following GRK2 siRNA, though knockdown of GRK5 resulted in reduced SV40 infection at both day 4 and day 7, each by approximately 50% (Fig. 3.12B). Collectively, these results suggest that the requirement of GRK2 for JCPyV infection occurs prior to viral transcription, confirming the effects on viral entry (Fig. 3.11), while GRK5 plays a role in both JCPyV or SV40 infection following localization of these viruses to the nucleus.

![Figure 3.12. Knockdown of GRK5 prevents polyomavirus infection post entry.](image)

(A and B) SVG-A cells were transfected with siRNA targeting either an irrelevant control (CTL), GRK2, or GRK5. Cells were then transfected with an infectious clone of either (A) JCPyV or (B) SV40. Cells were fixed at either 4 or 7 days posttransfection, stained with an antibody specific for VP1, and infectivity was measured by indirect immunofluorescence. Data are representative of the number of infected cells/10x visual field for 5 fields of view per sample in triplicate. Data are representative of results from three independent experiments. Error bars indicate SDs. A two-sample student’s T test was used to determine statistical significance. NS = no significance. *; P < 0.05.
3.4.7. **GRK2 Mediates β-arrestin-5-HT₂R Interactions for Viral Internalization**

GRK2 has been identified to phosphorylate the Ser residue within 5-HT₂R, and this phosphorylation is critical for β-arrestin engagement of the receptor and subsequent internalization and signaling [203]. Further, JCPyV infection of cells expressing 5-HT₂Rs relies on β-arrestin and GRK2 (Fig. 3.7, 3.8, 3.9, [181]) and, the conserved ASK motifs of 5-HT₂A, 5-HT₂B, and 5-HT₂C are critical for JCPyV entry and infection of glial and kidney cell types (Fig. 3.3 and 3.5, [181]). Additionally, mutagenesis of the ASK motif results in reduced β-arrestin engagement with 5-HT₂Rs (Fig. 3.6). To determine if either GRK2 or GRK5 plays a role in recruiting β-arrestin to 5-HT₂Rs during JCPyV infection, HEK293A cells stably expressing WT 5-HT₂Rs were transfected with siRNA targeting either an irrelevant control (CTL), GRK2, or GRK5 for 72 h. Serum starved, prechilled cells were incubated with either 5-HT or JCPyV, or were not treated, at 4°C for 1 h for synchronized attachment. Cells were then shifted to 37°C for 15 min for entry and subsequently harvested and lysed. Cellular lysates were incubated with magnetic agarose beads pre-conjugated to a GFP antibody for detection of YFP for immunoprecipitation of 5-HT₂R-YFPs and direct-interacting proteins. Bound protein fractions were resolved by SDS-PAGE and quantitation of the percentage of bound β-arrestin was employed using anti-GFP and anti-β-arrestin antibody detection utilizing LI-COR ImageStudio software (Fig. 3.13). Briefly, the borders of each protein band were defined by ROI, resulting in determination of the intensity of the band, the β-arrestin band intensity was then internally normalized to the 5-HT₂R band intensity to normalize for protein loaded/well. siRNA knockdown of GRK2 resulted in reduced β-arrestin interactions with 5-HT₂Rs when either 5-HT (approximately 60-65%) or JCPyV (approximately 65-70%) served as the agonist in comparison to CTL-siRNA samples (Fig. 3.13A, 3.13B, 3.13C). However, siRNA knockdown of GRK5 did not significantly alter β-arrestin binding to 5-HT₂Rs, regardless of agonist (Fig. 3.13A, 3.13B, 3.13C). Together, these results demonstrate the necessity of GRK2 in facilitating β-arrestin-receptor interactions, when either 5-HT or
JCPyV is the cellular stimulus, regardless of 5-HT₃R subtype, and reinforce the necessity of GRK5 following viral entry.

Figure 3.13. GRK2 is responsible for β-arrestin-receptor interactions during JCPyV entry.
Figure 3.13. (Continued)

HEK293A cells stably expressing 5-HT2AR-, or 5-HT2BR-, or 5-HT2CR-YFP were transfected with siRNA targeting an irrelevant control (CTL), GRK2, or GRK5. (A) 5-HT2AR-, (B) 5-HT2BR-, or (C) 5-HT2CR-YFP cells were incubated with either JCPyV (MOI = 12 FFU/cell), or serotonin (5-HT, 200 µM/well), or received no treatment (NT), at 4°C for 1 h for attachment followed by incubation at 37°C for 15 min. Cells were then removed from plates, lysed (whole cell lysate, WCL), and incubated with magnetic agarose beads conjugated to a GFP antibody for immunoprecipitation (IP) of 5-HT2Rs in fusion with YFP. WCL samples were stained for total protein. Bound samples for CTL, GRK2, and GRK5 siRNA samples were processed by SDS-PAGE followed by immunoblotting (IB) for detection of β-arrestin and GFP (corresponding 5-HT2R). The percentage of bound β-arrestin was determined using LI-COR ImageStudio software. Data represent the quantitation of the relative amounts of β-arrestin that are bound to 5-HT2Rs at 15 min post-internalization in the presence or absence of either GRK2 or GRK5 protein, normalized to 5-HT CTL samples (100%), and contain data from individual replicates performed in three independent experiments. Error bars indicate SDs. A two-sample student’s T test was used to determine statistical significance. *, P < 0.05.

3.5. Discussion

Viral utilization of a cellular receptor for functional internalization is a tightly controlled yet complex process. In this study we have further defined the mechanism by which JCPyV usurps human 5-HT2Rs for internalization. A β-arrestin binding domain within 5-HT2Rs, the ASK motif, is critical for viral entry and infection (Fig. 3.3 and 3.5). Further, G-protein coupled receptor kinases, GRK2 and GRK5, are important for JCPyV infection (Fig. 3.7, 3.8, and 3.9), with a specific role for GRK2 in facilitating JCPyV internalization (Fig. 3.11), while GRK5 promotes JCPyV and SV40 infection at a postinternalization step (Fig. 3.6). Additionally we have defined herein that upon JCPyV or 5-HT activation of 5-HT2Rs, β-arrestin directly engages the receptor. Further, mutagenesis of the Ser residue within the ASK motif of 5-HT2Rs reduces these β-arrestin-receptor interactions (Fig. 3.6), which are facilitated by GRK2 (Fig. 3.13). These results demonstrate a mechanism by which JCPyV activates β-arrestin and usurps its functionality to promote viral internalization, further defining and characterizing key host cell proteins involved in the utilization of 5-HT2Rs for entry of JCPyV (Fig. 3.14).
Figure 3.14. Model of JCPyV infectious cycle.

JCPyV infection is initiated by (1) binding to LSTc or glycosaminoglycans (GAGs) through capsid protein VP1. Entry then requires 5-HT2Rs, leading to (2) recruitment of GRK2 which, through GTPase activity, likely phosphorylates the ASK motif in the intracellular domains of the 5-HT2 receptors. Once phosphorylated, (3) activated b-arrestin can be recruited to the 5-HT2Rs. This interaction leads to the further activation of the receptors and (4) recruitment of intracellular proteins, clathrin and AP2, involved in receptor-mediated internalization. Through the formation of a clathrin-coated pit, the receptor and associated viral cargo could be internalized into an (5) early endosome (*, internalization of 5-HT2Rs results in delivery of receptors into an early endosome, but has not been demonstrated for JCPyV) and (6) transferred to a caveolin-1+ vesicle (demonstrated for 5-HT2AR), prior to delivery of the virus to the (7) endoplasmic reticulum (ER). Within the ER partial viral uncoating occurs allowing for nuclear delivery of the virion for (8) transcription and replication, followed by (9) eventual release of viral progeny.

