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Unraveling viral encephalitis in vivo : dynamic imaging of neuro-invasion and neuro inflammation processes in the zebrafish

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Université Pierre et Marie Curie

Complexité du Vivant

Macrophages et Développement de l'Immunité

Unraveling viral encephalitis *in vivo*:
Dynamic imaging of neuro-invasion and
neuro-inflammation processes in the zebrafish

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Thèse de doctorat de Biologie

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“Per aspera sic itur ad astra”
Seneca, Hercules furens, act II, v. 437

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RESUMÉ ÉTENDU DU PROJET DE THÈSE

De nombreux pathogènes sont neurotropes, posant un problème aigu de santé publique; on estime ainsi que près de la moitié des virus émergents peuvent causer des encéphalites ou d'autres atteintes neurologiques sévères. La compréhension des interactions qui sous-tendent le neurotropisme de ces pathogènes est d'une importance considérable, afin de guider les stratégies thérapeutiques et préventives pour limiter les conséquences de ces infections.

Le genre *Alphavirus* compte plusieurs virus neuro-invasifs et inclut par exemple le virus de l'encéphalite équine occidentale (WEEV), causant une mortalité d'environ 10%, et le virus du Chikungunya (CHIKV), qui s'est récemment propagé aux Caraïbes et en Polynésie, et qui cause de graves encéphalites chez les nouveaux-nés.

Le laboratoire où j'ai effectué une partie de ma thèse a décrit, par imagerie de l'organisme entier, la dissémination du CHIKV dans le modèle danio zébré (*Danio rerio*), pour mieux caractériser sa pathogénèse ainsi que les réponses immunitaires activées chez le danio. Le danio zébré est un modèle bien établi pour étudier la biologie du développement des vertébrés. Ses larves petites, transparentes et faciles à anesthésier, sont favorables à des approches de microscopie non invasive, qui permettent de réaliser des observations à l'échelle d'un individu entier à des niveaux de résolution cellulaire et subcellulaire. De surcroît, les outils génétiques disponibles chez cette espèce ont permis le développement de nombreuses lignées transgéniques exprimant des protéines fluorescentes spécifiquement dans certaines cellules (*e.g.* neurones, cellules immunitaires). De telles lignées constituent des outils de choix pour évaluer la dynamique de la réponse immunitaire et la mort cellulaire neuronale au cours de l'infection virale. Ces atouts font du danio zébré un excellent modèle pour étudier les infections virales *in vivo*.

Au cours de mon projet, j'ai étudié l'entrée et la colonisation du système nerveux central (SNC) par un *Alphavirus* de la même famille de CHIKV, le virus Sindbis (SINV) dans le modèle danio zébré. SINV est transmis chez l'homme par piqûre

de moustique et provoque une maladie avec des symptômes tels que fièvre, éruption cutanée, douleurs articulaires et musculaires. De plus, le virus induit une forte réponse de l'hôte, sous forme de production d'interféron de type I. Malgré toutes les études conduites jusqu'à présent, ni le mécanisme d'entrée ni la réponse induite par l'infection dans le cerveau n'ont pu être visualisés en temps réel à l'échelle d'un organisme entier. Mon projet présentait donc plusieurs axes: 1) développer un modèle d'infection du virus Sindbis chez le danio zébré, 2) caractériser l'invasion du SNC par le virus par des techniques d'imagerie à haute résolution, 3) définir la voie d'entrée du virus dans le SNC, 4) évaluer la dynamique de la réponse immunitaire innée par l'étude de la réponse IFN et, enfin, 5) établir les mécanismes de persistance du virus dans les cellules neuronales.

Les études du virus SINV réalisées dans les modèles murins reposent essentiellement sur des injections par voie intracrâniale (IC), ce qui ne permet pas d'identifier la voie d'entrée dans le cerveau. Par conséquent, j'ai réalisé des injections par voie intraveineuse (IV) afin de pouvoir observer le passage de la périphérie au cerveau. Le suivi de la propagation du virus a été rendu possible par l'utilisation d'un ARN viral recombinant dans lequel est intégré la séquence de la protéine fluorescente verte 'GFP' exprimée dans les cellules cibles de l'infection lors de la réplication virale. L'utilisation de cette construction m'a permis de caractériser la progression de SINV chez le danio zébré et d'identifier les organes/tissus cibles que sont le vitellus, le foie, le cœur et enfin, le cerveau. De plus, j'ai pu montrer par des techniques de biologie moléculaire que SINV provoque une réponse interféron très forte dans les larves infectées, tout comme dans les modèles d'infection chez les mammifères. L'importance de cette réponse a été corroborée par la mortalité plus élevée que l'on observe dans les larves où la réponse interféron a été inactivée.

Les données rassemblées jusqu'à présent m'ont aussi permis d'identifier le mécanisme par lequel SINV se propage vers le cerveau. J'ai pu constater que SINV ne détruit ni n'infecte la barrière hémato-encéphalique, après infection d'un lignée transgénique permettant l'étude du système vasculaire; j'ai aussi montré qu'il n'exploite pas l'entrée naturelle dans le cerveau des leucocytes circulant comme les macrophages (tactique du 'Cheval de Troie'), puisque la progression

de l'infection est inchangée lors de la déplétion sélective des macrophages. La primo-infection des neurones périphériques notamment au niveau des ganglions trigéminés semble indiquer que le virus se propage par un transport axonal du system nerveux périphérique vers le SNC.

Dans le cadre de la réponse immunitaire au niveau cellulaire, j'ai pu observer le rôle joué par les leucocytes, en particulier les neutrophiles, comme cellules productrices d'interféron: l'infection du cerveau, à partir d'une certaine taille du foyer, détermine le recrutement de ce type cellulaire. Cette réponse dans le système nerveux est particulièrement intéressante, si l'on considère que, chez le danio zébré tout comme chez les mammifères, les neutrophiles sont normalement exclus du cerveau.

Enfin, le danio zébré est devenu un modèle de choix courant pour réaliser des criblages pharmacologiques, et ces travaux permettent d'identifier de nouvelles molécules antibactériennes, antivirales ou anti-inflammatoires. Mon modèle est donc maintenant exploité pour évaluer les propriétés antivirales d'une drogue spécifique sélectionnée par des approches *in vitro*, en collaboration avec l'Unité « Populations Virales et Pathogénèse » à l'Institut Pasteur.

Compte tenu de la conservation entre les types cellulaires et les gènes de l'homme et du danio zébré, j'espère que ce travail de thèse permettra d'identifier certains mécanismes généraux impliqués dans le neurotropisme, ce qui pourrait à plus long terme servir à la mise en place d'une prévention des encéphalites virales.

SUMMARY

The zebrafish (*Danio rerio*) is an important model organism, particularly for studies of development, cancer, neuroscience and more recently host-pathogen interactions. As opposed to other vertebrate model organisms, its optical clarity and ease of genetic manipulations allow to visualize highly dynamic cellular processes *in vivo* at the whole-organism scale. These assets make the zebrafish a perfect model for the study of viral infections *in vivo*, such as those caused by neurotropic viruses.

Viral infections that involve the Central Nervous System (CNS) are particularly serious, especially due to the limited number of effective treatments or prophylactic drugs. Moreover, the CNS is a particularly vulnerable region. Encephalitis and meningitis are among the worst complications that can arise from a viral infection. This can be due to strong inflammatory responses, which are especially deleterious in the CNS, and must therefore be tightly and specifically regulated.

The aim of this project has been to gain insights in some of the interactions that determine encephalitis, by combining the optical properties offered by the zebrafish model with the neurotropic features of the widely-known Sindbis virus (SINV).

SINV is a single-stranded positive-polarity RNA virus belonging to the *Togaviridae* family, genus *Alphavirus*. Together with other medically important viruses (such as Dengue and Nipah virus), it is an arbovirus, thus transmitted to humans by arthropods – mostly represented by mosquito vectors. SINV has been extensively used in the laboratory, especially in murine models, with some highly encephalitogenic strains being developed for that host. Despite extensive studies in this model, the exact mechanism of entry in the CNS has never been fully elucidated. This is due mainly to difficulties in assessing the distribution of the virus and following the progression of the infection in real time in live animals.

This Thesis project has consisted therefore in: 1) the development of a SINV infection model in zebrafish larvae, 2) the characterization of SINV neuroinvasion

upon its inoculation in the bloodstream, thanks to the use of high-resolution microscopy, 3) the study of SINV mechanism of entry in the CNS, 4) the characterization of the innate immune response, both at the whole organism and organ-specific level, and 5) the characterization of SINV persistence mechanism in infected neuronal cells.

Thanks to the use of a SINV recombinant strain, engineered to express the green fluorescent protein “GFP” in infected cells upon viral replication, we have been able to follow the onset and the progression of the infection. We have identified several organs targeted by the virus, including the yolk, the heart, the liver, and eventually, the brain. Moreover, we have investigated the mechanism through which SINV reaches and infects the CNS. We have excluded propagation from the blood-brain barrier as well as infection of circulating leucocytes (“Trojan horse” mechanism) and have suggested infection of peripheral neurons and subsequent axonal transport to the CNS as SINV entry mechanism. These results have been reported in the submitted article. Furthermore, the activation of the host response at early times post-infection as well as CNS-specific responses have been investigated. In particular, at the cellular level, we have identified neutrophils as the main IFN-producing cells and are currently investigating their recruitment to the brain, upon extensive CNS infection in zebrafish larvae.

In addition to these *in vivo* analyses of infection, several tools have been developed for the project: production of various recombinant SINV viruses (leading to interesting findings of strain-dependent neuroinvasiveness), and *in vitro* culture of primary zebrafish neurons and glial cells.

Finally, we initiated a collaboration with the “*Viral population and Pathogenesis*” Unit at Pasteur Institute, to evaluate the effect of antiviral compounds on SINV-infected larvae.

Thanks to the conservation between zebrafish and human genes and cell types, we hope this project will shed more light on the mechanisms of viral neurotropism. This, in turn, will be helpful long-term to set up strategies for the prevention of viral-induced encephalitis.

LIST OF ABBREVIATIONS

Ab	Antibody
BBB	Blood-Brain Barrier
BHK-21	Baby Hamster Kidney-21
BMVECs	Brain Microvascular Endothelial Cells
Cas9	CRISPR-Associated Protein 9
CDC	Centers for Disease Control and Prevention
CHIKV	Chikungunya Virus
CNS	Central Nervous System
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Cerebrospinal Fluid
DENV	Dengue Virus
DFMO	Difluoromethylornithine
dpf	Day Post-Fertilization
dpi	Day Post-Inoculation
EEEV	Eastern Equine Encephalitis Virus
EID	Emerging Infectious Disease
GFP	Green Fluorescent Protein
HIV	Human Immunodeficiency Virus
hpf	Hour Post-Fertilization
hpi	Hour Post-Inoculation
HSV-1	Herpes Simplex Virus-1
HTLV	Human T Cell Leukemia Virus
IC	Intracerebral (Inoculation)
IFN	Interferon
IFNAR	Interferon- α/β Receptor
IFN ϕ	Fish Interferon
IHNV	Infectious Hematopoietic Necrosis Virus
IL	Interleukin
IRF	Interferon Regulatory Factor

ISG	IFN Stimulated Gene
ISGF	IFN Stimulated Gene Factor
IV	Intravenous (Inoculation)
JAK1	Janus Kinase 1
JCV	John Cunningham Virus
JEV	Japanese Encephalitis Virus
MAVS	Mitochondrial Antiviral Signaling Protein
MAYV	Mayaro Virus
MHC	Major Histocompatibility Complex
MO	Morpholino Oligonucleotide
NGN1	Neurogenin 1
nsP	Non-Structural Protein
NSV	Neuroadapted Sindbis Virus
ONNV	O’Nyong-Nyong Virus
ORF	Open Reading Frame
PKR	Protein Kinase R
PRR	Pathogen-Recognition Receptor
PV	Poliovirus
RdRp	RNA-Dependent RNA Polymerase
RGC	Retinal Ganglion Cell
RLR	RIG-I-like Receptor
RNAi	RNA Interference
RRV	Ross River Virus
RV	Rabies Virus
SFV	Semliki Forest Virus
SINV	Sindbis Virus
SINV1-GFP	SINV <i>pTE3’2J-eGFP</i> strain
SINV2-mCherry	SINV <i>pTR339-mCherry/2A</i> strain
SOCS	Suppressor of Cytokine Signaling Protein
sP	Structural Protein
STAT1	Signal Transducer and Activator of Transcription
TBK1	TANK Binding Kinase 1
TILLING	Targeting Induced Local Lesions In Genomes

TLR	Toll-like Receptor
TNF- α	Tumor Necrosis Factor- α
TRAF	TNF Receptor-Associated Factor
TRIM	Tripartite Motif-Containing Protein
TYK2	Tyrosine Kinase 2
UTR	Untranslated Region
VEEV	Venezuelan Equine Encephalitis Virus
VIG1	VHSV-Induced Gene
VSV	Vesicular Stomatitis virus
WEEV	Western Equine Encephalitis Virus
WNV	West Nile Virus
WT	Wild-Type

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INTRODUCTION

INTRODUCTION

CHAPTER I: Viral Infections of the Central Nervous System

ἰδρῶτες δὲ καὶ ἔπειτα⁶ ἐπὶ πολὺν
χρόνον τῆς κεφαλῆς ἐγίνοντο· ἐξηράνθη.

⁶ ἔπειτα III R

*“Riper matter broke forth towards the ear. Subsequently, there were sweats
(lesions) on the head for a long time.”*

*Hippocrates Volume VII, Epidemics II, Section 3 (v.112-113)
Harvard University Press, 1994*

In this passage extracted from Epidemics II, the Greek physician Hippocrates describes a disease of unknown origin affecting one of his patients and causing lesions spreading on the head. It is the beginning of the 4th century BC when Hippocrates first reports a case of a herpes-induced brain infection.

Despite being well known since ancient times, infectious diseases of the Central Nervous System (CNS) did not cease to pose a major health threat to the human population, as exemplified by the number of pathogens that can affect the CNS. Table 1 provides a general overview of neurotropic viruses, based on their classification and properties (Table II.1).

With the exception of a few specialized human viruses (*e.g.* Poliovirus and herpesviruses), persistent or “stable” neuroinvasion is generally rare. On the other hand, acute infections of the CNS are more often caused by zoonotic diseases, for which humans normally represent a “dead-end host” (Gubler, 2001).

It has recently been estimated that nearly 50% of emerging viruses are characterized by serious neurological clinical manifestations (Olival and Daszak, 2005). Contacts between humans and animals, especially wild ones, are an important factor for the onset and transmission of viral Emerging Infectious Diseases (EIDs), 75% of which are of zoonotic origin (Taylor *et al.*, 2001). It has

been shown, for example, that bushmeat hunters in Africa are at high risk of contracting novel viral zoonoses (Wolfe *et al.*, 2004).

Genome	Virus Family	Genus	Virus	CNS Disease
dsDNA	Herpesviridae	Simplexvirus	HSV-1, HSV-2, PRV	Encephalitis
		Varicellovirus	VZV	Encephalitis, Myelitis, Leuko-encephalopathy
		Lymphocryptovirus	EBV	Meningo-encephalitis
		Cytomegalovirus	HCMV	Encephalitis
	Polyomaviridae	Polyomavirus	JCV	Progressive Multifocal Leuko-encephalopathy
dsRNA	Reoviridae	Coltivirus	CTFV	Encephalitis
(+)ssRNA	Coronaviridae	Betacoronavirus	MHV	Encephalomyelitis
	Flaviviridae	Hepacivirus	HCV	Hepatic Encephalopathy
		Flavivirus	JEV, WNV, Saint-Louis virus	Encephalitis, Acute Flaccid Paralysis
	Picornaviridae	Enterovirus	PV, EV71, Coxsackie virus	Poliomyelitis, Encephalitis
	Togaviridae	Alphavirus	EEEV, WEEV, VEEV, CHIKV, SINV	Encephalitis
(-)ssRNA	Arenaviridae	Arenavirus	LCMV, JUNV, MACV	Meningo-encephalitis
	Bornaviridae	Bornavirus	BDV	Encephalopathy
	Bunyaviridae	Orthobunyavirus	LACV	Encephalitis
	Orthomyxoviridae	Influenza A virus		Encephalitis
	Paramyxoviridae	Morbillivirus	Measles virus	Meningo-encephalitis
		Rubulavirus	Mumps virus	Meningo-encephalitis
		Henipavirus	HeV, Nipah virus	Febrile Encephalitis
	Rhabdoviridae	Lyssavirus	RV, VSV	Acute Encephalitis
ssRNA-RT	Retroviridae	Lentivirus	HIV-1	Acute/Chronic Meningitis, Encephalitis
		Deltaretrovirus	HTLV-1, HTLV-2	Myelopathy

Table II.1. Classification and CNS disease of neurotropic viruses. dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; ssRNA-RT, RNA reverse transcribing. In bold, neurotropic arboviruses. Adapted from Koyuncu *et al.* (2013).

Changes in ecological, environmental, and demographic factors also contribute to the spreading of these diseases. Moreover, of all viral EIDs 40% are arboviruses (*Arthropod-Borne Viruses*) – pathogens transmitted by arthropods. Increased exposure to mosquitoes and other arthropod vectors, adjustment of a vector to new ecological niches (Roche *et al.*, 2015) or adaptation of a pathogen to new vectors greatly increase viral EIDs expansion. The adaptation of Chikungunya virus (CHIKV) to the mosquito *Aedes albopictus* since 2004, for example, greatly

contributed to its re-emergence in various parts of the world (Tsetsarkin and Weaver, 2011).