In hijacking a cellular receptor, viruses select for specific signaling networks to transverse the plasma membrane and ensure arrival at the appropriate compartment for replication [14]. Interestingly, polyomaviruses utilize different cellular attachment and entry factors [17, 25, 151, 167, 265, 341]. The majority of polyomaviruses studied to date, including SV40, relying on ganglioside receptors for internalization by caveolin- or non-clathrin/non-caveolin-mediated endocytosis [25, 340]. Internalization
of polyomaviruses results in delivery to the same destination [17, 167, 265, 341], the ER, for uncoating prior to nuclear translocation [24-29]. In contrast, while JCPyV also arrives at the ER for partial uncoating, after a low affinity binding event to sialic acid-containing LSTc receptors or GAGs, it usurps the proteinaceous 5-HT$_2$R family for functional internalization by CME [150, 151, 194]. Although this dichotomy in internalization mechanisms among polyomaviruses exists, it suggests that crosstalk occurs between endocytic pathways; this crosstalk is important for ensuring polyomavirus delivery to the appropriate intracellular compartment.

Agonist-mediated activation of GPCRs recruits specific subsets of proteins, ultimately resulting in a cascade of signaling within the cell [203, 209]. This receptor activation results in two signaling outcomes: either G-protein dependent or β-arrestin-dependent/G-protein-independent signaling pathways. GRKs are involved in the direct identification and phosphorylation of GPCRs, determining the signaling pathway activated by the receptor [363, 364]. Furthermore, due to the requirement of β-arrestin for internalization [181], JCPyV likely utilizes a pathway similar to the canonical 5-HT-activated signaling cascade [363, 364]. In phosphorylating GPCRs and mediating β-arrestin binding, GRKs sterically block the binding of G proteins from the receptor [209]. While seven GRKs have been identified to date [358], the implication of either GRK2 or GRK5 in facilitating the internalization of 5-HT$_2$Rs have yielded mixed results [203, 206, 327]. Through GRK2 phosphorylation of the Ser residue of 5-HT$_2$AR, β-arrestin then binds to the phosphate tag on the receptor initiating endocytosis [203]. Phosphorylation by GRKs and subsequent binding by β-arrestin commits the receptor to β-arrestin-dependent internalization [338, 343, 345, 349]. Interestingly, the rat-origin 5-HT$_2$AR contains an NCT motif in place of the ASK motif and, internalization of rat-origin 5-HT$_2$AR is independent of β-arrestin and GRK2 [203, 327]. However, expression of the human ASK motif in place of the NCT in the rat 5-HT$_2$AR confers a reliance on β-arrestin and GRK2 for internalization [203]. Collectively, this demonstrates the critical nature of the human-derived ASK motif in promoting 5-HT$_2$AR-β-arrestin-dependent internalization.
Following GRK phosphorylation of 5-HT$_2$Rs initiating receptor activation, β-arrestin is recruited to the site of the activated receptor [206, 336, 342, 343] where direct interactions between β-arrestin and the phosphate residues on the receptor occur (Fig. 3.6, 3.13, [203]). In addition to the ASK motif, there are several other identified binding sites for β-arrestin on the third intracellular loop and in the C-terminus including the Asn-Pro-X-X-Tyr (NPXXY) and the Asp-Arg-Try (DRY) [181, 199, 353, 354]. Interestingly, altering the ASK motif of 5-HT$_2$AR not only changes the β-arrestin bias of the receptor but also the recycling rate of the receptor, with the mutated receptor recycling more rapidly than the unmutated receptor, likely due to the contributions of β-arrestin [203]. JCPyV utilization of 5-HT$_2$Rs for internalization requires an intact ASK motif and disruption of this interaction either through receptor mutagenesis or siRNA of β-arrestin significantly reduces JCPyV internalization and infection (Fig. 3.3 and 3.5, [181, 199]), though surface expression of these receptors following mutagenesis is not significantly altered, suggesting that accessibility of the receptor by the virus is not hindered (Fig. 3.2, [181]). However, mutagenesis of the ASK motif does not completely ablate β-arrestin binding (Fig. 3.6), suggesting that multiple receptor interactions with β-arrestin may be necessary to support JCPyV internalization and infection. While mutagenesis of the ASK motif correlates with a reduction in JCPyV entry, infection, and binding of β-arrestin with 5-HT$_2$Rs (Fig. 3.3, 3.5, and 3.6), the location of the ASK motif differs between the different receptor subtypes. This suggests that the conservation of the ASK motif within the receptor, rather than the location of the motif, may be the critical driver for JCPyV utilization of this receptor subfamily. Moreover, while GRK2 had been previously identified to facilitate β-arrestin binding with the ASK motif of 5-HT$_2$AR for canonical 5-HT-stimulated signaling, we demonstrate herein that a similar mechanism occurs for the 5-HT$_{2B}$R and 5-HT$_{2C}$R, regardless of whether 5-HT or JCPyV serves as the receptor stimulus (Fig. 3.6 and 3.13). Collectively this suggests that JCPyV may be hijacking these cellular receptors, activating the typical 5-HT driven pathway, in a conserved manner, to ensure delivery of the virus within the cell. In addition, as β-arrestin binding does not
completely correlate with the reduction in JCPyV infection following GRK2 siRNA (Fig. 3.11 and 3.13), there may be additional roles for GRK2 in JCPyV entry aside from the identification of the ASK motif β-arrestin binding site in 5-HT2Rs. Alternatively, a recent study by Assetta et al. suggested that the Pro residue adjacent to the DRY motif is necessary for successful JCPyV infection, and may contribute to additional β-arrestin receptor binding [199]. However, the Pro residue is thought to contribute to phosphorylation-independent β-arrestin-mediated endocytosis [201]. Therefore, GRK2 may identify an additional β-arrestin binding site for internalization. Although GRKs 2 and 5 are both G-protein receptor kinases, they belong to differential GRK subfamilies, with GRK2 belonging to the βARK subfamily (GRK2 and GRK3) and GRK5 to the GRK4-like subfamily (GRK4, GRK5, and GRK6) [209]. Compensation between GRKs has been reported to occur within GRK subfamilies, with a compensation effect observed between GRKs 5 and 6 [365]. However, a GRK5/6 knockout mouse results in lethality, suggesting the inability of compensation for the loss of these proteins by GRKs from other subfamilies [365]. Furthermore, knockout of GRK2 in mice also results in a lethal phenotype, again highlighting the importance of distinct individual roles of these proteins within cells [366]. While both GRK2 and GRK5 siRNAs individually reduce JCPyV infection (Fig. 3.7 and 3.8), JCPyV infection is further reduced upon a combinatory siRNA of both GRK2 and 5, suggesting independent roles during infection (Fig. 3.7).

Although knockdown of GRK2 or GRK5 reduces JCPyV infection (Fig. 3.7 and 3.8), only GRK2 reduces JCPyV internalization, suggesting involvement of GRK5 in a postinternalization step in the infectious process (Fig. 3.10). Moreover, transfection of either JCPyV or SV40 infectious clones, bypassing viral attachment, entry, and trafficking, results in reduced infection following GRK5 siRNA (Fig. 3.12). Interestingly, previous studies have demonstrated a role for GRKs independent of GPCR kinase function. GRK5 has been identified to play roles in receptor tyrosine kinase activity [367], and reduced GRK5 levels correlate with diminished ERK signaling [367], both of which are necessary for productive JCPyV infection [180, 321, 356, 357]. GRKs, including GRK2 and GRK5, have also been implicated with
regulation of cytoskeletal dynamics and microtubule rearrangement through modulation of proteins in
the ezrin-radixin-moesin (ERM) family [368-371]. Furthermore, GRK5 has been associated with the
regulation of nuclear factors including activation of NF-kB signaling [372, 373], a DNA-damage response
pathway that not only promotes JCPyV replication [374], but also is activated by SV40 during infection
[375]. Moreover, GRK5 also plays a role in facilitating activation of NFAT [376], a transcription factor
downstream of the MAPK cascade. Importantly, NFAT has been associated with the promotion of SV40
and JCPyV transcription and replication [377, 378]. While SV40 does not utilize the MAPK cascade for
replication [321, 379], GRK5 may play a role in facilitating activation of NFAT outside of the MAPK
cascade during infection. Collectively, this may suggest potential roles for GRK5 in JCPyV and SV40
infection following attachment, internalization, and trafficking.

The importance of 5-HT2Rs, GRKs, and β-arrestin introduces an enticing target for the
development of GPCR-directed antiviral therapies [380]. Gaining a deeper understanding of the
mechanism by which JCPyV utilizes 5-HT2Rs to mediate entry will further elucidate targets for GPCR
therapies. Moreover, 5-HT receptor antagonists prevent the internalization of other viruses including
chikungunya virus, coronavirus mouse hepatitis virus, and Ebola virus [381, 382]. The common effect of
5-HT receptor antagonists suggests multiple viruses may target overlapping GPCR-mediated signaling
pathways. Thus, development of GPCR-mediated antiviral therapies could potentially be applied broadly
for the treatment of multiple significant viral pathogens. Developing therapies to prevent GPCR
activation through β-arrestin- or 5-HT2R-specific therapies represents an area for the development of
new antivirals [383, 384]. β-arrestin biased therapeutics, including carvedilol and TRV120027, have
demonstrated promising results when used as therapies for heart failure [385-388]. Furthermore, β-
arrestin has been shown to be upregulated in individuals with MS, a patient group that is at-risk for
JCPyV-induced PML, while taking immunosuppressive therapies like natalizumab [389], warranting
exploration of a potential role for β-arrestin in PML development.
Within this study we have identified that 5-HT$_2$Rs, capable of supporting JCPyV infection, bind to $\beta$-arrestin, and this interaction is critical for JCPyV internalization. Furthermore, we have identified that a key cellular kinase, GRK2, is required to mediate JCPyV internalization and infection. These results further define JCPyV-activated 5-HT$_2$R signaling pathways that are critical for JCPyV infection, contributing to our understanding of viral usurped internalization pathways. Collectively, conclusions from this work identify new potential targets for the design of therapeutic agents to treat or prevent JCPyV infection and development or progression of PML.
CHAPTER 4

JC POLYOMAVIRUS ACTIVATES THE MAPK CASCADE FOR VIRAL INFECTION


4.1. Chapter Summary

JC polyomavirus (JCPyV) establishes a lifelong asymptomatic infection in most of the population. Under conditions of prolonged immunosuppression, JCPyV may reactivate, traffic to the central nervous system, and cause the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). Infection of JCPyV is established through initial interactions with lactoseries tetrasaccharide c (LSTc) on the exterior surface of the target cell for viral attachment. Internalization of JCPyV is initiated through 5-hydroxytryptamine (5-HT) subtype 2 serotonin receptors (5-HT2Rs) by clathrin-mediated endocytosis (CME). 5-HT2Rs are GPCRs that can activate the mitogen-activated protein kinase (MAPK) cascade, thus, this cascade may become activated by virus-receptor interactions. Extracellular regulated kinase (ERK), the terminal kinase in the MAPK cascade, becomes activated at times consistent with viral internalization, though the necessity of ERK or other members of the MAPK cascade for the promotion of infection remains unclear. Utilizing small-interfering RNA (siRNA), the Maginnis Laboratory identified that ERK1/2, as well as cellular proteins upstream of ERK1/2, including bRaf, cRaf, and MEK1/2 are important for JCPyV infection. The experiments further define the necessity of the MAPK cascade in the promotion of JCPyV infection and illustrate that ERK is activated through the core kinase cascade of Raf-MEK-ERK. Further, these data illuminate cellular kinases as potential therapeutic targets for the treatment or prevention of PML.
4.2. Introduction

Largely, signaling events activated in response to viral infection are due to cellular receptor activation and signaling [14]. JCPyV infection is initiated through direct interactions between viral capsid protein 1 (VP1) and host cell receptor lactoseries tetrasaccharide c (LSTc) on the exterior surface of the cell [144, 151, 236]. However, interaction between JCPyV and glycosaminoglycan receptors has also been described [134, 150]. Importantly, while engagement of these receptors is sufficient to mediate viral attachment, JCPyV requires use of a secondary proteinaceous receptor for cellular internalization by clathrin-mediated endocytosis, the 5-hydroxytryptamine (5-HT) serotonin subfamily 2 receptors (5-HT2Rs) [180, 181, 194, 196, 199]. After entry within the host cell, JCPyV usurps the endosomal system [29, 180, 193] for trafficking to the endoplasmic reticulum (ER) [29]. Within the ER, JCPyV undergoes partial uncoating [29], followed by translocation from the ER to the nucleus for viral transcription and replication.

Comparable to other small DNA tumor viruses, JCPyV transcription occurs bidirectionally with transcription of early genes occurring first followed by replication of the genome and transcription of late gene products [33]. An example of an early gene product is large tumor antigen (T-Ag) while late genes comprise the viral capsid components, VP1, VP2, and VP3 [33]. As early genes like T-Ag accumulate within the cell over the course of infection, it drives the replicative cycle forward for genome replication and transcription of late genes, which are critical for capsid formation and release of infectious progeny [6]. As a DNA virus, JCPyV utilizes host-cell transcription factors, and it is hypothesized that transcription factors become activated in response to signaling events upon infection [374, 377, 390-394]. The mechanism by which JCPyV activates signaling pathways remains poorly understood, yet previous research revealed that JCPyV infection results in the activation of extracellular regulated kinase (ERK), as early as 15 min postinfection [180], a time consistent with viral attachment and internalization [29]. ERK is the terminal kinase in the mitogen-activated protein kinase (MAPK)
signaling cascade. However, the necessity of ERK in the infectious process is poorly understood. Moreover, as ERK activation was sustained for up to 6 h postinfection, JCPyV may usurp ERK signaling for multiple steps of the viral lifecycle [180]. Further, while ERK has been demonstrated to become active early on in infection, it would suggest the importance of proteins upstream of ERK in the MAPK cascade for JCPyV infection, which also remain enigmatic. Collectively this would suggest that JCPyV likely requires components of the MAPK cascade for productive infection, though its role in promoting this process is uncharacterized.