Reducing vector prevalence and vector avoidance are certainly the first line of defense against arboviral nervous system infections. However, new outbreaks of both new and old pathogens are almost certain to occur in the future, and novel treatments – possibly with broad relevance to many viruses – should also be part of our global health strategy. To do so, a thorough understanding of the mechanisms of disease is necessary and animal models play a key role in deciphering virus-induced pathogenesis.

The following sections include a brief overview of neurotropic arboviruses, the neurological pathogenesis they can induce, the several mechanisms of entry neurotropic viruses can employ to reach and spread in the CNS, and the defense mechanisms that are readily activated in the CNS upon infection.

1.1. Neurotropic Arboviruses

Arboviruses that can reach and affect the nervous system mostly include single-stranded RNA viruses belonging to various families. The DNA African Swine Fever virus represents the only exception. All other neurotropic arboviruses either belong to the *Togaviridae*, *Flaviviridae* (West Nile virus (WNV), Dengue virus (DENV)), *Bunyaviridae* (Hantavirus, Crimean-Congo Hemorrhagic Fever virus, Rift Valley Fever virus), *Rhabdoviridae* (Rabies virus (RV)), *Orthomyxoviridae* (Influenza virus), or to the *Reoviridae* (Colorado Tick Fever virus) families (Table I1.1).

Neurotropic viruses of the *Togaviridae* family include *Alphaviruses* such as CHIKV, Sindbis virus (SINV), Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), and Venezuelan Equine Encephalitis virus (VEEV). Their structure, life cycle, and pathology are further described in Chapter II.

Arboviruses are found worldwide and can be transmitted to several vertebrate hosts by blood-feeding arthropods, including mosquitoes, biting flies, ticks, mites

and lice. They are normally maintained in complex life cycles involving vertebrate hosts and arthropod vectors (Figure I1.1).

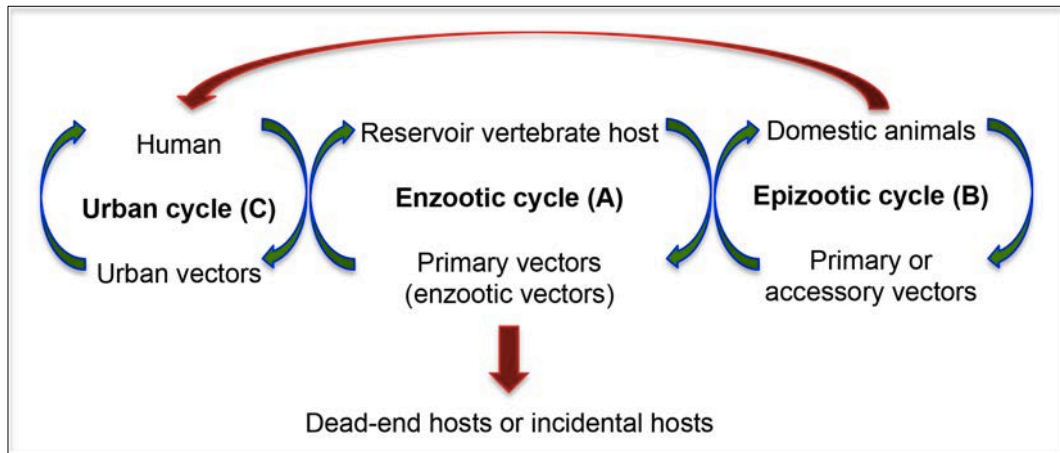


Figure I1.1. Vertebrate host and vector transmission cycles. (A) Enzootic, the natural transmission of virus between wild animals and primary vectors. (B) Epizootic, the transmission of virus between domestic animals and primary vectors. (C) Urban, the virus cycles between humans and insect vectors, due to high level of viremia. Adapted from Go *et al.* (2014).

Epidemics in humans and domestic animals occur when the virus comes in close contact with a bridge vector. Nevertheless, humans are generally dead-end hosts. They do not in fact contribute to the distribution/spreading of the disease, since they do not normally develop sufficient levels of viremia to infect an arthropod, such as in the case of EEEV (Figure I1.2).

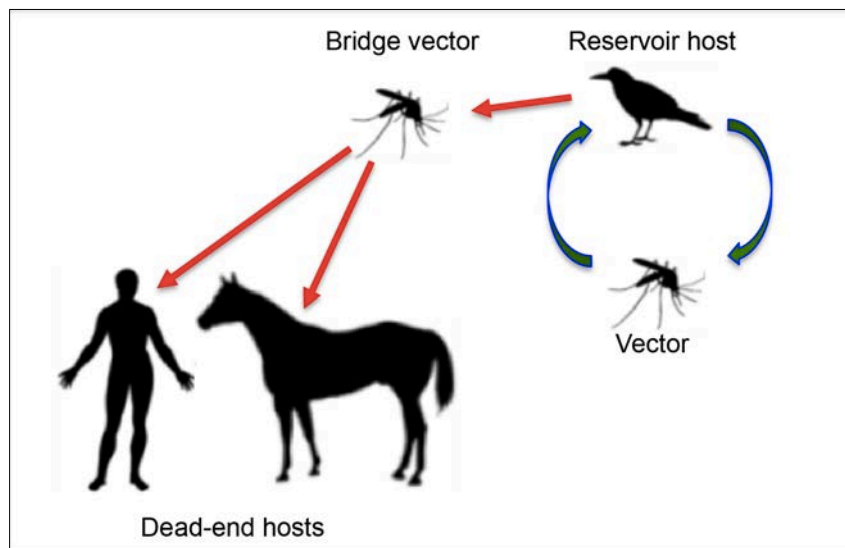


Figure I1.2. Transmission cycle of Eastern Equine Encephalitis virus. The enzootic cycle occurs in hardwood swamps where the vector transmits the virus among passerine birds. Other vectors are believed to transmit the virus to humans and horses. These develop little viremia and do not contribute to amplification. Adapted from Weaver (2005).

Viruses such as DENV or CHIKV represent important exceptions to this rule. They can reach high levels of viremia and can be transmitted from humans to other humans by mosquitoes (urban cycle) (Weaver and Barrett, 2004).

The incidence of arboviral-induced neuroinvasion varies annually depending on vector abundance, geographical location, climatic conditions, and human behavior. As disclosed by the Centers for Disease Control and Prevention (CDC), in 2013 over 2600 cases of arboviral diseases were notified in the United States, 53% of which were reported as neuroinvasive. WNV was the most prevalent neuroinvasive arbovirus, with over 2400 reported cases (Lindsey *et al.*, 2014). Preliminary data on WNV infection in 2015 are in accordance with previous reports, showing over 60% neuroinvasiveness (Figure I1.3).

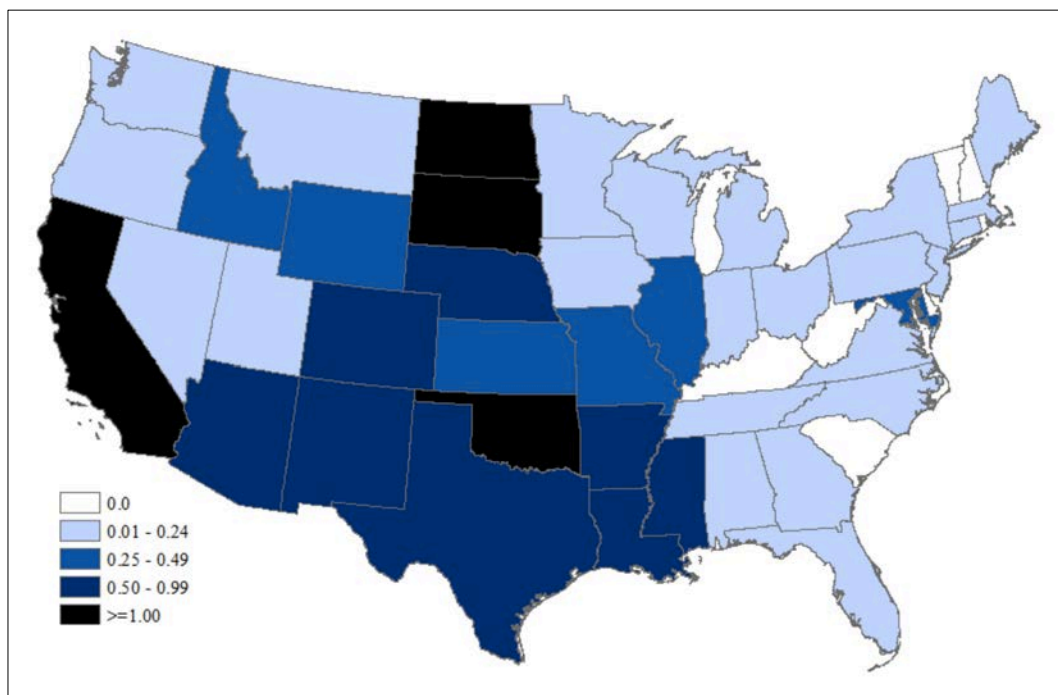


Figure I1.3. Incidence of human West Nile virus neuroinvasive disease by State as of November 2015 – United States. Ranging from 0.01 to greater than 1.00 per 100,000 population. Source: CDC.

1.2. Arbovirus-Induced Neurological Pathogenesis

As can be expected, morbidity and mortality from CNS arboviral infections are significantly high and, depending on the pathogen, can lead to fatality rates ranging between 1% and 70% (Hollidge *et al.*, 2010). When not fatal, these

infections lead to long-term sequelae in 90% of affected patients and include conditions such as cognitive deficits, blindness, epilepsy, flaccid paralysis of limbs, focal neurologic deficits, and permanent vegetative state (Rust, 2012).

The neurological manifestations that can arise upon infection of vector-borne viruses include myelitis, encephalomyelitis, neuritis, myositis, and more often meningitis or encephalitis. WNV, for example, most commonly induces encephalitis but can also lead, in some cases, to meningitis and/or acute flaccid paralysis (Tyler, 2009).

While meningitis involves inflammation of the tissues lining the brain, encephalitis is an acute inflammation of the brain parenchyma, and myelitis an inflammation of the spinal cord, both typically due to the infection of neurons belonging to the CNS. This in turn leads to neuronal cell death. *Human Enteroviruses* and *Alphaviruses* are known to induce apoptosis in infected neurons (Kuo *et al.*, 2002; Jan and Griffin, 1999). In the case of *Flaviviruses*, the induced cellular immune response upon CNS infection is instead considered to play a greater role in disease pathogenesis, such is the case of Japanese Encephalitis virus (JEV) (German *et al.*, 2006). Other neurotropic viruses (not restricted to arboviruses) can cause damage by disrupting the blood-brain barrier (BBB), inducing autoimmune attack on specific cell types, altering gene expression, causing cell fusion, altering neuronal migration, reducing neural progenitor replication, and blocking cerebrospinal fluid (CSF) generation.

Despite many years of study, it is still not completely understood why only a certain percentage of patients develop serious neurological symptoms after infection. A fundamental factor certainly involves the efficiency of one's immune system in eliminating the pathogen from the whole body. It has been shown, for example, that mutations in the *ccr5* gene (a chemokine receptor and co-receptor for Human Immunodeficiency virus (HIV)) increase the incidence of symptomatic WNV infection (Glass *et al.*, 2006). Another important aspect to consider is the age of the patient at the time of infection. Intrauterine and newborn infections are generally more deleterious as opposed to those affecting adults, most probably due to the incomplete maturation of the BBB. Maturation of the innate and adaptive immune system is also an important aspect to consider (Kollmann *et al.*, 2012). Moreover certain viruses selectively target dividing neuronal precursors. In

a neuronal cell culture model of *Alphavirus* infection, it was shown for example that terminally differentiated, mature neurons express higher levels of immunoregulatory factors that help them mount a more rapid antiviral response in contrast to undifferentiated neurons (Schultz *et al.*, 2015).

1.3. Mechanisms of Viral Entry in the CNS

Upon establishment of the infection at their entry site, most viruses spread locally in a cell-to-cell manner. After a first cycle of replication in target cells, the virus disseminates. Primary viremia – the presence of a virus in the blood – ensues. It is generally short-lived and of low titer and contributes to the spreading of the virus from peripheral tissues to other sites, such as to the lymphoid organs, leading to secondary viremia. This in turn lasts typically for longer periods of time and is higher in titer (Pekosz *et al.*, 1995) (Figure I1.4).

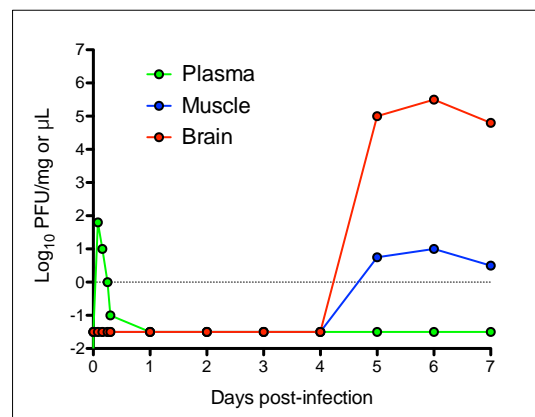


Figure I1.4. Primary viremia, after injection of La Crosse virus in weanling mice. Viremia appears as a sharp peak limited to the first 12 hrs. Even if transient, viremia is sufficient to initiate a lethal encephalitis after delivery of the virus to the target organ. Adapted from Pekosz *et al.* (1995).

After the onset of viremia, neurotropic viruses must exit the bloodstream to be able to reach and invade the CNS. The detailed mechanisms of exit from the bloodstream are poorly understood, mostly due to the difficulty in following the progression of an infection *in vivo*. Nevertheless, neurotropic viruses have been shown to reach the CNS either via the hematogenous route or via infection of peripheral nerves. In the former case, they may travel free in the plasma (plasma viremia) or in association with cells (cell-associated viremia), before crossing the

BBB. On the other hand, infection through peripheral nerves generally occurs upon binding of the virus to specific receptors found on axonal termini of peripheral neurons. Each of these mechanisms will be described in more details in the following sections.

One important aspect to consider when investigating mechanisms of viral entry in the CNS is the inoculation route used in experimental animal models. Certain viruses can in fact employ more than one mechanism; WNV, for example, has been shown to enter the CNS through infection of endothelial cells of the brain vasculature as well as through infection of olfactory neurons (Xu *et al.*, 2012; Monath *et al.*, 1983). Therefore, during sampling and analysis all cases should be taken into account and thoroughly investigated, to avoid missing important cues.

1.3.1. Infection of Brain Microvascular Endothelium

Several viruses are known to enter the CNS by infecting Brain Microvascular Endothelial Cells (BMVECs) of the BBB. Subsequently, they induce disruption of BBB integrity and unrestrained migration of cells of the immune system.

Under physiological conditions, the BBB is principally constituted by an interface of glial and vascular cells tightly joined to restrict the movement of solutes and cells from the bloodstream to the brain parenchyma. The endothelial cells lining the microvasculature within the CNS are adjoined by tight and adherens junctions, and receive support from pericytes and astrocytes for their homeostasis. Together with perivascular macrophages, microglia, and neurons these structures constitute the neurovascular unit (Figure I1.5).

Upon infection of BMVECs, several neurotropic viruses have been shown to alter junction expression and functionality as well as to induce expression of inflammatory mediators by other BBB cell types. Examples of these viruses include DNA viruses, such as John Cunningham virus (JCV), a human polyomavirus (Chapagain *et al.*, 2007), Epstein-Barr virus (Casiraghi *et al.*, 2011), Human Cytomegalovirus (Fish *et al.*, 1998), and Mouse Adenovirus (Gralinski *et al.*, 2009). RNA viruses, such as WNV (Xu *et al.*, 2012), Hepatitis C virus

(Fletcher *et al.*, 2012), and Human T cell leukemia virus (HTLV) (Afonso *et al.*, 2008), have also been shown to affect BBB integrity.

In some cases, viruses can access the CNS by crossing areas where the BBB is not completely sealed. These include the choroid plexuses and the circumventricular organs¹. Viruses can enter the CSF space and subsequently infect the underlying ependymal cells and surrounding brain tissues. Examples of such viruses are mumps virus (Herndon *et al.*, 1974) and HIV (Falangola *et al.*, 1995).

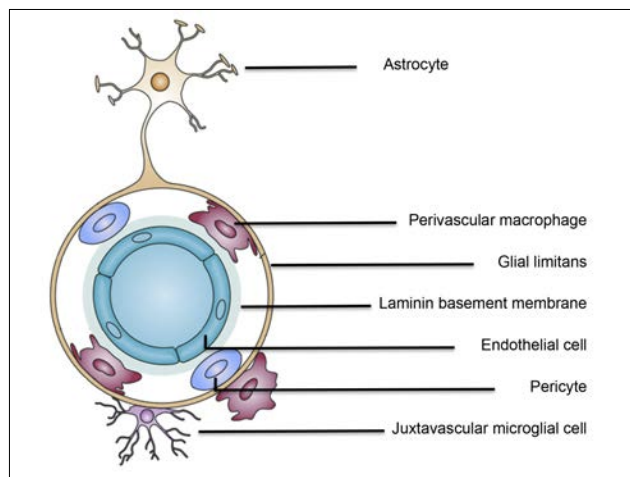


Figure II.5. Cellular components of the BBB. The barrier is formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular endfeet. Astrocytes provide the link to neurons. Adapted from McGavern and Kang (2011).

1.3.2. Infection of Leukocytes: the “Trojan Horse” Strategy

The second possible mechanism through which viruses can gain access to the CNS is the so-called “Trojan horse” strategy. Circulating leukocytes such as monocytes/macrophages become infected and are able to release the virus in the CNS after infiltrating the brain parenchyma, where they normally act as immune sentinels. Once they reach the CNS, they shed infectious virions that establish CNS infection. Examples of such viruses include HIV and HTLV (Kaul *et al.*, 2001). JCV has also been shown to employ this mechanism, although it rather infects B cells that infiltrate the CNS of immune suppressed patients, causing a

¹ Circumventricular Organs: Small structures bordering the subventricular spaces characterized by larger perivascular spaces and endothelial cells lacking tight junctions. They allow for greater exchange of fluids between the brain and the vasculature. From Johnson and Gross (1993).

fatal brain disease (Boothpur and Brennan, 2010). Moreover, in a previous report it was shown that human macrophages become infected by SINV *in vitro* (Assunção-Miranda *et al.*, 2010). This has been however linked to the onset of arthritis in humans. To our knowledge, their contribution to SINV neuroinvasion in *in vivo* models has not yet been addressed.

1.3.3. Infection of Peripheral Neurons

Several pathogens infect and replicate in local nerve endings. These can either be sensory or motor neurons, extending outside the confinement of the CNS. Nevertheless, they are connected to neurons of the CNS (through neurochemical synapses) and act as springboard for efficient entry and replication in the brain. According to their specific receptor and entry route, neurotropic viruses will bind to either sensory or motor neurons and use anterograde and/or retrograde axonal transport systems to reach neurons in the CNS.

RV (Ugolini, 2011) and Poliovirus (PV) (Racaniello, 2006) are well-known examples of viruses that infect peripheral motor neurons. Subsequently, they use dynein-based components to retrogradely move along the axons up to the CNS. On the other hand, herpesviruses use sensory-nerve endings as entry portal to the Peripheral Nervous System, where they maintain a quiescent state. Under certain conditions, such as stress stimuli, they can become reactivated and induce production of a large number of virus particles in a short amount of time (Camarena *et al.*, 2010) that can reach the CNS via retrograde transport.

Infection of the CNS can also occur from invasion of olfactory nerves, whose projections are found in the olfactory bulb of the CNS. Examples of such viruses include Influenza A virus, Herpes Simplex virus-1 (HSV-1), PV, Vesicular Stomatitis virus (VSV), RV, JEV, WNV, La Crosse virus (van Riel *et al.*, 2015). Munster *et al.* showed that Nipah virus infects the olfactory system of hamsters after intranasal inoculation (Munster *et al.*, 2012). CHIKV (Powers and Logue, 2007) and VEEV (Schafer *et al.*, 2011) have also been suggested to use this entry path, in a model where mice were inoculated intranasally. Additionally, SINV was shown to propagate along peripheral nerves from the foot to the nose or the spinal

cord prior to the brain of mice inoculated subcutaneously. This was determined by intravital imaging approaches using a luciferase-expressing recombinant SINV strain (Cook and Griffin, 2003).

1.4. Mechanisms of Spreading in the CNS

Localization of infection in the CNS, or viral neurotropism, is a direct consequence of cellular susceptibility and viral dissemination, which in turn is preceded by systemic spread (by viremia or the neural route). Upon entry in the CNS, cell-to-cell transmission ensues and the virus starts spreading. Neurotropic viruses may use more than one mechanism, although they generally exhibit cell tropism, by infecting one cell type more readily than others. Viral progression can develop in two prototypical ways:

1. Sequential cellular infection, via axonal transport of virions from one neuron to the connected one
2. Transit via periaxonal accessory cells that surround neurons

Axonal transport is by far the best characterized. In this case, viruses employ cellular transport systems to move along axonal projections in an anterograde and/or retrograde manner (Figure I1.6).

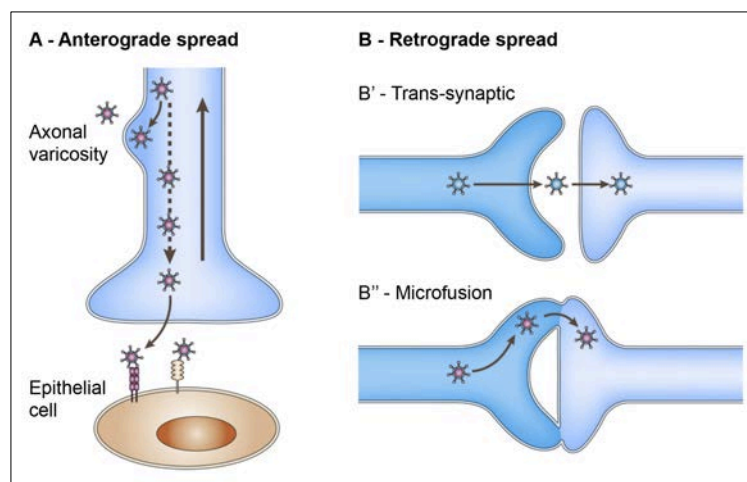


Figure I1.6. Viral axonal spread. (A) Anterograde spread involves movement of the virus from the soma to the synapse and infection of neighboring cells. (B') Trans-synaptic spread allows movement from the synapse to the cell body (*e.g.* RV, PRV). (B'') Microfusions between neighboring cells allow retrograde spreading of measles virus. Adapted from McGavern and Kang (2011).

Anterograde transport uses the kinesin motor proteins, to move cargo from the soma to the synapse. HSV-1, for example, undergoes anterograde transport to exit via axonal varicosities at axon termini and infect neighboring cells (Diefenbach *et al.*, 2008). On the other hand, retrograde transport involves movement of cargo and viral particles from the synapse to the soma. This type of transport relies on proteins of the dynein family. RV interacts with the LC8 dynein light chain (Raux *et al.*, 2000) and HSV-1 uses trans-synaptic release to reach connected neurons, while measles is thought to disseminate through microfusions between neighboring neurons (Young *et al.*, 2009).

1.5. Antiviral Immune Responses in the CNS

The brain is generally considered an immunologically privileged site, as neuronal cells are normally protected from the dangerous side-effects of inflammatory and immune responses. Infected cells are normally spared from immunological effector mechanisms if they are located in the brain. This in turn might contribute to virus persistence in the brain. Immune cell entry restriction exerted by the BBB and limited expression of Major Histocompatibility Complex² (MHC) class I molecules from neurons – that renders them unable to activate virus-specific cytolytic T lymphocytes – also account for virus persistence in this organ.

The brain must therefore employ virus-specific immune mechanisms for the clearance of the pathogen that do not involve cytolytic elimination of the infected cell and consequent CNS damage. While DNA viruses undergo replication in the nucleus and have the capacity to remain latent, most RNA viruses (such as neurotropic arboviruses) replicate in the cytoplasm and do not establish latency³. Unlike DNA viruses, they are therefore more susceptible to immune control mechanisms that are readily activated in the CNS upon detection of infected cells.

² Major Histocompatibility Complex: A set of cell surface proteins interacting with either CD4⁺ T cells or CD8⁺ T cells to mediate initiation of adaptive immunity.

³ Viral Latency: The ability of a virus to remain dormant within the host cell, sometimes establishing lifelong occult infection. The viral genome can remain latent either integrated in the host chromosome or as an episome. Stress cellular signals are often promoters of viral genome reactivation, termination of viral latency and re-establishment of infection.

Production of type I Interferon (IFN) cytokines constitutes the first line of defense against viral infections and it is vital for the survival of the infected host. Mice lacking the IFN receptors (IFNAR^{-/-}) show in fact a greater susceptibility to fatal disease, including to infections of the CNS (Muller *et al.*, 1994). Nevertheless, basal IFN activity seems to be low within the CNS, likely due to the toxicity of this cytokine to the brain (A detailed description of the IFN signaling pathway will be given in Chapter III).

Several studies have also shown the importance of molecules upstream or downstream of the IFN signaling pathways that become expressed in the CNS upon viral infection. In the case of upstream molecules, several Toll-like Receptors (TLRs) are widely expressed and have been shown to respond to neurotropic viruses (Carty and Bowie, 2011). Mutations in the endoplasmic reticulum UNC-93B protein (required for TLR3 signaling) (Casrouge *et al.*, 2006) and in TLR3 (Zhang *et al.*, 2007) have been in fact associated with onset of Herpes Simplex Encephalitis. Downstream of IFN expression, Interferon Stimulated Genes (ISGs) have been found to be either specifically expressed in the CNS (*e.g.* ISG54 in the case of VSV) or in specific regions of the CNS (*e.g.* Ifi27 and Rsad2/viperin/vig1, against WNV) (Fensterl *et al.*, 2012; Cho *et al.*, 2013).

Depending on the virus, IFN can be produced by several cell types. In the case of La Crosse virus infection, for example, neurons are mostly responsible for IFN production (Delhaye *et al.*, 2006), while Mouse Hepatitis virus induces IFN production mainly in macrophages and/or microglial cells (Roth-Cross *et al.*, 2008).

IFN production is fundamental to counteract viral replication and spread as well as for the subsequent induction of an adaptive immune response. The activation of the IFN signaling pathway in fact contributes to the upregulation of MHC class I molecules on the surface of microglial cells and of adhesion molecules by BMVECs. Subsequently, several induced cytokines (*e.g.* Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-12 (IL-12), Tumor Necrosis Factor- α (TNF- α)) are transcytosed to the endothelial lumen, where they are presented to attract circulating leukocytes (Chang *et al.*, 2000; Middleton *et al.*, 2002). Infiltration of leukocytes begins at late stages of infection. Cells normally accumulate in

perivascular areas of the brain and later infiltrate the brain parenchyma in regions of virus infection, to subsequently induce the activation of the adaptive immune response. The peak of inflammatory cell accumulation generally occurs within two weeks after infection and, unless the virus persists, they are gradually eliminated (Rowell and Griffin, 1999). It has been shown, for example, in a mouse model of SINV infection that the virus can persist in the brain of mice that recovered from viral acute encephalitis for long periods of time in a non-productive form. Moreover, viral reactivation was associated with low levels of humoral immunity, suggesting the importance of antibody-mediated clearance (Levine and Griffin, 1992).

Summary of Chapter I

- The majority of EIDs are characterized by serious neurological manifestations and are mostly caused by zoonotic pathogens.
- Neurotropic arboviruses are kept in complex enzootic cycles but can sometimes escape, due to adaptation to a different vector. When humans or other vertebrates become infected, they are generally dead-end hosts.
- Neurotropic viruses often cause encephalitis, myelitis or meningitis.
- They can reach the CNS either via the bloodstream (by disrupting the BBB or by hijacking leukocytes) or via infection of peripheral nerves.
- Once in the CNS, they can spread via several mechanisms, including anterograde or retrograde transport along axons of infected neurons.
- Antiviral immune responses activated in the CNS differ from those in the periphery due to the non-renewable features of neurons. IFNs are the first cytokines to be expressed, in order to induce the antiviral state.

CHAPTER II: Alphaviruses – A Public Health Threat

“Chikungunya Hits U.S. Mainland...

The first locally acquired cases of chikungunya were reported in Florida on July 17, 2014. These cases represent the first time that mosquitoes in the continental United States are thought to have spread the virus to non-travelers. Though CDC does not expect widespread cases this year, Americans infected when traveling to the Caribbean, South America, or the Pacific Islands may continue to return and bring the virus with them.”

Centers for Disease Control and Prevention, USA

Together with *Rubiviruses*, *Alphaviruses* are members of the *Togaviridae* family. As the etymology of the name suggests (from the Latin name for cape, *toga*), they are characterized by the presence of a lipid envelope, which is perfectly adherent to an icosahedral capsid, and containing a single-stranded positive-polarity RNA genome.

While the *Rubivirus* genus only contains the agent responsible for rubella, rubella virus, the *Alphavirus* genus is divided into seven antigenic complexes based on serological cross-reactivity and contains 29 recognized species (Powers *et al.*, 2001) (Figure I2.1).

Depending on where they occur, they have been classified as either belonging to Old World or New World types. Most Old World *Alphaviruses* are found in Africa, Europe, Asia, and Australia, and include Semliki Forest virus (SFV), Ross River virus (RRV), O’Nyong-Nyong virus (ONNV), Chikungunya virus (CHIKV), and Sindbis virus (SINV). New World *Alphaviruses* are generally found in the Americas, and include viruses such as Mayaro virus (MAYV), Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), and Venezuelan Equine Encephalitis virus (VEEV). This geographic distinction, however, does not always correlate with similar disease symptoms. For example, MAYV is limited geographically to Latin America while ONNV virus has never been identified outside of Africa. Nevertheless, they cause almost identical clinical signs and symptoms. This raises interesting questions regarding the evolution and expansion of the genus.

New World *Alphavirus* infections result in a flu-like syndrome, that often progresses into encephalitis. Old World *Alphaviruses* are generally more benign, resulting in a disease characterized by arthralgia, fatigue and rash. However, RRV, CHIKV and SINV have sometimes been associated with encephalitis. Neurovirulence of these viruses is associated with rapid and efficient spread throughout neurons of the Central Nervous System (CNS), resulting in death of infected neuronal cells (Griffin, 2007).

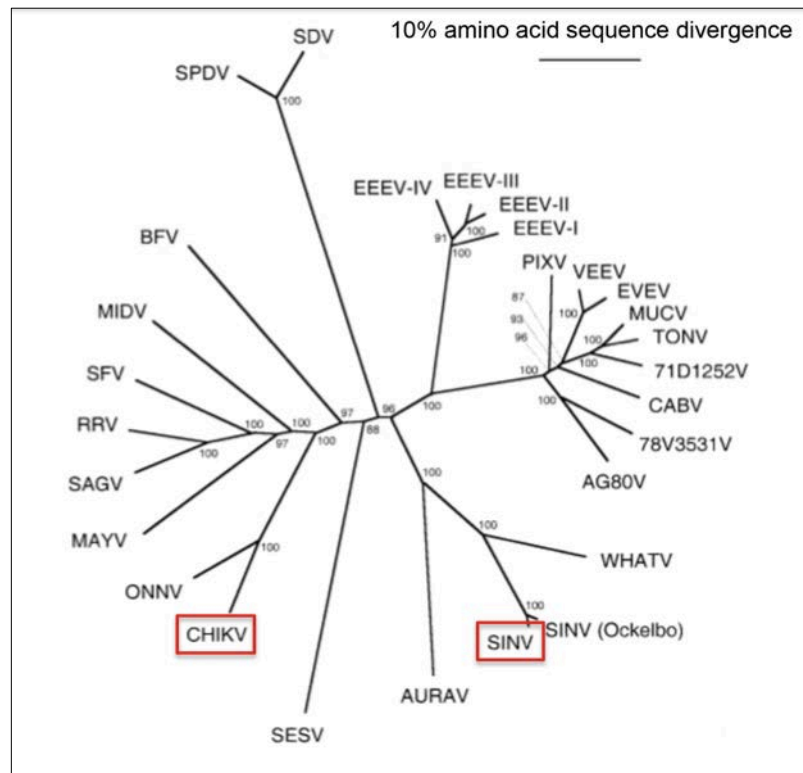


Figure I2.1. Phylogenetic tree of *Alphaviruses* from complete structural polyprotein amino acid sequences. From Powers *et al.* (2011).

As can be expected, their pathogenicity, the global spread of the mosquito vectors as well as the lack of effective treatments and vaccines, make them a significant public health threat as both emerging infectious diseases and possible agents of bioterrorism (Weaver, 2005).

This introductory Chapter includes an overview of the molecular biology of *Alphaviruses*. Subsequently, it focuses on several important aspects related to Old World *Alphaviruses*, including epidemiology, pathogenesis and immune responses. SINV, the pathogen used in this Thesis as a model to study viral-induced encephalitis in zebrafish embryos, is described in more details in the

last section. Its importance for the study of viral-induced pathogenesis is well exemplified by the number of articles in which it appears as a model – over 60 articles published within the last year (*e.g.* ranging from studies on mechanisms of viral assembly and viral immune evasion, distinct immune responses, potential therapeutics). Nevertheless, many unanswered questions related to its neurotropism constitute the driving factor for the study presented in this Thesis.

2.1. Alphavirus Structure, Genome Organization and Replication Cycle

The virions of *Alphaviruses* are spherical and measure ~70 nm in diameter. They are composed of a host cell-derived lipid bilayer, two membrane-associated glycoproteins (E1 and E2) bound with E3, and a nucleocapsid enclosing the viral genome. 240 monomers of capsid protein are arranged together to form a T = 4 icosahedral structure, studded with 80 heterotrimeric glycoprotein spikes.