The MAPK cascade is responsible for activating cellular response pathways due to extracellular stimuli [395, 396]. The MAPK cascade is comprised of Raf, MEK, and ERK, each of which in turn become activated through a cascade of phosphorylation [397]. Activation of the MAPK cascade involves activation of Ras, which, through inherent GTPase function activates a phosphorylation cascade beginning with Raf (MAPKKK), which in turn results in phosphorylation of MEK1/2 (MAPKKs), then activating ERK1/2 [397-401]. Active, phosphorylated, ERK1/2 can then bind to a number of intracellular targets resulting in their activation, to induce cellular reprogramming [402]. ERK1/2 can target cytosolic or nuclear factors resulting in their activation, including transcription factors, or can become dephosphorylated, resulting in its deactivation, by cellular phosphatases [402].

In promoting the activation of ERK1/2, cellular kinases Raf and MEK1/2 play significant roles in the cellular response to stimuli. Raf, a kinase that comprises 3 subtypes, aRaf, bRaf, and cRaf, differentially activate MEK1/2, with bRaf and cRaf the predominate subtypes responsible for phosphorylation of MEK1/2 [403, 404]. Moreover, previous reports suggested an importance of bRaf and cRaf for JCPyV infection, through the utilization of the selective chemical inhibitor Bay43-9006 [180, 405]. However, Bay43-9006 has been established to also target cellular factors aside from bRaf and cRaf, including receptor tyrosine kinase activity (RTK) [405]. Because RTK signaling is also required for JCPyV
infection [180], a more direct analysis of the role of these kinases and the kinase downstream of them, MEK1/2, in JCPyV infection is necessary.

The MAPK cascade has been identified to be important for multiple RNA and DNA viruses, as many of them usurp this cascade to promote viral replication [406], including polyomaviruses [170, 407, 408]. Replication of BKPyV has been demonstrated to also require the MAPK cascade [407], in contrast to what has been described for SV40, which instead usurps a MAPK-independent signaling cascade linked to PKC [409]. Together, this suggests that polyomaviruses may differentially utilize the MAPK cascade, adding to our understanding of signaling cascades that are important for infection of this virus family.

Collectively the work described within this chapter further defines the necessity of components of the MAPK cascade for JCPyV infection. Though not serving as the first-author of the publications containing this work, the experiments described herein were completed in response to reviewer comments after the first-author had left the laboratory. Thus, this work was critical for the importance and publication of these manuscripts. From these studies, we have a deeper understanding of the signaling networks linked with JCPyV infection, which could serve as the basis for improved antiviral therapeutics for the debilitating disease PML.

4.3. Materials and Methods

4.3.1. Cell Types, Virus Strains, Reagents, and Antibodies

SVG-A cells [317] were cultured in Minimum Essential Medium (MEM) (Corning) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) (Mediatech, Inc., Corning, NY, USA) and 0.2% Plasmocin prophylactic (Invivogen) (cMEM). Cells were grown in a humidified incubator at 37°C with 5% CO2. SVG-A cells were generously provided by the Atwood laboratory (Brown University) and authenticated by ATCC. JCPyV strain Mad-1/SVEΔ (provided from the Atwood laboratory (Brown
University) was purified as described [410] and used for infectivity experiments as indicated. Antibodies used for detection of viral proteins include PAB597 (provided by Ed Harlow) and PAB962 (provided by the Tevethia laboratory) hybridoma supernatants that produce a monoclonal antibody against JCPyV VP1 or large T Antigen (T-Ag), respectively.

Antibodies used for ICW analysis of protein knockdown or phosphorylation include: ERK1/2 (1:500, CST), bRaf (1:300, CST), cRaf (1:500, CST), and MEK1/2 (1:500, CST). Additionally, CellTag 700 (cell normalization stain) (1:500, LI-COR), as well as, secondary LI-COR 800 anti-rabbit or anti-mouse antibodies (1:10,000, LI-COR) were used for ICW protein detection analyses.

4.3.2. siRNA Treatment

SVG-A cells were plated in 12-well plates (Greiner Bio-One) to 50% confluency in media containing 10% FBS (lacking antibiotics). Cells were transfected with siRNA targeting unconjugated scrambled (non-targeting control, CST), EGFR (irrelevant control, CST), ERK1/2 (CST), bRaf (CST), cRaf (CST), or a combination of MEK1 and MEK 2 (CST), using RNAiMax (Invitrogen) per the manufacturer’s instructions, as described in [181]. Briefly, RNAiMax transfection reagent and siRNAs (30 pmol/siRNA/well (bRaf, cRaf, MEK1/2) or 7.5 pmol/siRNA/well (ERK1/2)) were prepared in incomplete media (no FBS and antibiotics), and incubated for 10 min at RT. MEK1 and MEK2 combinatory siRNAs (15 pmol/each) were combined for a final concentration of 30 pmol/siRNA/well. siRNA complexes were added to SVG-A cells and incubated at 37°C for 72 h. At 72 h post-transfection cells were either infected with JCPyV or SV40 (MOI indicated in figure legend) at 37°C for 1 h or fixed with 4% PFA, washed three times with 1X PBS, and analyzed to confirm siRNA protein knockdown by ICW or western blot analysis (indicated in figure legends). Infected cells were fed with complete media (containing FBS and antibiotics) and were incubated at 37°C for 72 h. At 72 hpi, cells were fixed with 4% PFA, washed with
three times with 1X PBS, and stained for VP1 followed by quantitation of viral infection by indirect immunofluorescence.

4.3.3. Quantitation of Viral Infection by Indirect Immunofluorescence

Following PFA fixation, siRNA-treated SVG-A cells were incubated with 1X PBS-1% Triton X-100 for 15 min for permeabilization, followed by incubation with 10% goat serum (Vector Labs) in 1X PBS at RT for 1 h, rocking. Cells were stained with antibodies for detection of either JCPyV or SV40 VP1 (PAB597, 1:40) in 1X PBS at 37 °C for 1 h. Cells were then washed three times with 1X TBS and incubated with an anti-mouse Alexa Fluor 488 antibody (Thermo Fisher Scientific) at 37°C for 1 h and nuclei were counter stained with DAPI at RT for 5 min (Thermo Fisher Scientific). Using a Nikon Eclipse Ti epifluorescence microscope (Micro Video Instruments, Inc.), the number of VP1-expressing cells per 10x visual field for 5 visual fields (per well) was quantified. Percent infection was determined by dividing the number of VP1+ infected cells/visual field by the total number of DAPI-positive nuclei/corresponding visual field as previously described [321]. The average percent infection was normalized to the indicated control (100%). Statistical significance was determined utilizing a two-tailed Student’s t-test, *, P < 0.05 was considered statistically significant.