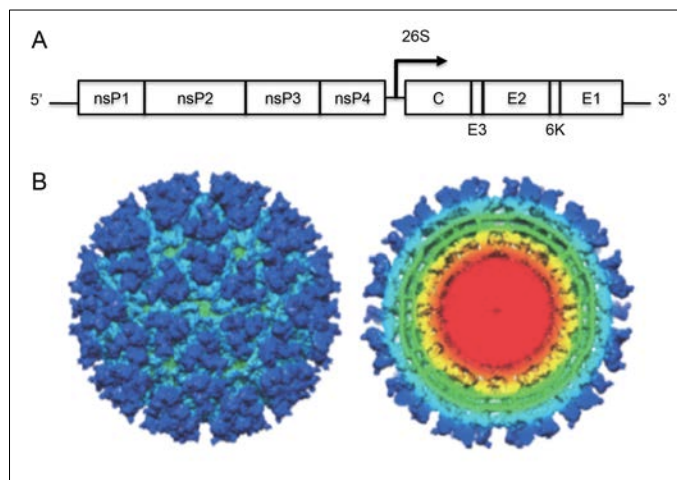


Figure I2.2. *Alphavirus* genome and virion structure. (A) The genome is single-stranded positive-sense RNA, encoding two Open Reading Frames. (B) Left, surface shaded view of SINV at 9Å resolution. Trimeric spikes are seen in blue, the small portions of the lipid bilayer are seen in green. Right, central cross-section of the virus showing in blue the glycoproteins, in turquoise the envelope, in green the lipid bilayer, in yellow the capsid, and the RNA region in red. Adapted from Jose *et al.* (2009).

The genome is roughly 12 kb long, it includes a 5' terminal cap and a 3' terminal poly(A) tail, and it is divided into two Open Reading Frames (ORFs) separated by a well-conserved Untranslated Region (UTR) of ~120 nucleotides. Each ORF codes for multiple proteins generated by proteolytic cleavage. The 5' ORF, which

is immediately translated upon virus entry, codes for four non-structural Polyproteins (nsP1-nsP4) required for transcription and replication. By contrast, the 3' ORF is not translated from the full genome, but from the subgenomic 26S RNA, which is made later in the viral cycle; it codes for the structural proteins (sPs): the capsid (C), the three envelope proteins (E3, E2, and E1) and a membrane-associated protein, 6K (Figure I2.2).

The *Alphavirus* nsP1 possesses both guanine-7-methyltransferase and guanylyl transferase activities, required for capping and methylation of newly synthesized genomes. nsP2 acts as triphosphatase and helicase on its N-terminal domain, and as protease for the nsPs on its C-terminal domain. nsP3 has been shown to act as phosphatase and is involved in modulation of pathogenicity in mice, while nsP4 is the RNA-dependent RNA-polymerase (RdRp).

While E3 is required for efficient particle assembly, E2 is known to be responsible for receptor binding as well as a determinant of neurovirulence, as demonstrated by studies on amino acid changes (Ubol *et al.*, 1994). 6K is essential for viral particle assembly at the plasma membrane and E1 is the *Alphavirus* fusion protein.

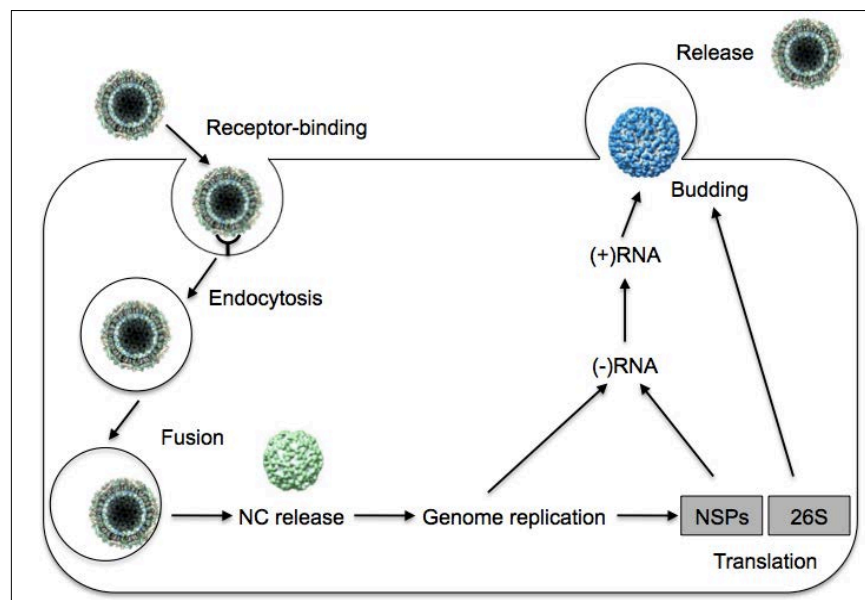


Figure I2.3. *Alphavirus* life cycle. Upon receptor binding and clathrin-mediated endocytosis, the viral particle fuses with the endosomal membrane. The resulting nucleocapsid is transported to the cytoplasm, where the RNA is released after disassembly. Genomic RNA is used both for translation of proteins from the genomic and subgenomic RNA, as well as for transcription of nascent RNA. Lastly, the structural proteins are translated and encapsidate nascent genomic RNA before budding and release. Adapted from Leung *et al.* (2011).

Upon receptor-mediated binding to the cell surface and subsequent entry, viral particles undergo disassembly to release the genome into the cytoplasm (Figure I2.3). The viral genome is then translated to generate the nsPs and sPs. After complete cleavage of the nsPs, they form a stable complex for the synthesis of negative strand antigenomes, then used as a template for synthesis of positive-strand genomic and subgenomic RNAs. Except for E2-E3, cleavage of the sPs occurs simultaneously with their translation. The C protein subsequently associates with newly synthesized RNA genomes. The E1/(E2+E3) complexes are then transported from the endoplasmic reticulum; in the Golgi, furin cleaves the E2-E3 bond. At the plasma membrane, the mature E1-2-3 heterotrimers interact with the C protein and form an envelope around nucleocapsid-like particles. Thus, upon release from the infected cell, virions have acquired a membrane bilayer from the host cell plasma membrane (Leung *et al.*, 2011) (Figure I2.4).

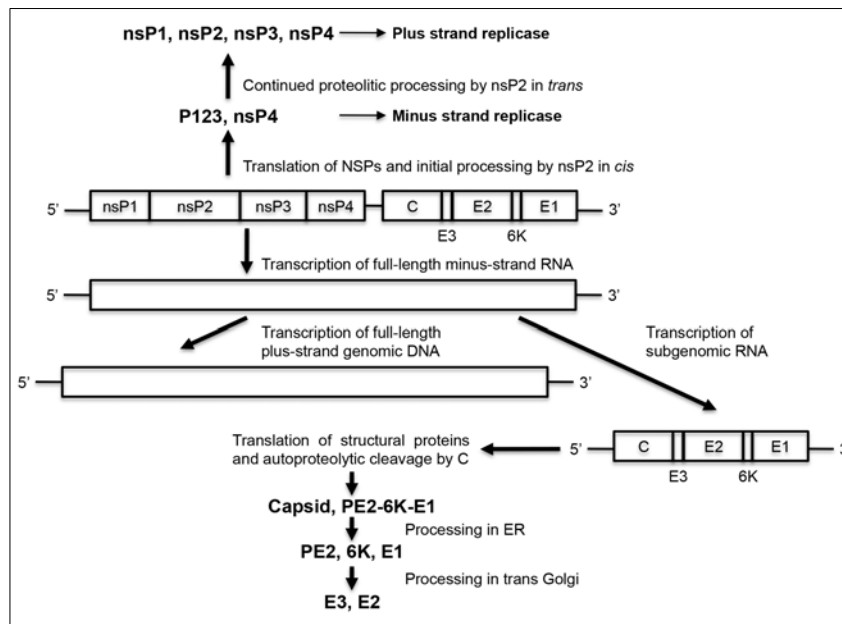


Figure I2.4. Schematic diagram of *Alphavirus* genome replication. Non structural proteins are translated and processed first. Later, after transcription of the subgenomic RNA, the structural proteins can be translated and processed. Adapted from Ryman and Klimstra (2008).

2.2. Old World Alphaviruses: Epidemiology and Vector Distribution

Alphaviruses are kept in enzootic and epizootic cycles between blood-feeding arthropods and vertebrates. Vertebrate hosts can be divided into two groups: 1. the natural vertebrate host acting as the primary source of mosquito infection in which

the virus replicates, but no symptomatic disease occurs and 2. vertebrate hosts that develop symptomatic disease.

For encephalitic *Alphaviruses* such as SINV, SFV, EEEV, and WEEV, the primary host is typically avian. In epizootic or epidemic cycles, infected mosquitoes transmit the virus to a secondary host, usually human or equine. Large outbreaks of disease occur when infected secondary hosts reach a high titer viremia, permitting further infection of mosquitoes and host populations.

Recurrent epidemics of both Old and New World *Alphaviruses* have been reported since the 20th century. WEEV was the first encephalitic *Alphavirus* isolated from the brain of an infected horse (Meyer *et al.*, 1931).

CHIKV was first isolated in 1953 in Tanzania, Africa and has since then caused frequent epidemics in Africa, Islands of the Indian Ocean, and Asia (Powers and Logue, 2007). Between 2004-2011, the largest epidemic of CHIKV ever recorded took place. It began in Kenya, spread to Islands in the Indian Ocean, India, and South East Asia and was attributed to a new adaptation of the virus to the mosquito *Aedes albopictus*, a vector with a wide geographic distribution. In late 2013, local transmission of CHIKV was reported for the first time in the Caribbean countries, and has ever since moved to the United States (Florida, Puerto Rico, and the U.S. Virgin Islands) and Polynesia.

SINV was first isolated in 1952 in Cairo, Egypt and is most commonly found in Africa, the Philippines, and Australia. SINV is widespread also in Finland, Sweden, and Russia, where it has been associated with Pogosta, Ockelbo and Karelian fever diseases respectively. SINV epidemics in Finland have been reported in approximately seven-year cycles. Specific climatic conditions and density of the primary host, the black grouse (*Tetrao tetrix*), are considered important factors for the occurrence and incidence of SINV infections in humans, together with the presence of the primary vector (Jalava *et al.*, 2013). This has been identified in members of the mosquito genera *Culex* and *Culiseta*. However also *Aedes* mosquitoes (Lvov *et al.*, 1985) and, in a few cases, ticks have been found to carry and transmit the virus (Gresikova *et al.*, 1978).

A study conducted on the phylogeographic evolution of SINV has shown that over 50 different SINV strains exist (Lundström and Pfeffer, 2010) but only half

of them are pathogenic in humans. SINV strains have been grouped into five genotypes, based on E2 sequence divergence (Figure I2.5).

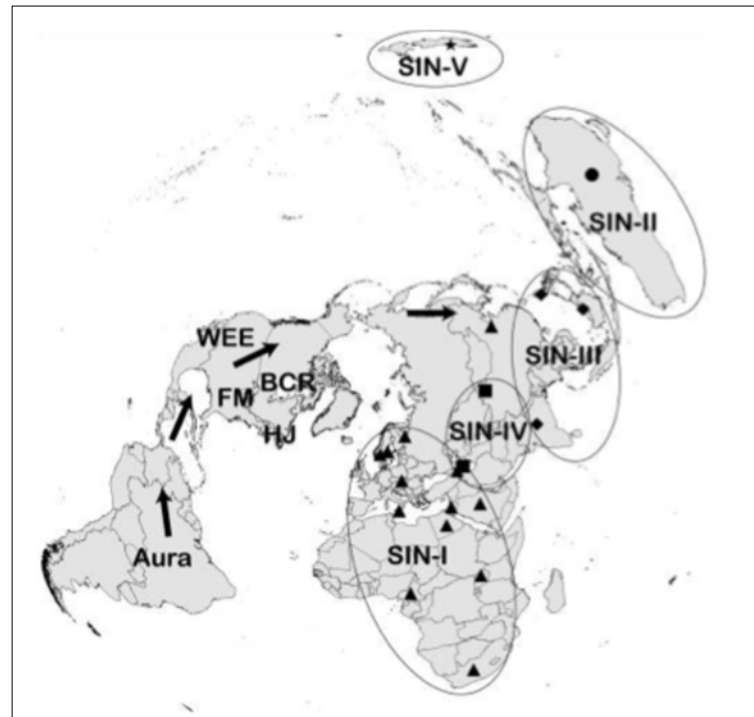


Figure I2.5. Geography of SINV genotypes (I-V), and suggested route of ancient SINV dispersal from South America to East Asia. Other *Alphaviruses* included: Aura, Aura virus; WEEV, Western Equine Encephalitis virus; HJV, Highland J virus; BCV, Buggy Creek virus; FM, Fort Morgan virus. From Lundström and Pfeffer (2010).

Their geographic distribution has been attributed to major bird migration patterns, validating the hypothesis that migrating birds are involved in large-scale viral dissemination (Herrera, 1978). Moreover, SINV-associated disease outbreaks in South Africa, Sweden, Finland and Russia, are in accordance with the distribution of one of these five genotypes (Brummer-Korvenkontio *et al.*, 2002).

2.3. Pathogenesis of Old World Alphaviruses

Old World *Alphaviruses* are generally associated with the development of long-lasting arthritis, as opposed to their New World counterparts, for which severe neuropathogenic diseases often result from the infection. After the 2005-2006 epidemic of CHIKV, however, there has been much debate as to whether Old World viruses can induce neurological symptoms, due to reports of

severe cases of encephalitis, neuropathies, and deaths in infants and elderly patients (Economopoulou *et al.*, 2009). Moreover, experimental models for other *Alphaviruses*, such as SFV, RRV, EEEV and SINV, have shown similar neurological signs reminiscent of those observed in CHIKV infected patients (Fazakerley *et al.*, 2006; Mims *et al.*, 1973; Deresiewicz *et al.*, 1997). Among these, SINV is the best characterized and has been widely used in mice to perform studies on neural pathogenesis of *Alphaviruses*, upon which it has been shown that it replicates in neural tissues causing encephalitis (Lewis *et al.*, 1996; Cook and Griffin, 2003).

The disease progression in humans has been characterized as typically consisting of two phases: first, patients generally experience a mild, flu-like illness associated with replication at the primary site of infection, viremia, and initiation of the immune response. The second phase involves a second febrile episode directly linked with dissemination of the virus to other tissues (Zacks and Paessler, 2010). During the acute phase of the disease, several symptoms other than fever can occur, such as headache, eye pain, generalized myalgia, arthralgia, diarrhea, vomiting, and rash (Taubitz *et al.*, 2007; Powers and Logue, 2007).

During this phase, a number of patients develop neurological manifestations associated with mortality. Even though rare, Old World *Alphavirus*-induced neurological symptoms reported during the 2005-2006 CHIKV outbreak include encephalopathy associated with lymphopenia (Lemant *et al.*, 2008), seizures, encephalitis, optic neuritis (Mittal *et al.*, 2007), bilateral frontoparietal white matter lesions (which are considered as early signs of encephalitis), and lymphocyte infiltration in areas of demyelination and microglia activation in the gray matter with neuronal degeneration (Ganesan *et al.*, 2008).

Moreover in some cases, dual infections of *Alphaviruses* with other endemic viruses can occur. Co-circulation of CHIKV and Dengue virus (DENV), for example, was reported in various States of India in 1964 and more recently in 2010. Data from the 2010 outbreak showed that many of the observed clinical symptoms were common in both monotypic and dual infections (Taraphdar *et al.*, 2012). This not only increases the chances of a wrong diagnosis but also reduces the likelihood for the patient to reach a full recovery.

2.4. Role of Innate Immunity against Alphaviruses

Most of the data relative to the host response elicited from *Alphavirus* infections come from studies in small animal models and, in a few cases, non-human primates. It is general agreement that, upon onset of infection, type I Interferons (IFNs), mostly IFN α and IFN β , are readily expressed and followed by production of virus-specific neutralizing antibodies at later time points.

The protective role played by type I IFNs against *Alphaviruses* has been demonstrated in several studies. Ryman *et al.* showed that adult mice lacking the IFN receptors (IFNAR^{-/-}) infected with a neurovirulent SINV strain (NSV) succumb to the infection, as opposed to wild-type (WT) mice. Moreover, they were able to show that the lack of a fully functional IFN signaling pathway modifies the viral cell and tissue tropism and renders more cell types susceptible to the infection (Ryman *et al.*, 2000). Very similar results have been obtained in the case of CHIKV infection. Couderc *et al.* showed in fact that IFNAR^{-/-} adult mice are highly susceptible to CHIKV infection and that the clinical signs observed were in accordance with the cell types and tissues infected (Couderc *et al.*, 2008). Labadie *et al.* developed a cynomolgus macaque (*Macaca fascicularis*) model of CHIKV, in which infection was detected in several organs, such as in the liver, the spleen, the lymph nodes, the joints, the muscle, the skin and the meninges (Labadie *et al.*, 2010). Moreover, the authors showed that macrophages can act as a CHIKV reservoir and suggested that viral persistence accounts for the chronic CHIKV infection symptoms that are often observed in patients.

As can be expected, this group of viruses has developed evasive strategies to counteract the effects of IFNs. An *in vitro* study on SINV-infected cell cultures has shown that this role is played by nsP2 proteins, as they induce host cell transcription and translation shutoff (Frolova *et al.*, 2002). Studies performed on CHIKV-infected Human Embryonic Kidney-293 cell cultures have shown that the virus promotes activation of the autophagy machinery to enhance its own replication (Krejchich-Trotot *et al.*, 2011). In a comparative study between mouse and human cell cultures, it was shown that ubiquitinated capsids are bound to p62 (in both human and mice cells) and transported to the autophagolysosomes, thereby protecting infected cells from virus-induced death (Judith *et al.*, 2013). In

contrast, human (but not mice) NDP52 interacts with nsP2 and favors viral replication. In the case of SINV, on the other hand, it was shown that autophagy-mediated clearance of SINV proteins is cytoprotective in neuronal cell types, via p62 and Atg5 (Overdahl and Levine, 2008).

2.5. Sindbis Virus

Sindbis virus, the prototypical *Alphavirus*, has been studied mainly in mice and *Drosophila*. In particular, studies in *Drosophila* have been directed towards a better understanding of RNA interference (RNAi) upon viral infections. Even though mosquitoes develop infection upon virus inoculation, RNAi has been shown to protect the arthropod from an otherwise lethal infection (Cirimotich *et al.*, 2009). In fact, the combined action of cellular reverse-transcriptase activity and RNAi was shown to inhibit viral replication in favor of viral persistence (Goic *et al.*, 2013).

On the other hand, the mouse model has helped in the characterization of virus-induced encephalitis. Despite its relatively low pathogenicity in humans, SINV is known to preferentially infect neurons and causes age-dependent encephalomyelitis in mice (Tucker *et al.*, 1993). The increased survival of adult mice has been attributed, rather than to an incomplete maturation of the immune system, to a lower susceptibility of mature neurons to the virus. These have been found to express higher levels of IFN regulatory factors 3 (IRF3) and 7 (IRF7) molecules and to further induce them upon infection (Schultz *et al.*, 2015). The genetic background of infected animal models has also been shown to determine different immune responses, which correlate with the different susceptibility observed between hosts (Kulcsar *et al.*, 2015).

SINV neurovirulence has been directly linked to specific amino acid changes in the E1 and E2 glycoproteins. In particular, Ala-72-Val and Asp-313-Gly of E1 (from the NSV to a less virulent strain) and His-55-Gln in E2, have been shown to considerably decrease the associated virulence of the strain (Lustig *et al.*, 1988). As a consequence, strains with specific E1 and E2 mutations have been shown to

induce significant levels of apoptosis in brains and spinal cord of weanling but also of older mice (Lewis *et al.*, 1996).

Studies on persistence of the virus after recovery from acute encephalitis have demonstrated that the virus can linger in a non-replicative form for long periods of time and reactivation is dependent on a decrease in the humoral response (Levine and Griffin, 1992). The mechanism through which the virus can be cleared from the CNS is not fully understood, although it has been suggested to be dependent on tissue injury and neuronal cell death induced by infiltrating macrophages/microglia and T helper cells (Kimura and Griffin, 2003).

One important aspect to consider when studying SINV-associated neurovirulence involves the viral strain being used. The comparison of different strains can provide important information not only into the genetic determinants that control the virulence phenotype but also into the observed differences in pathogenesis. Different variants can exhibit great divergence in terms of organ, tissue and cell tropism as well as in the induced antiviral response. Brodie *et al.* showed that infection of mouse astrocytes with either a non-neurovirulent or with the NSV strains resulted in differential cell proliferation and expression of cytokines, which in the case of NSV were associated with changes characteristic of astrogliosis (Brodie *et al.*, 1997).

Moreover, several SINV laboratory strains have been derived over the years. These include for example the NSV strain used in the study of age-dependent virulence in mice (Tucker *et al.*, 1993) as well as recombinant strains, in which fluorescent reporter proteins have been inserted to follow various aspects of the infection process *in vitro* or *ex vivo*. SINV genome is in fact suitable for the insertion and consequent expression of genes of interest (Geiss *et al.*, 2007). In particular, two SINV strains have been employed in this Thesis: 1. *pTE3'2J-eGFP*, in which a second subgenomic promoter has been included at the 3' end for the insertion of specific genes of interest (*i.e.* GFP) (Hahn *et al.*, 1992) and 2. *pTR339-mCherry/2A*, derived from the original African isolate AR339 (Taylor *et al.*, 1955) and modified to include the mCherry reporter gene between the capsid and the E3 genes. Their differences in terms of genomic sequence and associated virulence have been addressed in Chapter II of the Results.

Summary of Chapter II

- *Alphaviruses* are single-stranded RNA enveloped viruses, transmitted to humans after the bite of an infected mosquito. They are normally divided into “arthritogenic” Old World *Alphaviruses* and “encephalitic” New World *Alphaviruses*, although several reports indicate that Old World viruses can sometimes induce neurological manifestations.
- Their genome is divided into two ORFs, coding for the non-structural proteins and structural proteins, respectively. Among the latter, several studies have identified specific amino-acid sequences of the E2 glycoprotein as determinants of neurovirulence.
- The geographical distribution of their vector is an important factor for the onset of an outbreak, as well as adaptation of the virus to new vectors.
- A functional IFN signaling pathway is essential for the survival of *Alphavirus*-infected hosts, although these viruses have evolved evasion mechanisms to subvert the immune response.
- SINV is encephalitogenic in mice and has therefore been employed as a model of *Alphavirus*-induced encephalitis.

CHAPTER III: Zebrafish – Host-Pathogen Interactions Unveiled

“Seeing is believing.”

John Clarke, from Paræmiologia Anglo-Latina (1639)

“I wouldn’t have seen it, if I hadn’t believed it.”

Marshall McLuhan (1911-1980)

The zebrafish (*Danio rerio*) is well established for the study of developmental biology in vertebrates. Studies of embryogenesis in the zebrafish date back to the 1930s and have been followed in the 1980s by works aimed at adapting this organism to forward genetic screens. It was thanks to the works of George Streisinger and Charles Kimmel that the zebrafish became recognized as a valid vertebrate model for the study of gene function and human genetic disease (Streisinger *et al.*, 1981; Kimmel *et al.*, 1989; Lieschke and Currie, 2007). Furthermore, the completion and release of the full zebrafish genome assembly in 2002 and its comparison to the human genome in 2013 (Howe *et al.*, 2013) have greatly expanded the possibility of understanding the role of human genes for which no function has yet been assigned.

More recently, the zebrafish has emerged as an alternative vertebrate model to be used in a wide range of research fields including pharmacology and toxicology, cancer, neuroscience, and disease modeling (Davis *et al.*, 2002; Neely *et al.*, 2002; Phelan *et al.*, 2005; Ludwig *et al.*, 2011). Its main experimental advantage, beyond its genetic tractability, consists in its transparency during early life that considerably facilitates *in vivo* imaging. It is therefore relatively easy to follow the distribution of fluorescent-protein-tagged pathogens in the whole organism in real time. At the same time, small regions with subcellular details can be visualized with modern confocal microscopy techniques and very little invasiveness (Palha *et al.*, 2013).

Its small size, large number of eggs per clutch, and relatively low husbandry costs are also important factors that make zebrafish a valuable model organism. The following Chapter is therefore dedicated to some general aspect of zebrafish biology, with a particular focus on its Central Nervous System (CNS)

development. Subsequently, it discusses some important aspects regarding the development of the zebrafish immune system. Finally, it gives a brief overview of the advantages and disadvantages of the model.

3.1. Zebrafish – General Aspects on Biology and Development

The zebrafish belongs to the family *Cyprinidae*, infraclass *Teleostei*, class *Actinopterygii*. Teleost fish constitute a monophyletic, very successful group of vertebrates with more than 20,000 species, that likely originated 350 million years ago and underwent an additional event of whole-genome duplication, called teleost-specific genome duplication (Meyer and Schartl, 1999).

Zebrafish is a tropical freshwater fish. It is indigenous to the south-eastern Himalayan region and is commonly found in India, Pakistan, Bangladesh, Nepal and Burma. In the wild, zebrafish are thought to be a largely annual species, although when kept in captivity they can survive up to 3.5-5 years. They normally feed on zooplankton and insects, and can breed all year round, although the main period of reproduction occurs just before the onset of the monsoon season (Spence *et al.*, 2007). When kept in captivity, they can easily be led into mating by simulating night/day circadian cycles, with light onset being the trigger. This has become common practice in zebrafish facilities worldwide.

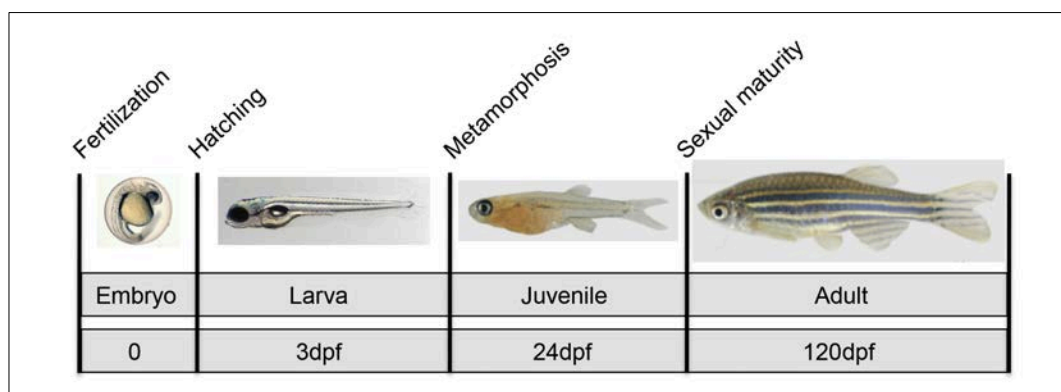


Figure I3.1. Schematic of zebrafish life stages. Adapted from Levraud *et al.* (2014).

The zebrafish is a fast-developing organism. By 3 days post-fertilization (dpf), the embryo has normally hatched and most organs have developed; circulation begins around 26 hours post-fertilization (hpf). Temperature is a very important factor to

assure the proper development of zebrafish. The acceptable range is between 23°C and 32°C and the optimal temperature is 28.5°C (83.3°F), used as the reference for staging (Kimmel *et al.*, 1989). After 3 dpf, zebrafish are generally referred to as larvae; the swim bladder inflates shortly after and by 4-5 dpf, they exhibit autonomous feeding behaviour. At around 24 dpf, zebrafish are considered juvenile (coinciding with the development of scales) and reach sexual maturity around 3-5 months, depending on the environmental conditions, after which are considered adults (Parichy *et al.*, 2009) (Figure I3.1).

3.2. Zebrafish Central Nervous System: Organization and Development

The small size and transparency of the zebrafish larva make it an attractive model to study the formation and function of the vertebrate brain. The fast-growing importance of the zebrafish for the neuroscience field is well exemplified by the increasing number of available bioinformatic resources. Recently, Ullmann *et al.* have developed a three-dimensional digital atlas of the zebrafish brain, which provides detailed spatial information of all neuronal structures (Ullmann *et al.*, 2010). Researchers from University College London and King's College London, in the United Kingdom, have launched *zebrafishbrain.org*, a website with updated neuroanatomical data and resources for the zebrafish research community. Ahrens *et al.* showed that light-sheet microscopy is a powerful tool to detect single-cell neuronal activity while imaging the whole brain *in vivo* (Ahrens *et al.*, 2013). These studies, together with the work performed by Wullimann *et al.*, greatly contributed to our understanding of the zebrafish brain, its organization, and its development (Wullimann *et al.*, 1996).

3.2.1. Zebrafish Brain Anatomy

The zebrafish brain is very similar in its general structures to that of higher animals and can be divided into five regions: the telencephalon, the diencephalons, the mesencephalon, the metencephalon, and the myelencephalon (Speare and Frasca, 2006; Roberts and Ellis, 2001) (Figure I3.3).

Together, the telencephalon and the diencephalon constitute the forebrain, the most anterior region of the brain. The midbrain or mesencephalon is the brain region between forebrain and hindbrain. Finally, the hindbrain, which includes the metencephalon and the myelencephalon, is the most posterior region of the brain.

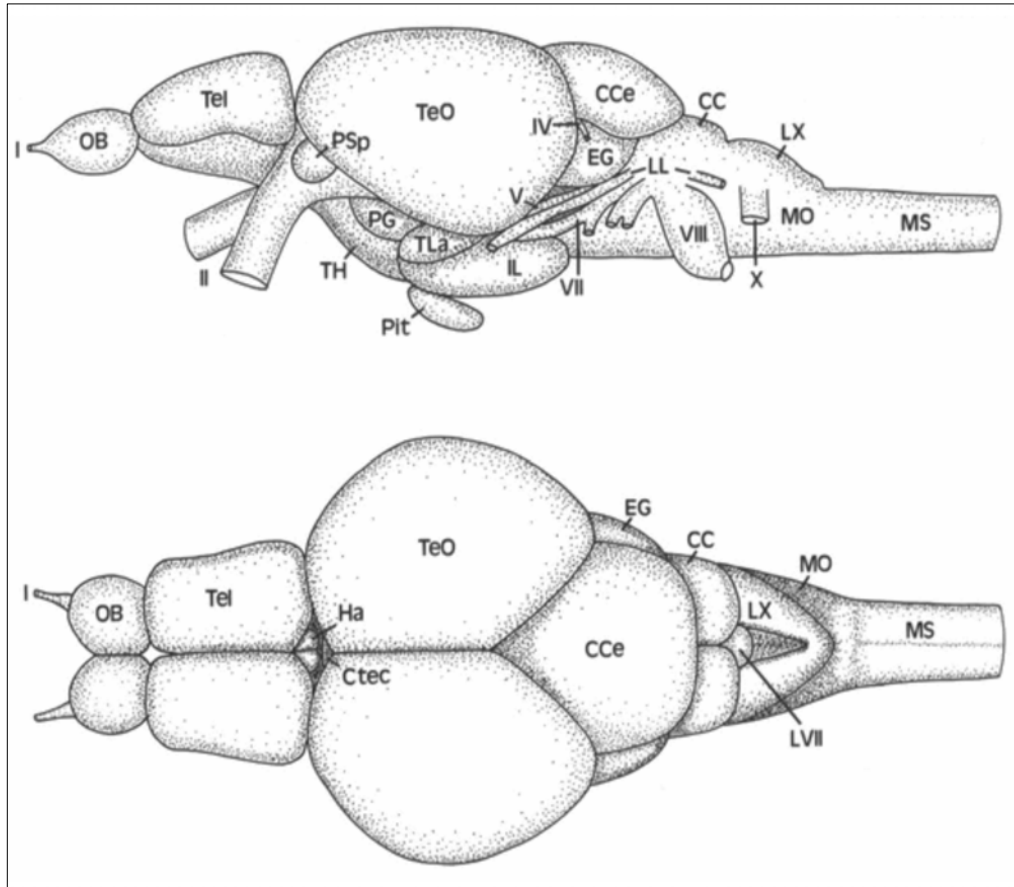


Figure I3.2. (Top) Lateral and (bottom) dorsal view of the adult zebrafish brain. CC, crista cerebellaris; CCe, corpus cerebelli; Ctec, commissura tecti; EG, eminentia granularis; Ha, habenula; IL, inferior lobe of hypothalamus; LL, lateral line nerves; LVII, facial lobe; LX, vagal lobe; MO, medulla oblongata; MS, medulla spinalis; OB, olfactory bulb; PG, preglomerular area; Pit, pituitary; PSp, parvocellular superficial pretectal nucleus; Tel, telencephalon; TeO, tectum opticum; TH, tuberal hypothalamus; TLa, torus lateralis; I, olfactory nerve; II, optic nerve; IV, trochlear nerve; V, trigeminal nerve; VII, facial nerve; VIII, octaval nerve; X, vagal nerve. From Roberts and Ellis (2001).

- The telencephalon controls olfaction, memory, reproductive behavior, feeding behavior, and color vision. The olfactory organ is directly connected to the telencephalon via the olfactory bulb.
- The diencephalon can be subdivided into three components: the epithalamus, the thalamus, and the hypothalamus. The thalamus and the hypothalamus act as correlation centers for sensory inputs such as olfaction and gestation; however, the complete function of the thalamic

region has not yet been identified. Several features of the mammalian thalamus, such as connection to the isocortex, are absent in zebrafish and more studies are needed in order to better define evolutionary differences (Mueller, 2012).

- The mesencephalon is anatomically subdivided into the optic tectum, at the roof, and the tegmentum, at the floor. The optic tectum is relatively large and is divided longitudinally into two globular structures – the corpora bigemina – involved in the reception and the coordination of optic nerve inputs. The zebrafish eye is similar to that of all other vertebrates and consists of three regions: 1. the tunica fibrosa, which encompasses the cornea and the lens 2. the tunica vasculosa, 3. the retina. In the retina, several layers can be distinguished: a. the pigment epithelium, which contains photoreceptor cells, b. the external nuclear layer, with the nuclei of photoreceptor cells, c. the bipolar cells that connect photoreceptor cells to d. the retinal ganglion cells (RGCs). These send their axons through the optic fibers into the optic nerve (Diekmann *et al.*, 2015).
- The cerebellum, or metencephalon, is associated with reception and coordination of proprioceptive and balance stimuli.
- The myelencephalon is the most posterior region of the brain, from which the medulla oblongata develops. The medulla connects the higher regions of the brain to the spinal cord.

3.2.2 Zebrafish Neuronal and BBB Development

The CNS of vertebrates develops from the neuroectoderm, a specialized region of the ectoderm that matures at the end of the gastrulation phase (~10 hpf in zebrafish embryos). Neurulation in zebrafish resembles the secondary neurulation observed in higher vertebrates, characterized by condensation of the mesenchyme to form a rod, which then undergoes an epithelial transition from the neural plate into the neural tube⁴ (Figure I3.3).

⁴ As opposed to Primary neurulation: It involves columnarization of an existing epithelium, and then rolling or folding of the epithelium.