4.3.4. Detection of Protein Knockdown by ICW Analysis

For detection of protein knockdown by in cell western (ICW), following siRNA treatment (for MEK1/2, bRaf, and cRaf) and PFA fixation, cells were washed 1X PBS three times, and incubated with 1X PBS-1% Triton X-100 at RT for 15 min to permeabilize. Cells were then incubated with TBS Odyssey Blocking Buffer (LI-COR) at RT for 1.5 h, rocking. Cells were stained with primary antibodies for bRaf (1:300), cRaf (1:500), or MEK1/2 (1:500) in TBS Odyssey Blocking Buffer at 4°C O/N, rocking. After incubation with the primary antibody, cells were washed with 1X TBS-T three times and then incubated with LI-COR 800 secondary antibody (1:10,000) and CellTag 700 (1:500) at RT for 1 h, rocking. Cells were
washed with 1X TBS-T three times and liquid was aspirated prior to scanning. Plates were read at a resolution of 42 μM, medium quality, 3.0 mm focus offset. After scanning, channels were aligned using the Image Studio software version 5.2 (LI-COR) equipped with In-cell Western module. After scanning, the ICW analysis grid (Image Studio) was applied to the image to outline each well and images were processed using ImageJ (NIH) [322] as previously described [357] for intensity of target protein and CellTag. Following quantitation, the percent response was determined using R (version 3.6.1) equipped with RStudio (version 1.2.1335, 2019) for three independent experiments.

4.3.5. Measuring Protein Knockdown Following siRNA by Western Blot Analysis

Following siRNA, cells were washed with 1X PBS, removed from plates by scraping, and pelleted by centrifugation at 376 X g at 4°C for 5 min. 1X PBS was aspirated and cells were resuspended in lysis buffer (Tris-HCl) containing protease (1:10) and phosphatase (1:100) inhibitors and were incubated on ice for 20 min. Cellular membranes were pelleted by centrifugation at 18,600 X g at 4°C for 10 min. Cellular lysates were combined 1:1 with Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol (Bio-Rad) and were promptly boiled at 95°C for 5 min. Samples were processed by SDS-PAGE using a Bio-Rad gel (4-15%) and were transferred to a nitrocellulose membrane in a semi-dry Transblot Turbo transfer system (Bio-Rad) for 30 min at 10V. The membrane was incubated in blocking buffer (5% non-fat dry milk, 1X-PBS-T (0.05%)) O/N at 4°C, rocking. Membranes were washed three times with 1X PBS prior to incubation with primary antibodies for detection of total ERK1/2 protein (1:500) and tubulin (1:450) O/N at 4°C, rocking. Membranes were washed three times with PBS-T, incubated with secondary anti-rabbit-800 antibody (1:5,000, LI-COR) and secondary anti-mouse-680 antibody (1:1,000, LI-COR) diluted in 5% non-fat dry milk PBS-T. Images were captured using a LI-COR Odyssey CLx.
4.3.6. Statistical Analyses

Experiments were performed in triplicate each containing at least triplicate samples. Matlab and Statistics Toolbox (MATLAB 2014a, The MathWorks, Natick, MA) were used to analyze data, determining the appropriate statistical test/experiment. Samples that were normally distributed were analyzed using a two-sample, Student’s t-test assuming either equal variance to compare the means of triplicate samples. Statistical significance was determined for $p$-values < 0.05. Distribution of populations was determined using the Anderson–Darling test and variation between sets of populations was determined using the F Test for Equal Variance. The Wilcoxon rank sum test was used in populations that were not normally distributed. Statistical significance was determined for $p$-values < 0.05.

4.4. Results

4.4.1. ERK1/2 are Required for JCPyV Infection

Early in JCPyV infection ERK1/2 are activated at approximately 15 min postinternalization [180]. Although ERK1/2 are activated at times consistent with entry it is unclear whether these kinases are important for JCPyV infection. To determine the necessity of ERK1/2 for JCPyV infection SVG-A cells were transfected with siRNA targeting Scrambled, a non-targeting control, or EGFR, an irrelevant control (CTL) [181], or an siRNA that recognizes and results in the knockdown of both ERK1/2 proteins. Cellular lysates were processed by SDS-PAGE and immunoblotted to confirm knockdown of ERK1/2 (Fig 4.1A) and then infected with either JCPyV or SV40 (Fig 4.1B). SV40 replication has been identified to occur independently of the ERK/MAPK cascade, instead requiring Ca$^{2+}$ signaling and receptor tyrosine kinase (RTK) activity due to protein kinase C (PKC) [409, 411]. Cells treated with ERK1/2 siRNA resulted in a 97% decrease in JCPyV infection while SV40 infection was not significantly reduced (Fig. 4.1B). Collectively these data indicate that ERK1/2 is critical for productive infection of glial cells by JCPyV while not required for the closely-related polyomavirus SV40.
Figure 4.1. siRNA knockdown of ERK1/2 reduces JCPyV infection.

(A and B) SVG-A cells were transfected with siRNA targeting EGFR (CTL), Scrambled (a non-targeting control), or siRNA targeting ERK1/2 for 72 h. Following incubation cells were either harvested for western blot analysis (A) or infected (B). (A) Cellular lysates were lysed and processed by SDS-PAGE followed by immunoblotting for either tubulin (housekeeping protein, red) or total ERK1/2 (green). (B) Cells were infected with either JCPyV (MOI = 0.1 FFU/cell or SV40 (MOI = 0.001 FFU/cell). 72 h postinfection infectivity was determined through nuclear detection of newly synthesized VP1 protein utilizing an antibody that detects both JCPyV and SV40 VP1. Data represent the percentage of JCPyV or SV40 infected cells (VP1+)/10x visual field for triplicate samples. Samples were normalized to the respective CTL siRNA sample (100%). Data are representative of three independent experiments. Statistical significance was determined using a two-sample student’s T test. Error bars = SD. *, P < 0.05 [321].

4.4.2. Knockdown of Raf Prevents JCPyV Infection

ERK1/2 is activated at times consistent with viral internalization [180]. Further, infection of glial cells by JCPyV is critically dependent upon ERK1/2 (Fig. 4.1). However, the mechanism by which ERK1/2 becomes activated for JCPyV infection remains uncharacterized. Thus, investigation of the other kinases of the MAPK cascade in the promotion of JCPyV infection warrants further exploration. In the MAPK
cascade, the initial kinase in the signaling chain is Raf. Activation of Raf through Ras-GTP results in a phosphorylation cascade that ultimately leads to ERK1/2 activation. Three isoforms of Raf are expressed ubiquitously: aRaf, bRaf, and cRaf. However, bRaf and cRaf are the isoforms that predominately phosphorylate the next kinase in the MAPK sequence, MEK [403, 404]. Further, a selective inhibitor that targets bRaf and cRaf, Bay43-9006, sorafenib, reduces JCPyV infectivity in SVG-A cells [180, 357]. However, Bay43-9006 has also been demonstrated to inhibit receptor tyrosine kinase activity, which has also been demonstrated to be important for JCPyV infection [180, 405]. Due to the determination that Bay43-9006 targets proteins aside from bRaf and cRaf [405], the specific involvement of these proteins in JCPyV infection was revisited using siRNA treatment and infectivity assays.