In zebrafish, the first neurons become post-mitotic in the neural plate shortly after gastrulation; therefore embryos start twitching as soon as 1 dpf (Eisen *et al.*, 1986). At 2 dpf, they start to respond to touch, thanks to the action of a few differentiated neurons and a simple scaffold of axons (Kimmel, 1993).

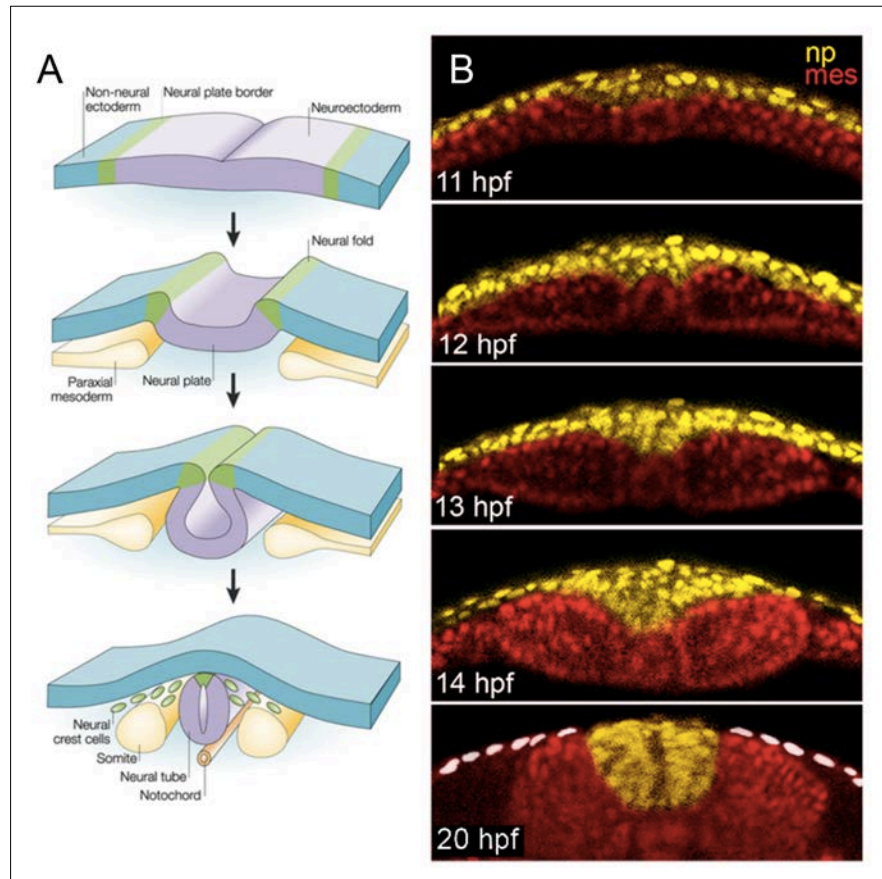


Figure I3.3. Schematic representation of (A) neural crest development and (B) primary neurulation in zebrafish. In (B), mesodermal nuclei are in red, and neural nuclei in yellow. Adapted from Gammill and Bronner-Fraser (2003), and Araya *et al.* (2014).

Early neurons are distributed in three longitudinal columns of the neural plate. Within these columns, only a subset of cells express the HuC marker, typical of differentiated neurons. Expression of the neurogenin1 (NGN1) transcription factor helps define the longitudinal proneuronal domains where cells have the potential to become neurons (Korzh *et al.*, 1998). However, only a subset of cells maintains high levels of NGN1 (Haddon *et al.*, 1998) that allows for the stable adoption of a neuronal fate. In the spinal cord, motor neurons occupy central and ventrolateral positions, interneurons are found at intermediate ones, and sensory neurons develop dorsally (Figure I3.5). Somatosensory neural circuits in the developing

hindbrain are constituted by different trigeminal sensory neurons. These are groups of ~60 neurons, all extending a single axon along the lateral line of the hindbrain and spinal cord. Later they form numerous branches that innervate nuclei along the antero-posterior axis of the hindbrain and spinal cord (Pan *et al.*, 2012).

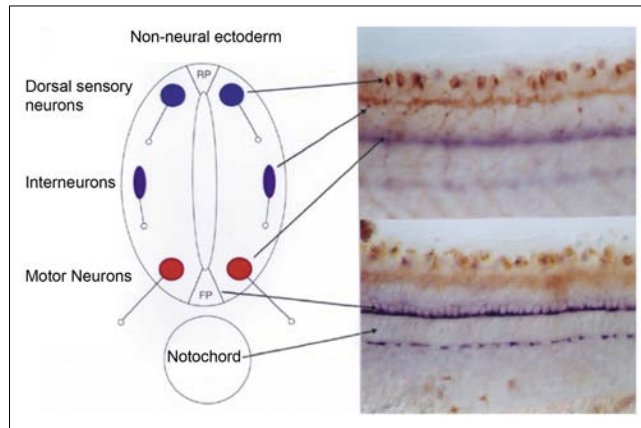


Figure I3.4. Organization of the neural tube of a 24 hpf zebrafish embryo. Left panel, schematic cross-section, indicating: non-neural ectoderm, RP, roofplate; dorsal, inter- and motor neurons; FP, floorplate; notochord. Right panel, lateral view of a zebrafish embryo in two planes of focus. From Blader and Strähle (2000).

With respect to the development and maturation of the blood-brain barrier (BBB) in zebrafish larvae, expression of tight junction proteins such as claudin-5 and ZO-1 has been detected in cerebral microvessels already at 2-3 dpf. Dye exclusion experiments have shown that high molecular weight dyes (> 900 Da) are excluded from the brain already at 3 dpf while low molecular weight compounds (> 350 Da) start to be retained from 5 to 10 dpf, time at which the BBB is fully formed (Fleming *et al.*, 2013).

3.3. Zebrafish Antiviral Innate Immune System

The following section is an extract from: “The Antiviral Innate Immune Response in Fish: Evolution and Conservation of the IFN System” (J. Mol. Biol., 2013). This review article represents a collaborative effort between the two groups in which I did my Thesis work. The full article is included at the end of this Thesis in Annex I.

Interferons (IFNs) represent the first line of defense against viral infections. Their vital role is testified by the conservation of their structure among vertebrates (Hamming *et al.*, 2011). IFNs belong to the class II helical cytokine family and, in mammals, can be divided into three different groups based on biological and structural features as well as receptor usage (Pestka *et al.*, 2004): mammalian IFNs have been classified as type I (α , β , ω , ϵ , and κ), type II (γ), and type III (λ) IFNs. Actually, only type I and type III IFNs (often grouped under the label “virus-induced IFNs”) are truly specialized as innate antiviral cytokines; IFN γ is rather a regulatory cytokine of innate and adaptive immunity, mostly active against intracellular bacteria.

Zebrafish possess four IFN genes (IFN ϕ 1-4) structurally similar to mammalian type I IFNs (Zou and Secombes, 2011; Aggad *et al.*, 2009). This number is unlikely going to change considering the quality reached by the zebrafish genome assembly.

At the cellular level, macrophages and neutrophils represent the first immune cell types activated at the larval stage in response to pathogens. In fact, it was shown that by 3 dpf the innate immune system is fully functional, with macrophages being able to phagocytose bacteria as early as 30 hpf (Herbomel *et al.*, 1999) and neutrophils from 52 hpf (Le Guyader *et al.*, 2008). Moreover, in the case of CHIKV infection in zebrafish, neutrophils (together with hepatocytes) were shown to be the main IFN-producing cell type (Palha *et al.*, 2013).

3.3.1. Upstream IFN Signaling Pathway

The activation of the IFN system is dependent on the specific recognition of conserved pathogenic motifs – known as PAMPS, for pathogen-associated molecular patterns – by the numerous pathogen-recognition receptors (PRRs) found on intracellular vesicles (Kawai and Akira, 2011). Antiviral PRRs include for example RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs). *In silico* analyses have revealed that they are highly conserved between mammals and teleost fish (Rajendran *et al.*, 2012). Sequence analyses have also suggested a fair conservation of signaling pathways downstream of RLRs, with a critical role

played by the mitochondrial antiviral signaling protein (MAVS) (Zou *et al.*, 2009; Biacchesi *et al.*, 2009; Simora *et al.*, 2010; Su *et al.*, 2011, Xiang *et al.*, 2011). Association of MAVS with TRAF3 (tumor necrosis factor (TNF) receptor-associated factor 3) and activation of the pathway by TBK1 (TANK binding kinase 1) via phosphorylation of IFN regulatory factor 3 (IRF3) and 7 (IRF7) transcriptional factors have also been shown in fish (Xiang *et al.*, 2011; Sun *et al.*, 2010). Nuclear translocation of these factors induces the transcription of different cytokines including IFN genes (Figure I3.5).

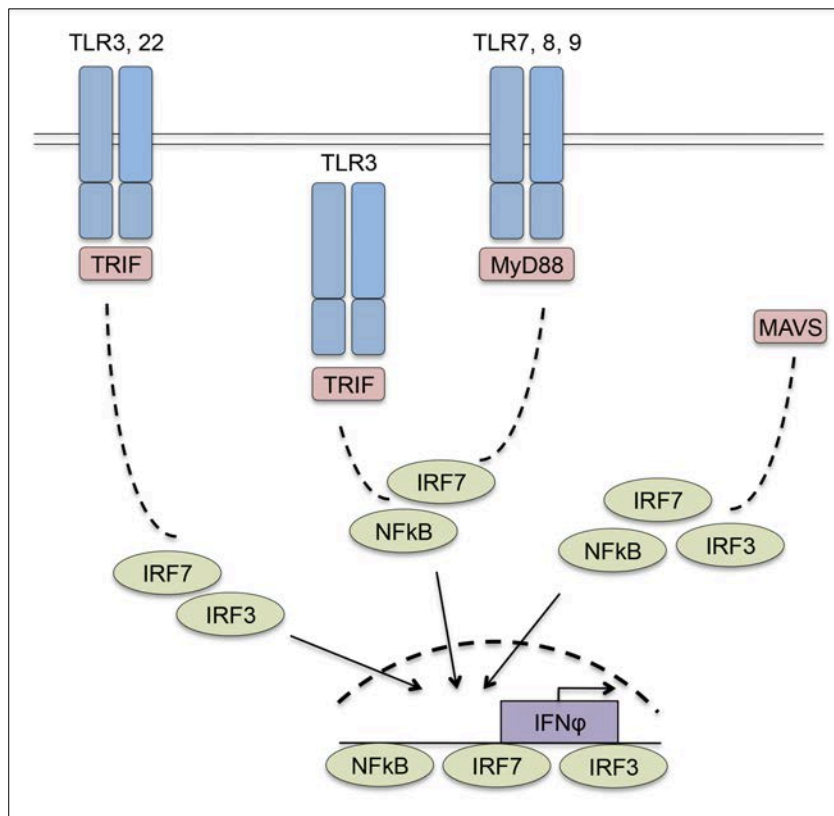


Figure I3.5. Schematic representation of upstream IFN signaling pathways in zebrafish. Adapted from Langevin *et al.* (2013).

3.3.2. Downstream IFN Signaling Pathway

In zebrafish, the two groups of IFNs signal via two different receptors. IFN ϕ s of the first group (IFN ϕ 1 and ϕ 4) bind to the cytokine receptor family B (CRFB) CRFB1-CRFB5 complex while the receptor for group II (IFN ϕ 2 and ϕ 3) consists of the association between the CRFB5 and CRFB2 chains (Levraud *et al.*, 2007; Aggad *et al.*, 2009) (Figure I3.6).

In mammals, IFN binding to their membrane receptors (IFNAR) triggers recruitment and binding of the kinases TYK2 and JAK1 to IFNAR1 and IFNAR2, respectively. Subsequently, these kinases promote the phosphorylation of STAT1 and STAT2 proteins preceding their oligomerization. Conjugation of cytoplasmic IRF9 to the STAT1/2 oligomers generates the complex ISGF3 (IFN-stimulated gene factor 3), which induces the transcription of IFN stimulated genes (ISGs) after binding nuclear IFN-stimulated responses elements on their promoter. The zebrafish genome encodes two different paralogues, *stat1a* and *stat1b* (Stein *et al.*, 2007). The kinases JAK1 and TYK2 as well as STAT2 and IRF9 are also present in fish genomes (Stein *et al.*, 2007).

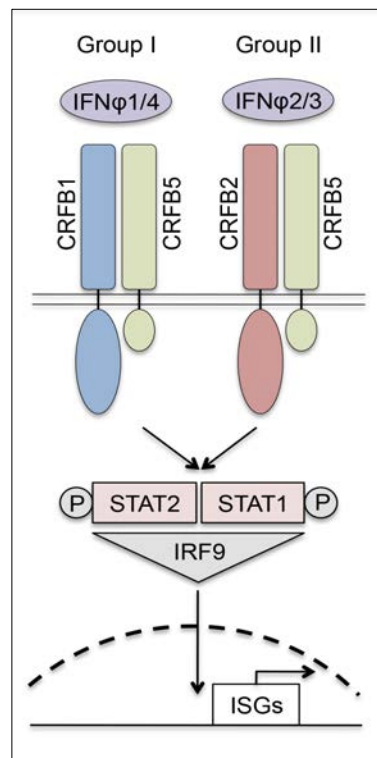


Figure I3.6. Schematic representation of virus-induced IFNs and their receptors in zebrafish. Adapted from Aggad *et al.* (2009).

Type I IFNs do not possess antiviral activity *per se* but rather interfere with viral infection through induction of a vast repertoire of ISGs via the JAK/STAT pathway. A few hundred ISGs have been identified in humans (Sadler and Williams, 2008; Schoggins *et al.*, 2011), with a rich diversity of molecular functions. Some ISGs exert a direct antiviral activity such as MX, RSAD2/VIPERIN/VIG1, ISG15, PKR, and TRIM5. However, the connection of

most ISGs to antiviral mechanisms, and even their role in the biology of the cell, remain unknown.

While ISGs are intrinsically located downstream of IFN in the antiviral pathways induced by viral infections, a number of them are able to up-regulate type I IFNs and are therefore involved in positive feedback regulatory loops (*e.g.* RSAD2) (Saitoh *et al.*, 2011), while some also feedback negatively on IFN signaling (*e.g.* SOCS1 and 2). Furthermore, the recognition of viral compounds by cellular sensors can up-regulate some ISGs directly, that is, independently of IFN induction; such bypass has been shown for example for MX (Goetschy *et al.*, 1989; DeWitte-Orr *et al.*, 2007) and for RSAD2 in humans and fish (Zhu *et al.*, 1997; Boudinot *et al.*, 1999).

In a recent study performed by our lab, zebrafish larvae were shown to possess ~100 ISGs orthologs to human ISGs (Briolat *et al.*, 2014), although their function has yet to be characterized. In this comparative study between the effects of Infectious Hematopoietic Necrosis virus (IHNV) and Chikungunya virus (CHIKV) on ISGs induction, it was shown that ISGs followed the same kinetics of IFNs while being expressed in a tissue-specific manner. Moreover, several were induced by CHIKV and repressed by IHNV, suggesting the presence of antagonist pathways.

3.3.3. Models of Viral Infections in the Zebrafish

Free-swimming larvae, which have to rely on innate immune defenses before maturation of adaptive immunity, are highly suited for the study of innate immunity and inflammation; as previously described, the overall similarity of the cytokine networks between fish and mammals as well as their immune cells (Traver *et al.*, 2003) make their use relevant for many diseases. Moreover, characterization of IFNs and ISGs pathways in zebrafish larvae has been the focus of the aforementioned study, performed in our labs (Briolat *et al.*, 2014). This has greatly contributed to our understanding of viral-specific immune responses and provides a useful reference for studies of host-virus interactions.

Several human viruses have already been studied in zebrafish, such as Hepatitis B and C viruses (Liu *et al.*, 2012; Ding *et al.*, 2011), Herpes simplex virus-1 (HSV-1) (Antoine *et al.*, 2014), and recently by our lab, CHIKV (Palha *et al.*, 2013). Fish virus models include for example Viral Hemorrhagic Septicemia virus (Novoa *et al.*, 2006), Spring Viremia of Carp virus (Encinas *et al.*, 2013), and IHNV (Ludwig *et al.*, 2011).

Moreover, a variety of tools including various transgenic reporter fluorescent lines have been developed to follow inflammatory cell types and responses in zebrafish. Among these, several were employed for this Thesis to follow *in vivo* the activity of macrophages (*mpeg1:Gal4/UAS:NfsB-mCherry* transgenic larvae (Ellett *et al.*, 2011)), neutrophils (*LyZC:DsRed* larvae (Hall *et al.*, 2007)) and IFN-producing cells (*ifn ϕ 1:mCherry* larvae (Palha *et al.*, 2013)) in the context of SINV infection.

3.4. Concluding Remarks

Several factors have contributed to the considerable expansion of the zebrafish as an animal model in medical research, with its small size, its transparency, and the high similarity of its immune system to that of mammals being only a few (Renshaw *et al.*, 2012).

Thanks to the complete annotation of the zebrafish genome, several reverse genetics techniques have become available, such as retrovirus-mediated insertional mutagenesis (Chen *et al.*, 2002), mutant identification using Targeting Induced Local Lesions In Genomes (TILLING) technology (Wienholds *et al.*, 2002) or the most recent one, CRISPR/Cas9-mediated knock-in approach for genome editing (Hwang *et al.*, 2013; Auer and Del Bene, 2014). As previously mentioned, generation of new transgenic reporter lines has almost become routine, and gene knock-down is made easy by microinjection of antisense Morpholino Oligonucleotides (MOs) at the 1 to 4 cell stage. Zebrafish are also regularly used in chemical screens, since they allow for testing of a large number of compounds *in vivo* by simply adding these into the water (Baker, 2011).

However, this model presents a few disadvantages as well. The maximum temperature at which zebrafish can be raised is 32°C (89.6°F), which impairs the

study of those pathogens that replicate at higher temperatures. The standard laboratory zebrafish lines are not fully inbred and the genetic background of mutants and transgenic lines is generally poorly characterized. There is a limited amount of available biochemical tools (*i.e.* antibodies) or of established *in vitro* cell lines, and it is extremely challenging to establish cell cultures. As a matter of fact, part of this Thesis has consisted in the development of a primary neuronal cell culture system from zebrafish brain homogenates. The work is still in its infancy but preliminary data have shown that this could be a valuable new tool to address questions such as neuronal survival and gene expression changes in response to pathogens.

Summary of Chapter III

- Zebrafish is a tropical freshwater fish, originally employed for studies of embryogenesis and more recently also in cancer, neuroscience, pharmacology, and disease modeling fields.
- Its main advantage lies in the optical clarity of its embryos and larvae. Transgenic lines expressing fluorescent reporter proteins in cells or tissues of interest are now being developed routinely.
- The zebrafish brain consists of five major regions: the telencephalon, the diencephalons, the mesencephalon, the metencephalon, and the myelencephalon, each one well-characterized anatomically, although functional knowledge is unequal.
- The zebrafish antiviral immune system shares overall similarity with the human one, relying heavily on the expression of IFNs and ISGs.
- The replication, tropism, and pathogenesis of several human and fish viruses have been characterized and/or are being currently characterized.
- Despite a few disadvantages, the zebrafish is a very powerful model to visualize in real time at the whole-organism level events taking place at the cellular or sub-cellular level.

AIM OF THE WORK

AIM OF THE WORK

The laboratories where I worked during my PhD Thesis previously pioneered the use of zebrafish to study viral infections. The groups within the Units “*Virology and Molecular Immunology*” at INRA and “*Macrophages and Development of Immunity*” at Pasteur Institute led by Pierre Boudinot and Jean-Pierre Levraud respectively, have been able to follow the progression of the infection of a lethal fish rhabdovirus (Ludwig *et al.*, 2011), and more recently of the human *Alphavirus* related to SINV – Chikungunya virus (CHIKV) (Palha *et al.*, 2013). Moreover, they have developed various transgenic zebrafish lines to follow antiviral responses, such as the *ifn ϕ 1:mCherry* that labels the main cell populations producing type I IFNs during the infection, and more recently the *MxA:mCherry*, that labels IFN-responding cells. It has thus been possible to image in real time the progression of the infection in the host, at cellular resolution in the whole body, while simultaneously imaging the host response to the virus.

A notable finding in the study of the model of CHIKV infection in zebrafish was the entry of the virus in the brain, despite the fact that the virus is inoculated in the bloodstream at a stage where the blood-brain barrier is mature enough to prevent passive passage of proteins. Remarkably, this virus was then shown to persist in the brain much longer than in other tissues. Moreover, neutrophils appeared to be the main cell population activated in zebrafish larvae to clear the infection but they were excluded from the brain, suggesting other mechanisms are involved in the control of the infection in this particularly sensitive organ (Palha *et al.*, 2013). These results laid the foundation for my project.

The aim was in fact to gain better insights into the mechanism through which *Alphaviruses* persist longer in the CNS than in other infected organs. To do so, Sindbis virus was the obvious choice. The virus is markedly neurotropic in mice but is nevertheless regarded as a BSL2 pathogen, and as such allowed us to use our confocal microscopy equipment for both *ex vivo* and *in vivo* imaging, unlike CHIKV, which is classified as a BSL3 pathogen.

We therefore proceeded to characterize the general course of SINV infection in zebrafish larvae, with a special attention towards its possible mechanism of entry in the CNS. The data collected on this part of the project constitute the submitted article included in the first part of the Results section.

At the same time, we surveyed the IFN-related immune response, from its onset to later times post-infection. The results are still very preliminary but indicate that depending on the level of brain infection – mild in the case of CHIKV and extended in the case of SINV – different responses may be activated in the CNS. This differential immune response has also been observed after the production and comparison of different SINV strains (2nd part of the Results section).

Part of this Thesis project has also been devoted to the development of a protocol to obtain neuronal cell cultures from zebrafish larvae, with the aim to determine the mean survival rate of isolated neurons and glial cells upon SINV or CHIKV infection and to possibly visualize the spreading mechanism within infected neurons (anterograde and/or retrograde movement).

Finally, we set up a collaboration with the group “*Viral populations and Pathogenesis*”, led by Marco Vignuzzi at Pasteur Institute, with the aim to exploit our SINV infection model to test potentially antiviral compounds.

RESULTS

RESULTS

The results obtained during this Thesis have led to the characterization of the infection course of Sindbis virus (SINV) in zebrafish larvae, with a special attention towards its neurotropism. Thanks to the combined use of a green fluorescent protein (GFP)-recombinant SINV strain (SINV-GFP) and zebrafish transgenic larvae, we have been able to follow *in vivo* and at cellular resolution SINV infection course as well as the elicited immune response. Data collected on SINV mechanism of entry in the Central Nervous System (CNS) have been included in the submitted paper and constitute the 1st Chapter of this section.

The 2nd Chapter of the Results involves the studies we performed on the differential immune responses observed in the periphery and in the CNS. Moreover, we have produced a second recombinant SINV strain (SINV2-mCherry) and have used it to characterize modulations in the antiviral Interferon (IFN)-mediated response by different virus strains. We have also set up a protocol to obtain zebrafish neuronal and glial cells in culture (Chapter 3). These will represent a valuable tool for the growing zebrafish research community, to study mechanisms of pathogenesis and cell survival under several stimuli, such as viral infections.

The final Chapter of the Results includes preliminary data on the potential antiviral properties of difluoromethylornithine (DFMO) on the pathogenicity of SINV. These in turn could be useful in the future to screen for and identify other potential broad-spectrum antiviral drugs.

CHAPTER I. Intravital High-Resolution Microscopy in a Vertebrate Model
System Unveils Sindbis Virus Neuroinvasion

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Summary

Alphaviruses, such as Chikungunya virus (CHIKV) and Sindbis virus (SINV), are vector-borne pathogens that cause acute illnesses in humans and are sometimes associated with neuropathies, especially in infants and elderly patients. Little is known about their entry mechanism in the central nervous system (CNS). Here we used the optically transparent zebrafish larvae to image *in vivo* the onset and the progression of the infection caused by SINV. Testing the possible mechanisms of entry in the CNS, we excluded infection of brain endothelium or transport via macrophages, and identified axonal transport from the periphery as the most likely route of access to the CNS. We also found a prominent role of Interferon (IFN) in the control of SINV pathogenesis but not neuroinvasion. These results will facilitate testing therapeutics for the control and prevention of *Alphavirus*-induced encephalopathy.

Introduction

The outcome of an infection critically depends on the organs reached by the invasive pathogen, which entails a precarious balance between the pathogen dissemination strategy and systemic and local host defenses. The central nervous system (CNS) is especially vulnerable, being highly susceptible to both virus induced cytopathic effects and to the inflammatory response itself (Swanson and McGavern, 2015). The CNS is however protected by specialized barriers, notably the blood-brain barrier (BBB). Neuroinvasion is generally rare but some viruses have evolved strategies to enter and spread within the CNS. Rabies virus is a well-known example of a neuroinvasive – and fatal – pathogen in nearly 100% of human cases (Dietzschold et al., 2008). Poliovirus can reach neurons in the spinal cord, brain stem or motor cortex, inducing characteristic paralysis, but only in 1-2% of infected individuals (Racaniello, 2006). Herpesviruses reach the peripheral nervous system and establish a lifelong persistent infection. While generally quiescent, they can revert under certain stress stimuli to produce infectious particles and in rare cases reach the CNS (Tirabassi et al., 1998). Persistent or “stable” infection of the CNS is also a rare occurrence, except for some specialized viruses. On the other hand, acute CNS infections are more often caused by zoonotic pathogens, for which humans normally represent a “dead-end host” (Gubler, 2001). In particular, among the arboviruses – viruses transmitted by arthropod vectors – *Alphaviruses* constitute a major source of viral zoonotic diseases, and often induce encephalitis. Eastern (EEEV) and Western Equine Encephalitis virus (WEEV) but also Chikungunya virus (CHIKV) have been associated with serious neurological manifestations.

Despite extensive studies in the field, the factors that determine the onset of neuroinvasion are not well understood. Infants are clearly more prone to CNS invasion than adults, generally attributed to an immature BBB. Among adults, genetic factors have been identified in a few cases. Mutations in the *ccr5* gene (a chemokine receptor and co-receptor for Human Immunodeficiency Virus – HIV), for example, have been shown to increase the incidence of symptomatic West Nile virus (WNV) infection (Glass et al., 2006). With respect to cellular determinants, maturation of neurons has been shown to impair viral replication of

certain arboviruses in contrast to non-differentiated neurons (Schultz et al., 2015). As of today, some studies have addressed ways to limit neurological symptoms of encephalitis (*e.g.* Brison et al., 2014) but no strategy exists to specifically prevent CNS invasion during outbreaks of neurotropic viruses.

The mechanisms of entry of viruses in the CNS have been characterized using a variety of approaches both *in vitro* and *ex vivo*, and fall within three categories. Some viruses are able to infect cells of the BBB (*i.e.* endothelial cells that form brain microvessels) or other components of the brain-periphery interface, such as epithelial cells of choroid plexuses. This allows their release in the brain parenchyma by the infected cells and/or disruption of the barrier causing leakage of blood-borne virions. Another strategy is known as the “Trojan horse” entry: viruses hide in monocytes/macrophages and exploit the ability of these cells to cross the barrier to enter the brain. Finally, various viruses travel within the numerous axons of motor or sensory neurons that connect the CNS to the periphery, using either anterograde or retrograde transport (Koyuncu et al., 2013). Despite the development of virus mutants built to express reporter genes (*e.g.* luciferase, GFP, mCherry), intravital imaging of virus infection in mammals is still a real challenge (Rameix-Welti et al., 2014). A major hindrance in detailed kinetic analyses of CNS viral invasion lies with observing these events in real time. The zebrafish larva offers a powerful system to follow such events in a vertebrate, being small, transparent, and tolerant for long anesthesia (Levraud et al., 2014). In a recent study, we performed real time imaging of zebrafish larvae inoculated with a green fluorescent protein (GFP) recombinant CHIKV (Palha et al., 2013). CHIKV is a re-emerging arbovirus, which has gained attention after the epidemic of 2005-2006 in Islands of the Indian Ocean (Powers and Logue, 2007) and, more recently, due to its spread to the Caribbean, the United States and Polynesia. In our previous study in zebrafish larvae, the majority of infected cells was found in various peripheral organs; however, infection of some brain cells always occurred. Remarkably, infected cells in the CNS survived much longer than cells infected in the periphery. Importantly, those experiments were performed at a developmental stage where the BBB of the zebrafish larva is mature enough to block the passage of proteins from the blood to the CNS (Fleming et al., 2013), excluding passive diffusion of viruses to the CNS. This

work demonstrated that it is possible to image neuroinvasion in zebrafish larvae from the earliest stages of the infection. However the BSL3 status of CHIKV limited our practical options for *in vivo* microscopy.

To better characterize *Alphavirus* neuroinvasiveness, we decided to employ in this work Sindbis virus (SINV), the *Alphaviridae* prototype and a BSL2 pathogen markedly neurotropic in mice (Lewis et al., 1996; Lustig et al., 1988). SINV has often been used for studies of viral spreading in the CNS after intracerebral (ic) inoculation, rather than to understand its mechanism of neuroinvasion. However, its entry into the CNS has been suggested to occur from infection of peripheral nerves (Cook and Griffin, 2003), although the resolution achievable in mice did not permit to distinguish details below tissue or organ level, and alternative hypotheses were not tested.

Here, we show that upon intravenous (iv) inoculation in zebrafish larvae SINV infects various cell types and replicates efficiently. Moreover, in a substantial fraction of infected larvae the virus reaches the CNS, where its progressive spread can be documented by *in vivo* imaging. We tested the different possible routes of invasion of the CNS and identified axonal transport via infected peripheral neurons as the most likely route of entry. In addition, we followed the elicited host response and highlighted the importance of type I interferons (IFN) in preventing spreading of the virus in the periphery, but not for neuroinvasion.

Results

SINV shows a broad organ tropism in infected zebrafish larvae

Wild type (WT) zebrafish larvae were inoculated intravenously (iv) at 3 days post-fertilization (dpf) (Figure 1A) with $\sim 10^2$ TCID₅₀ of a recombinant SINV strain: SINV-GFP (hereon referred to as SINV), engineered to induce GFP expression in infected cells (Hahn et al., 1992). Viral growth was determined by qRT-PCR of the viral E1 gene from whole larvae lysates (Figure 1B). Intense viral replication occurred during the first 48 hours of the infection and subsequently leveled off and declined over time.

We observed infected larvae with a dissecting stereomicroscope; relatively mild and usually transient signs of disease appeared as early as 2 days post-inoculation (dpi), including edema, irregular heartbeat, and yolk opacification. In a minority of larvae ($\sim 15\%$), these signs persisted and led to death around 4 dpi (Figure 1C). Live fluorescence microscopy revealed the presence of GFP-positive (GFP⁺) cells scattered or in clusters. These infected cells were mostly observed in peripheral organs including the liver, the heart and the yolk (Figure 1D). In addition, the infection often reached the CNS (Figure 1E).

Fixation and whole-mount immunohistochemistry (WIHC) with anti-GFP and anti-SINV capsid antibodies confirmed that GFP⁺ cells were infected and actively undergoing viral protein translation and assembly (Figure S1A. Related to Figure 1).

These results indicate that SINV replicates efficiently in zebrafish larvae and that its spread through the whole organism can be monitored over time via *in vivo* imaging (**Figure 1; Figure S1**).

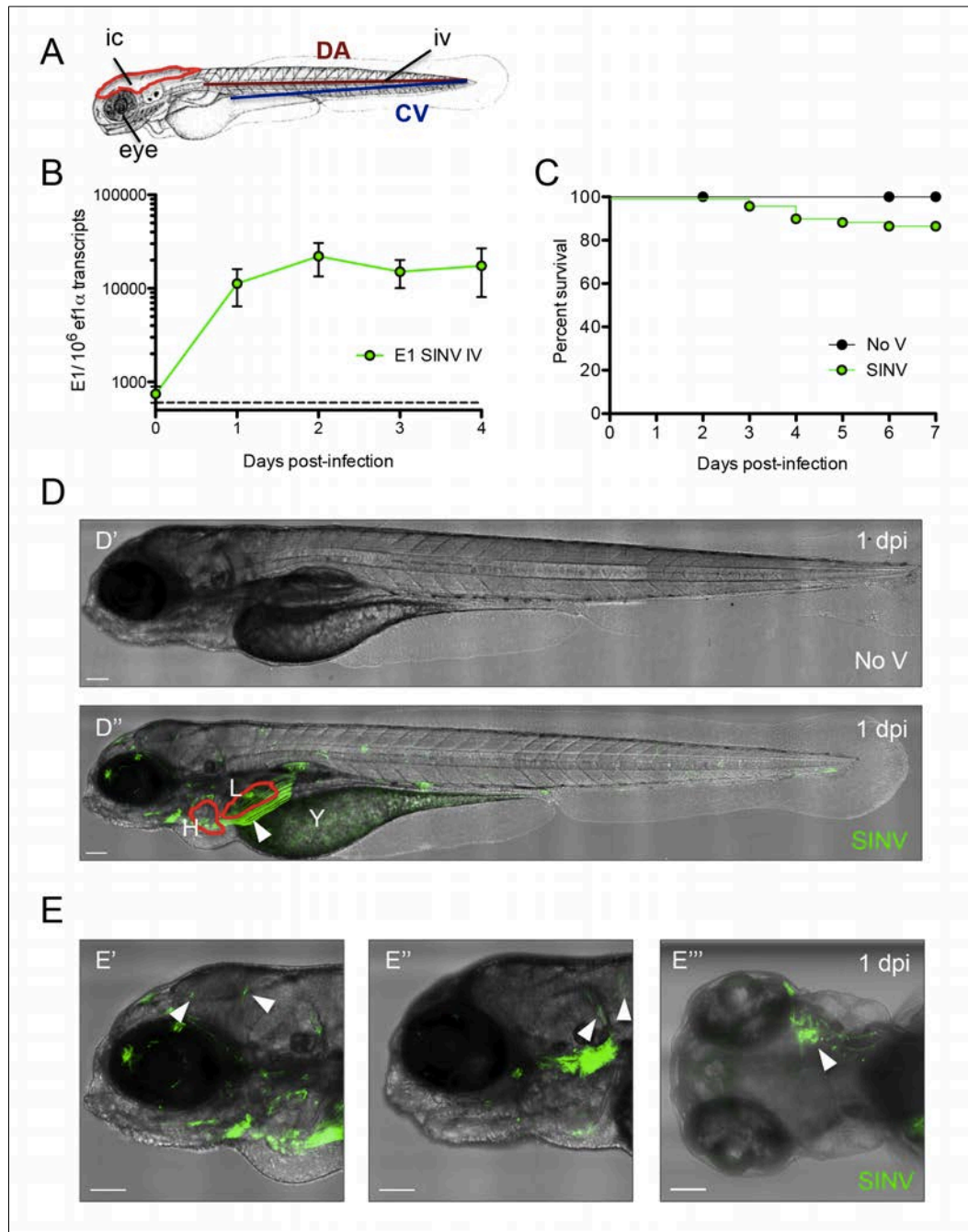


Figure 1. SINV replicates in zebrafish larvae and exhibits a broad organ tropism. (A) Scheme of a 72 hours post-fertilization (hpf) larva, showing the sites of injection: iv, intravenously in the dorsal aorta or in the caudal vein; ic, intracerebrally in the optic tectum; eye, in the retina. (B) Virus titers in iv infected zebrafish larvae. Data represent mean \pm s.e.m. of 5 larvae per time-point, from 2 experiments pooled. Dotted line: threshold level from uninfected control larvae. (C) Survival curves of control uninfected (No V) and iv infected zebrafish larvae (SINV). Data pooled from 5 independent experiments. N = 12 larvae per group. (D–E) Superposition of transmitted light and GFP fluorescence. (D) Confocal *in vivo* imaging of control uninfected (No V) and iv infected (SINV) larvae at 1 day post-infection (dpi). H, heart; L, liver; Y, yolk; white arrowhead: infection in the left pectoral muscle. (E) Confocal *in vivo* imaging of 3 infected larvae at 1 dpi, illustrating the pattern variability. (E', E'') Lateral views; (E''') Dorsal view; note that E' is a magnification of D''. White arrowheads point to sites of CNS infection. Scalebars: 50 μ m. See also Figure S1. ***In this and all following figures, anterior to left, dorsal to top for lateral views.***