Because the two isoforms inhibited by Bay43-9006 are bRaf and cRaf [405], and they are the two isoforms that predominantly phosphorylate MEK leading to activation of ERK1/2 [403, 404], their necessity during JCPyV infection was determined through utilization of infectivity assays following siRNA treatment. SVG-A cells were transfected with siRNA targeting a scrambled non-targeting control (CTL) [181] or an siRNA that recognizes and results in the knockdown of either bRaf or cRaf. Efficient protein knockdown was determined through utilization of ICW (Fig. 4.2B), which determined that bRaf and cRaf were knocked down by approximately 60% and 75%, respectively (Fig. 4.2C). Cells treated with siRNA targeting either bRaf or cRaf were infected with JCPyV and infectivity was determined by indirect immunofluorescence of newly synthesized VP1. Cells treated with bRaf siRNA resulted in reduced JCPyV infection by approximately 50% while cells treated cRaf yielded a nearly 90% reduction in infection (Fig. 4.2A). Together, this emphasizes the importance of bRaf and cRaf, the initial kinases in the MAPK cascade, in the promotion of JCPyV infection.
Figure 4.2. Both bRaf and cRaf are required for infection of JCPyV.

(A and B) SVG-A cells were transfected with siRNA targeting a scrambled non-targeting control (CTL), bRaf, or cRaf for 72 h. Following incubation cells were either infected (A) or fixed and stained for ICW analysis (B and C). (A) Cells were infected with JCPyV (MOI = 1 FFU/cell) and 72 h postinfection infectivity was determined through nuclear detection of newly synthesized VP1 protein utilizing an antibody that detects JCPyV VP1. Data represent the percentage of JCPyV infected cells (VP1+)/10x visual field for triplicate samples. Samples were normalized to the respective CTL siRNA sample (100%). (B) For ICW analysis to determine protein knockdown, following fixation cells were incubated antibodies detecting either bRaf or cRaf (green) and CellTag (red). Expression of bRaf and cRaf were determined for both CTL and target siRNA-treated samples and (C) knockdown was determined through calculation of the % response of either bRaf or cRaf using ([(Total bRaf or cRaf intensity/CellTag intensity)x100] as determined by ImageJ. Box and whisker plots represent the distribution of data for three independent experiments performed in triplicate. Whiskers represent 1.5 times the interquartile range. Data are representative of three independent experiments. Statistical significance was determined using a two-sample student’s t-test. Error bars = SD. *, P < 0.05.
4.4.3. MEK1/2 are Necessary for JCPyV Infection

As ERK1/2 is activated at times consistent with viral entry [180] and ERK1/2 are required for JCPyV infection (Fig. 4.1). The requirement of the Raf kinases upstream of ERK1/2 in the MAPK cascade was demonstrated through siRNA silencing of bRaf and cRaf, the initial kinases responsible for activation of MEK1/2, reducing JCPyV infection (Fig. 4.2). Within the MAPK cascade, bRaf and cRaf are linked to ERK1/2 through the intermediate kinases MEK1/2 which is in turn responsible for activation of ERK1/2 through phosphorylation [399, 412]. As ERK1/2 is the only known substrate of MEK1/2, the activity of MEK1/2 within the cell is critical for overall cell health. Interestingly, MEK1/2 are activated early in JCPyV infection at 10 min postinternalization [356]. Furthermore, chemical inhibition of MEK1/2 either through PD98059 or U0126, results in diminished JCPyV replication [321, 408], further implicating the importance of this kinase for JCPyV infection. To better understand the importance of MEK1/2 for JCPyV infection, these cellular kinases were targeted with selective silencing siRNA, resulting in their knockdown in glial cells. After 72 h, these cells were analyzed by ICW analysis for determination of total MEK1/2 knockdown (Fig. 4.3B), resulting in nearly 60% reduction in total MEK1/2 protein (Fig. 4.3C). Alternatively, cells were subsequently infected with JCPyV (Fig. 4.3A). After infection had progressed for 72 h, viral infection was determined through quantitation of newly synthesized nuclear VP1 protein (Fig. 4.3A). Cells that received MEK1/2 siRNA demonstrated an approximately 80% decrease in JCPyV infection in comparison to CTL siRNA-treated samples (Fig. 4.3A). Collectively, this data suggests that the middle kinases in the MAPK cascade, MEK1/2, are important for JCPyV infection, in line with previously published work [408].
Figure 4.3. MEK1/2 is necessary for JCPyV Infection.

(A and B)SVG-A cells were transfected with siRNA targeting a scrambled non-targeting control (CTL) or MEK1/2 for 72 h. Following incubation cells were either infected (A) or fixed and stained for ICW analysis (B and C). (A) Cells were infected with JCPyV (MOI = 1 FFU/cell) and 72 h postinfection infectivity was determined through nuclear detection of newly synthesized VP1 protein utilizing an antibody that detects JCPyV VP1. Data represent the percentage of JCPyV infected cells (VP1+)/10x visual field for triplicate samples. Samples were normalized to the respective CTL siRNA sample (100%). (B) For ICW analysis to determine protein knockdown, following fixation cells were incubated antibodies detecting MEK1/2 (green) and CellTag (red). Expression of MEK1/2 was determined for both CTL and target siRNA-treated samples and (C) knockdown was determined through calculation of the % response of MEK1/2 using [(Total MEK1/2 intensity/CellTag intensity)x100] as determined by ImageJ. Box and whisker plots represent the distribution of data for three independent experiments performed in triplicate. Whiskers represent 1.5 times the interquartile range. Data are representative of three independent experiments. Statistical significance was determined using a two-sample student’s t-test. Error bars = SD. *, P < 0.05.

4.5. Discussion

At times consistent with viral entry, JCPyV activates both receptor tyrosine kinase (RTK) activity and ERK1/2 of the MAPK cascade [29, 180]. However, the necessity of these proteins for viral infection remained unclear. The work described in this chapter reinforces the requirement of the MAPK cascade for JCPyV infection, establishing a role for each kinase in the cascade for JCPyV infection. Knockdown of ERK1/2 through siRNA resulted in severely diminished JCPyV infection (Fig. 4.1) while SV40, which does
not utilize MAPK-ERK for replication [409], was not impacted (Fig. 4.1). Further, silencing of additional MAPK kinases upstream of ERK1/2 including bRaf, cRaf, and MEK1/2 also proved to be important for infection of glial cells by JCPyV (Fig. 4.2 and 4.3). Collectively, this work demonstrates the importance of the MAPK signaling cascade during JCPyV infection.

Many of the signaling pathways activated over the course of JCPyV infection are poorly understood. Herein, we have defined the requirement of MAPK signaling for infection, likely due to the capacity of ERK1/2 to regulate cellular signaling networks. Collectively, ERK1/2 can interact with and activate over 200 cellular substrates [413]. Thus, activating this diverse signaling network would be advantageous to a virus for the promotion of infection, likely through activation of transcription factors important for replication of JCPyV [413]. Importantly, transcription factors activated and usurped during infection by JCPyV have also been linked to the MAPK cascade, including NFAT4, NF-κB, SMADs, Sp1, cJun, and cMyc [374, 377, 390-394]. Moreover, SV40 replication also requires the transcription factor NFAT4 [378], though replication occurs independently of activity of the MAPK cascade (Fig. 4.1, [409]). However, whether JCPyV activation of this transcription factor is a result of activation of the MAPK cascade remains unclear.

Early during JCPyV infection, ERK1/2 becomes activated [180], with timing that correlates with attachment, entry, and trafficking of JCPyV [29]. Importantly, JCPyV internalization involves the utilization of 5-HT₂Rs for endocytosis [181, 194, 196]. Activation of 5-HT₂Rs results in the recruitment of β-arrestin [203], facilitating internalization of 5-HT₂Rs by clathrin-mediated endocytosis. Moreover, internalization of 5-HT₂Rs utilizing β-arrestin results in activation of the MAPK cascade [206, 338, 351, 352]. However, whether JCPyV activation of ERK1/2 at early time points is due to activation of β-arrestin for endocytosis is uncharacterized, yet is hypothesized that JCPyV activation of β-arrestin for
internalization leads to the subsequent activation of ERK1/2 at this time, regardless of ERK1/2 utilization for JCPyV entry.

Infection of other viruses also relies on the MAPK cascade including HIV-1, severe acute respiratory syndrome coronavirus (SARS-CoV), Epstein-Barr virus, Kaposi’s sarcoma-associated herpesvirus (KSHV), and influenza A virus (IAV), among others [406, 414]. Collectively, viruses can activate the MAPK cascade to drive specific components of the viral infectious lifecycle. Adenovirus has been demonstrated to activate the MAPK, p38, and JNK pathways to promote viral replication [415-417]. Utilization of the MAPK cascade has also been shown for Vaccinia virus, as use of the MEK1/2 inhibitor PD98059 resulted in decreased replication and production of newly synthesized infectious virions [418, 419] and is thought to occur through viral activation of the epidermal growth factor receptor (EGFR) through interactions with a viral polypeptide [420]. This further supports the importance of host cell receptor signaling for the promotion of viral infection. Another closely related polyomavirus, BK polyomavirus (BKPyV) also has been demonstrated to use the MAPK cascade for replication, treatment of cells with either PD98059 or U0126 reduced BKPyV infectivity [407]. BKPyV may further usurp ERK1/2 to regulate cyclin D1 expression, suggesting the implication of this cascade for viral-manipulation of the cell cycle [407]. In contrast to JCPyV and BKPyV, SV40 does not activate the MAPK cascade for viral replication [321, 356], instead using MAPK signaling for viral egress, through virus-induced vacuolization [379]. Collectively, viruses may activate the MAPK cascade for numerous processes during infection, including replication, manipulation of the cell cycle, and egress, highlighting the critical importance of this signaling network for infection by numerous viruses.

Together, the work described within this chapter advances our understanding of the Raf/MEK/ERK pathway for the promotion of JCPyV infection. Silencing of any of these proteins severely diminishes JCPyV infection, while the MAPK-ERK network appears to be irrelevant for SV40 infection.
Further work should define the necessity of these kinases for the activation of substrates usurped during infection, likely involving the activation of select transcription factors. Moreover, the mechanism by which JCPyV attachment, entry, and trafficking promotes the activation of the MAPK pathway is unclear, requiring further investigation. The implication of the MAPK signaling network on aspects of the viral lifecycle should be further elucidated, as knowledge gained could impact therapies that could be broadly applied to viruses that coopt this pathway.
CHAPTER 5

FUTURE DIRECTIONS AND CONCLUDING REMARKS

The research that I have completed has elucidated a mechanistic understanding of JCPyV internalization within target host cells. In hijacking the clathrin-mediated endocytosis (CME) pathway, JCPyV is orchestrating the activation of select host factors that collectively drive the internalization of the virus. In doing so, JCPyV likely activates the necessary signaling cascades that facilitate delivery of the virus to the appropriate intracellular compartment, driving infection forward. To allow for viral entry JCPyV activates specific cellular endocytic proteins for entry, including clathrin, AP2, β-arrestin, and dynamin. Importantly, activation of these proteins appears to be linked with phosphorylation of 5-HT$_2$Rs by GRK2. Further, these proteins are critical for uptake of JCPyV in cell types relevant to sites of persistence and lytic infections within the body, highlighting the critical contributions and conservation of each protein to this process. In promoting the activation of β-arrestin during internalization, JCPyV may be selecting for the activation of signaling networks within the cell that are critical for the promotion of viral infection, including the MAPK cascade, which is required for viral replication [321].

While this work significantly enhances our understanding of how JCPyV usurps the cellular serotonin receptors to drive viral internalization, much of this process is poorly understood. JCPyV localization with 5-HT$_2$Rs has been demonstrated to occur early during internalization [199]; however, the residues responsible for interactions, if an interaction occurs, remains enigmatic. It’s currently unclear how JCPyV-mediated activation of 5-HT$_2$Rs occurs, and how this activation results in the recruitment of select cellular factors that are necessary for viral entry and productive infection. While this dissertation research has uncovered the necessity of β-arrestin in driving this process, the detailed mechanisms of 5-HT$_2$R activation have yet to be elucidated. For example, while the ASK motif is a conserved β-arrestin binding site in the internal loops or C-terminus of 5-HT$_2$Rs, other β-arrestin binding sites exist and have been suggested to play a role in JCPyV internalization [199]. The dynamic nature of
β-arrestin binding to multiple residues of 5-HT₃Rs, the sequence that these interactions take place, and the overall impact of multiple β-arrestin interactions for the promotion of JCPyV internalization and infection remain unclear. Further, how the direct interactions between β-arrestin and 5-HT₃Rs coordinates recruitment of other endocytic scaffolding proteins like clathrin, AP2, and dynamin is not clear. It is likely that 5-HT₃R requires multiple interactions with β-arrestin for endocytosis to occur. This is supported by evidence demonstrated within this work that mutagenesis of a β-arrestin binding motif does not ablate β-arrestin binding, nor does siRNA of the GPCR kinase GRK2. This also suggests that interaction between β-arrestin and 5-HT₃Rs could occur at other sites independent of phosphorylation. Moreover, interaction with β-arrestin at select residues could tailor the activation of the receptor, and signaling networks could differ by cell type.

A link between serotonin receptors and specific signaling networks has been established [396], including activation of the MAPK cascade due to β-arrestin interactions with 5-HT₃Rs. It is unclear how JCPyV-mediated activation of the serotonin receptor results in activation of downstream signaling cascades and networks, including the MAPK cascade. However, as ERK is activated at times consistent with JCPyV internalization and β-arrestin-5-HT₃R binding, it would suggest that this may be the link to the activation of this cascade early in infection and requires further experimentation. JCPyV activation of ERK1/2 early in infection results in a cyclic, sustained activation [180, 321]. This may result in the subsequent activation of transcription factors or other components of the cell necessary for viral replication, resulting in their translocation to the nucleus for replication of the viral genome. Moreover, JCPyV activation of MAPK signaling by β-arrestin may be necessary for crosstalk of intracellular signaling networks to control multiple cellular processes including the cell cycle, proliferation, mobility, and apoptosis; manipulation that could be initiated as early as viral entry [421].
Interestingly, β-arrestin has been directly linked with manipulation of signaling networks, aside from the MAPK cascade, which could be relevant for the promotion of JCPyV infection. One of these networks is the activation of the AKT/m-Tor signaling cascade, activated in response to extracellular stimuli, resulting in the initiation of this cascade through recruitment of PI3K and PTEN. Importantly, PI3K has been linked with JCPyV infection in SVG-A glial cells [422]. Manipulation of this pathway by β-arrestin results in activated AKT leading to increased protein translation, inhibition of cellular apoptosis, and inhibition of p53, thereby decreasing cell-cycle arrest [423]. Interestingly, β-arrestin is also linked with the modulation of transcription factors. IκBα, an inhibitory protein in NF-κB signaling, maintains NF-κB in the cytoplasm, preventing nuclear translocation and activity as a transcription factor [423]. In the context of β-arrestin manipulation of this pathway, through interaction with IκBα, β-arrestin can stabilize this protein within the cytoplasm. Stabilization of IκBα can result in retention of NF-κB in the cytoplasm and downregulation of NF-KB signaling [423]. However, NF-κB signaling has been associated with JCPyV infection [374], suggesting that JCPyV may selectively regulate the activation of β-arrestin for signaling. It is important to consider that some of these cascades, including negative regulation of NF-κB may be activated independently of GPCR stimulation, and thus, may not be influenced during JCPyV-utilization of β-arrestin for endocytosis [424]. Whether β-arrestin manipulates the AKT/m-Tor or the NF-κB pathway during infection should be explored further, as this may be directly impacted by β-arrestin activation during viral internalization.

The importance of defining the pathway JCPyV uses to enter host cells cannot be underscored. Further characterizing the utilization of 5-HT2Rs for entry not only further defines the signaling capabilities of this receptor family, but improves our understanding of why JCPyV may be usurping this specific class of receptors for infection. It is approximated that nearly 40% of drugs available on-market target GPCRs [425]. Moreover, antagonists of serotonin receptor signaling prevent infection and entry of
other important viral pathogens including Ebola, Marburg virus, and Chikungunya virus [382, 426]. While these viruses have not been demonstrated to use the serotonin receptor for entry, this would suggest that there may be overlapping signaling mechanisms that occur for multiple host cell receptors [427]. The implication of this for the generation of antivirals may suggest that therapies that target these signaling mechanisms could be used to prevent or treat infection by other significant viruses.

Although much of the literature highlighted in this work underscores the importance of the usurpation of specific cellular receptors and their necessity for dictating downstream signaling events, EVs appear to contradict this evidence. The manner in which viral utilization of EVs results in the activation of signaling networks that are necessary for the promotion of viral infection, while bypassing the activation of cellular receptors, remains unclear. However, the likelihood is that polyomaviruses hijack EVs for maintaining viral persistence within the host and for immune system avoidance. In releasing infectious particles packaged into EVs, polyomaviruses may avoid cellular lysis, which may trigger clearance of the infection [428]. This is supported by evidence that there is low spread of BKPyV particles from infected cells to naive cells during persistent infection [429]. However, the mechanisms by which polyomaviruses may utilize EVs to target particular tissues or organs, previously thought not to be susceptible, is not clear. In continuation, utilizing this method may result in increased spread of viral infection and heightened development of polyomavirus-associated disease [428]. Further investigation into the contribution of EVs to spread of polyomavirus infection, in conjunction with the CME internalization pathway defined herein, and their implications for facilitating the necessary trafficking and signaling networks within cells in order to promote infection is warranted. Collectively, entry of viruses results in the targeting of the virus to intracellular compartments that promote infection.

For many years it was not well-understood why polyomaviruses target the ER upon internalization inside the cell; this is by far the most unique feature of polyomavirus infection. Through
significant advances we now better understand the importance of the ER in viral disassembly for polyomavirus infection. However, many key questions remain to be addressed. It remains unclear why polyomaviruses target a broad diversity of cellular attachment and entry factors to eventually arrive at the same destination, especially when the overall conformation of the cellular attachment protein, VP1, is highly conserved. In evolving from a common viral ancestor, it is easy to speculate that the utilization of differing receptors is due to evolutionary divergence, resulting in differences in targeted tissues. Regardless, classically, tissue tropism correlates with expression of receptors that support viral infection [49]. Further, the mechanism of polyomavirus intracellular movement to the ER upon internalization remains largely uncharacterized, including transit from intracellular vesicles into the ER itself. In addition, it is important to highlight that much of our understanding of polyomavirus usurpation of the ER relies on what has been identified for SV40. While disassembly machinery and mechanisms utilized by polyomaviruses in the ER may demonstrate some conservation it remains possible that there are unique factors and pathways utilized, as is the case for PyV entry. While the necessity of the ER for other non-human polyomaviruses and their human counterparts has been determined, differences have already emerged in the involvement of specific components of the ERAD machinery. Clearly, there is still much to learn about the redox environment of the ER for specific polyomaviruses and the impact this plays for progression of infection. Further, the role of EVs in this process remains uncharacterized. Largely thought to be attributed to use of cellular receptors, it is unclear how endocytosis of EVs may result in the activation of downstream networks including ERAD for virus capsid disruption. Moreover, whether polyomaviruses aside from JCPyV and BKPyV use EVs for spread of infection remains to be determined. Investigation of alternative polyomaviruses including SV40, mPyV, and MCPyV, will undoubtedly advance our understanding of this process.

While significant progress has been made to unravel the complexities of polyomavirus infection multiple roadblocks still exist in our understanding of these multifaceted processes. The implication of
cell-type specific differences as well as the utilization of multiple receptors and endocytic pathways during infection further complicate our understanding of polyomavirus infection. Further, the utilization of virus-like particles (VLPs) in comparison to whole virions and their potentially differing attachment, entry, and trafficking properties cannot be ignored as much of these potential differences remain elusive. These studies may be further convoluted by the notion that viral infection could occur by receptor-mediated attachment and entry of virions and EV-mediated uptake simultaneously, thus complicating our understanding of these processes. Further, while these experiments were performed in multiple cell types, they were performed with the utilization of immortalized cells to understand these complex processes. The necessity of β-arrestin, other CME proteins, and the MAPK cascade during JCPyV infection should be further analyzed in the context of relevant primary cell types including renal proximal tubule epithelial cells (RPTEC), normal human astrocytes (NHA), and primary oligodendrocyte cell lines. The use of primary cell types could better recapitulate infection occurring within the human host.

Improved insight into the complex interactions that result in JCPyV uptake within the cell and how these events mediate activation of signaling networks for the continuation of the viral lifecycle will undoubtedly inform the development of improved therapeutics for the treatment or prevention of JCPyV and PML. There is a critical need to develop therapies for this devastating disease as cases of PML are increasing, particularly due to the use of immunomodulatory therapies. Moreover, studies of virus host-cell interactions will broaden our understanding of these processes could uncover targets for antiviral therapies for other significant viral pathogens, such as the novel coronavirus SARS-CoV-2, responsible for the COVID-19 pandemic. Utilization of selective serotonin reuptake inhibitors (SSRI) has shown promise in lessening the serious illness associated with SARS-CoV-2, through modulation of cytokine production [430]. Ultimately, the work described here contributes to our understanding of the
mechanisms by which viruses hijack cellular signaling to promote infection, thereby contributing to the
development of improved antiviral therapies.
CHAPTER 6

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CHAPTER 7

BIOGRAPHY OF THE AUTHOR

Colleen Mayberry was born in Waterville, Maine in July 1992. She was raised in Unity, Maine and graduated from Mt View High School in 2010. She attended the University of Maine and graduated in 2015 with a Bachelor’s of Science degree in Biochemistry with a minor in Microbiology. During this time, while Colleen was commuting to UMaine in the snow, she encountered numerous wildlife on the backroads of Maine. Their lives were lost in pursuit of education and science. She entered in the Molecular and Biomedical Sciences graduate program at The University of Maine in Fall of 2015. Colleen is a candidate for Doctor of Philosophy in Microbiology from the University of Maine in December 2020.