SINV is neuroinvasive in zebrafish larvae

Under the fluorescence microscope, a prominent feature of a number of SINV inoculated larvae was the presence of GFP⁺ cells in the brain or the spinal cord (Figure 1E). Approximately half of the larvae had obvious CNS infection by 3 to 7 dpi, although this was less prominent at early time-points. The course of the infection was therefore evaluated every 24 hours by following individual larvae with confocal imaging during several consecutive days. Unlike infection foci in the periphery, those observed in the brain were either persisting or progressively increasing over time (Figure 2A). Co-immunolabeling to stain SINV-positive cells and axonal projections confirmed that CNS neurons were infected (Figure 2B). Peripheral neurons, such as trigeminal ganglion cells and retinal ganglion cells, were also targeted by the virus (Figure 2C; Figure S2A. Related to Figure 2). Although the pattern of brain infection was very variable between individuals, analysis of a large panel of samples revealed that infection foci were commonly found in the olfactory bulb, the optic tectum, the vestibulolateralis lobe of the corpus cerebelli and the medulla (Figure S2B and S2C. Related to Figure 2).

To follow infection of neurons *in vivo*, we used *elavl3:Gal4/5xUAS:RFP* double-transgenic larvae (from hereon called *HuC:G/U:RFP*), in which RFP is expressed in postmitotic neurons. Observations from early stages of infection (~8 hpi) showed that the first infected cells to appear after iv injection were in the periphery (*e.g.* muscle cells, cells in the heart and in the liver), followed at later time-points by neurons of the peripheral and the central nervous system (1dpi) (Figure 2D).

To exclude the possibility that neurons may simply require longer to express GFP once infected, we performed intracranial (ic) inoculation of SINV in *HuC:G/U:RFP* larvae (Figure 1A). GFP⁺ neurons were detected as soon as 8 hpi (Figure 2D), indicating that late appearance of CNS infection in iv injected larvae is a result of late infection of neurons. Ic injection resulted in a more severe disease with over 50% of larvae dying between 5 and 7 dpi (Figure S2D. Related to Figure 2). Viral growth kinetics measured by viral transcripts from whole larvae homogenates was slightly higher in ic injected larvae than in iv inoculated ones (Figure S2E. Related to Figure 2). Many foci of GFP⁺ cells were

systematically found in various regions of the brain and spinal cord, but little spreading occurred at the periphery (*e.g.* eye) (Figure 2E).

Overall, these results show that after inoculation of the virus in the bloodstream, peripheral cells are infected first, followed by invasion of the CNS (**Figure 2; Figure S2**).

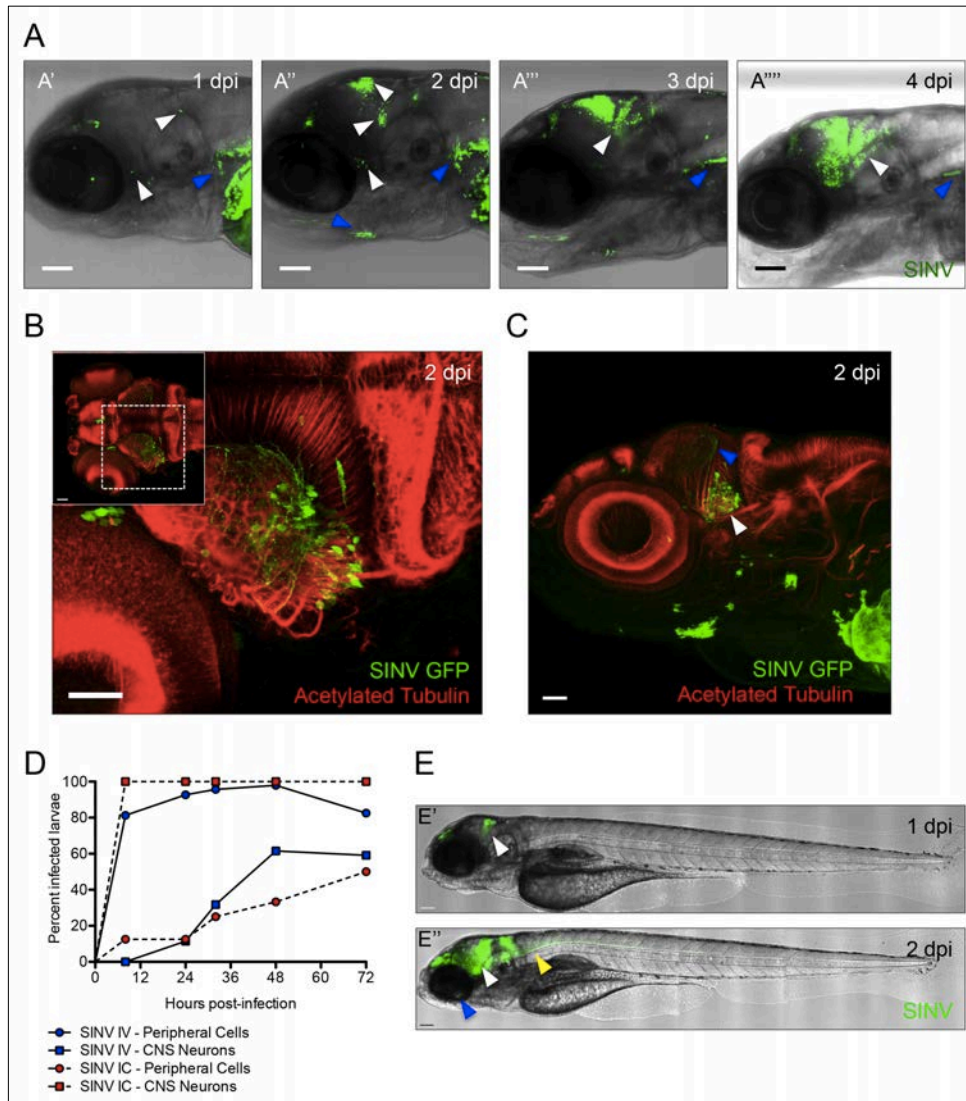


Figure 2. SINV is neurotropic in zebrafish larvae. (A'–A''') Confocal *in vivo* imaging of the same infected larva from 1 to 4 dpi. Blue arrowheads: infected peripheral cells; white arrowheads: infected cells in the CNS. (B, C) Confocal images of whole-mount immunohistochemistry (WIHC) processed SINV-infected larvae, GFP staining (SINV-infected cells) in green, acetylated tubulin (axons) in red. (B) Dorsal view, with inset showing a wider field of the head; dotted square corresponds to the area imaged at higher magnification. (C) Lateral view. White arrowhead: neurons of the trigeminal ganglion. Blue arrowhead: axon from a trigeminal ganglion neuron reaching the optic tectum. (D) Quantification of appearance of infected cells in the periphery and in the CNS, from *in vivo* observation of *HuC:G/U:RFP* larvae, infected *iv* or *ic*. N = 12-24 from 2 independent experiments pooled. *In vivo* confocal imaging at (E') 1 dpi and (E'') 2 dpi of a larva infected *ic*. White arrowheads: CNS infected cells; yellow arrowhead: spinal cord infection; blue arrowhead: infected cells in the eye. Scalebars in (A): 100 μ m, in (B, C, E): 50 μ m. See also Figure S2.

SINV does not target the blood-brain barrier endothelium

We then proceeded to test the possible mechanism of SINV entry in the CNS. A number of DNA and RNA viruses have been shown to gain access through infection of endothelial cells of the BBB, including for example Epstein-Barr virus (Casiraghi et al., 2011), Hepatitis C virus (Fletcher et al., 2012), and WNV (Xu et al., 2012). Moreover, our laboratory previously established a zebrafish infection model of a fish rhabdovirus, Infectious Hematopoietic Necrosis Virus (IHNV), in which we demonstrated that IHNV primarily infects endothelial cells of the vasculature. Infection of these cells in turn causes a rapid disruption of blood vessels and virus dissemination to neighboring cells, including cells in the brain (Ludwig et al., 2011). It was therefore used as a positive control to visualize BBB disruption.

To evaluate changes in the vascular endothelium, we inoculated *fli1a:GAL4FF/5xUAS:RFP* transgenic fish (hereafter referred to as *fli:G/U:RFP*), which express RFP in endothelial cells of the brain as well as of peripheral vessels. IHNV-infected fish were fixed at 1 and 2 dpi and immunolabeled using the 19B7 mAb directed against the G protein of IHNV. As opposed to control uninfected fish (Figure 3A), all stained fish showed disrupted vessels and scattered likely apoptotic RFP-positive cells (Figure 3B; Figure S3A; Movie S1. Related to Figure 3). On the other hand, we monitored SINV-infected *fli:G/U:RFP* fish over a period of 7 days and overall, the vasculature looked intact, even in larvae showing an extensive infection in the CNS (Figure 3C; Figure S3B; Movies S2 and S3. Related to Figure 3) and was comparable to control uninfected fish (Figure 3A).

To ensure optimal imaging of even deep brain vessels, SINV-infected *fli:G/U:RFP* larvae were processed using the recently developed Clarity protocol and later imaged at high resolution. Imaging revealed no apparent disruption of brain microvasculature (Figure 3D). Infected cells were sometimes adjacent to cells of the vasculature, especially in the periphery, but no co-localization was ever observed (for 15 animals examined in the brain, in 3 independent experiments, and for 13 animals examined in the periphery) (Figure S3C; Movie S4. Related to Figure 3).

In conclusion, our data show that SINV does not infect nor disrupt the BBB endothelium to gain access to the CNS (**Figure 3; Figure S3; Movie S1; Movie S2; Movie S3; Movie S4**).

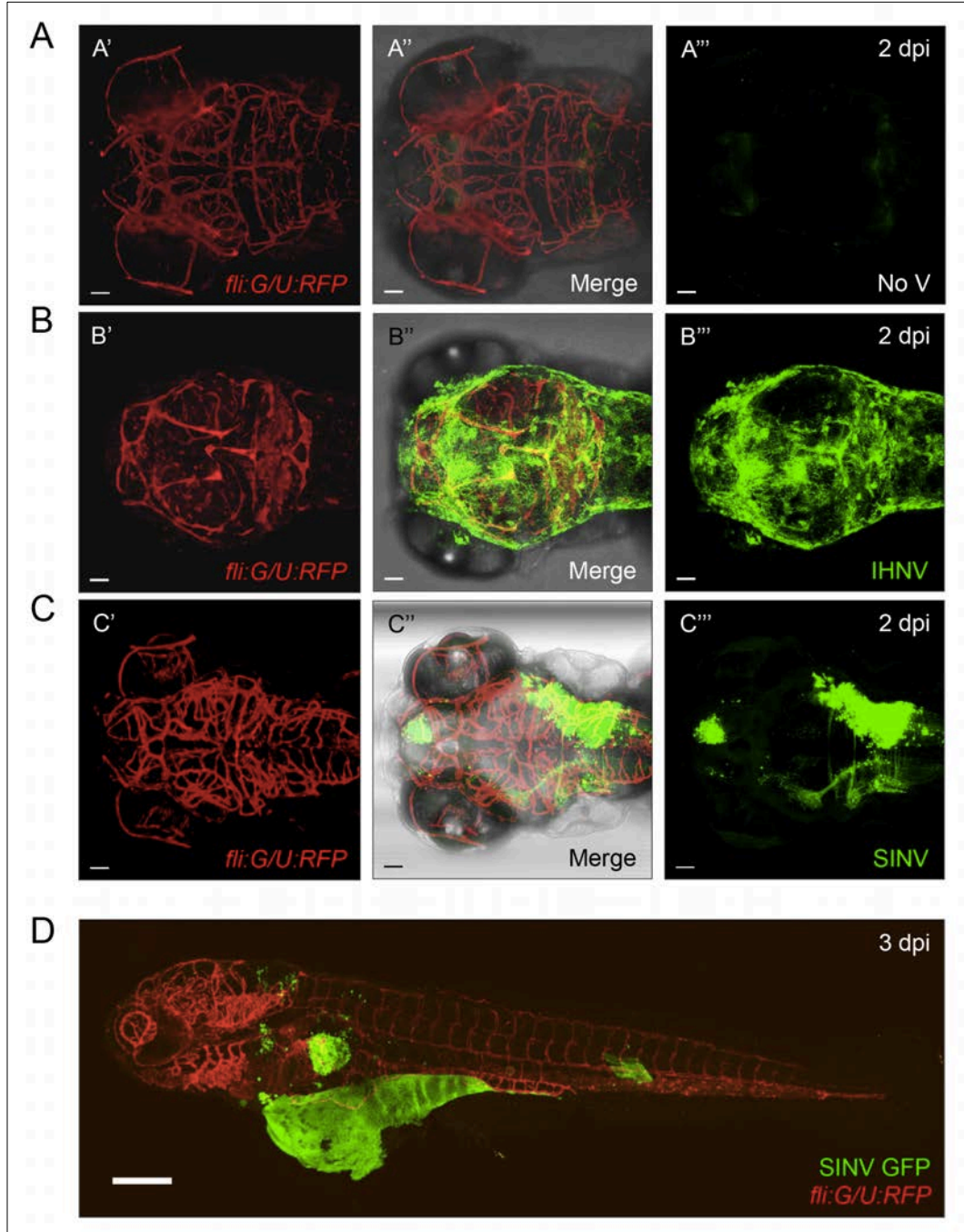


Figure 3. SINV does not infect the brain microvascular endothelium. Confocal imaging of (A, B) fixed and (C) *in vivo* *fli:G/U:RFP* larvae at 2 dpi. (A) No V. (B) IHNV-infected larva. (C) SINV-infected larva. (A', B', C') RFP staining (vasculature) in red. (A'', B'', C'') Merge with transmitted light, vasculature in red, virus in green. (A''') No V in green. (B''') G protein of IHNV in green. (C''') SINV-infected cells in green. Scalebars: 50 μm. (D) Confocal imaging from a Clarity-treated *fli:G/U:RFP* SINV-infected larva at 3 dpi. Scalebar: 250 μm. See also Figure S3.

Macrophages do not become infected and are not required for SINV entry in the CNS

A second possible mechanism through which viruses can enter the CNS is the so-called “Trojan horse” strategy, where viruses infect macrophage/monocytes and exploit their natural ability to cross the BBB. To test this possibility, we inoculated SINV in *mpeg1:GAL4FF/UAS-E1b:Eco.NfsB-mCherry* transgenic larvae (from hereon called *mpeg:G/U:Nfsb-mCherry*), in which macrophages (including microglia) express a cytosolic red fluorescent protein. Confocal microscopy showed no co-localization of cells displaying both green and red fluorescence (Figure 4A), thus macrophages were not productively infected by SINV. They displayed their characteristic shape with no evidence of apoptosis.

In addition, we depleted them specifically through metronidazole treatment of *mpeg:G/U:Nfsb-mCherry* larvae, exploiting the catalytic ability of the NfsB-mCherry fusion protein as previously described (Davison et al., 2007) (Figure S4A and S4B. Related to Figure 4).

Macrophage depleted SINV-infected larvae displayed a slight increase in disease severity – assessed using the scoring system previously developed (Palha et al., 2013) – (5.2 ± 1.1 N=24 compared to 3.6 ± 1.0 N=24 in controls) but no significant increase in mortality (Figure 4B). By 7 dpi, both controls and treated fish displayed a similar rate of infection (~50%) in the CNS (Figure 4C; Figure S4C. Related to Figure 4), although treated fish showed a bigger variability in terms of appearance of infected cells in the CNS.

We could therefore exclude entry of SINV in the CNS via infection of macrophages (**Figure 4; Figure S4**).

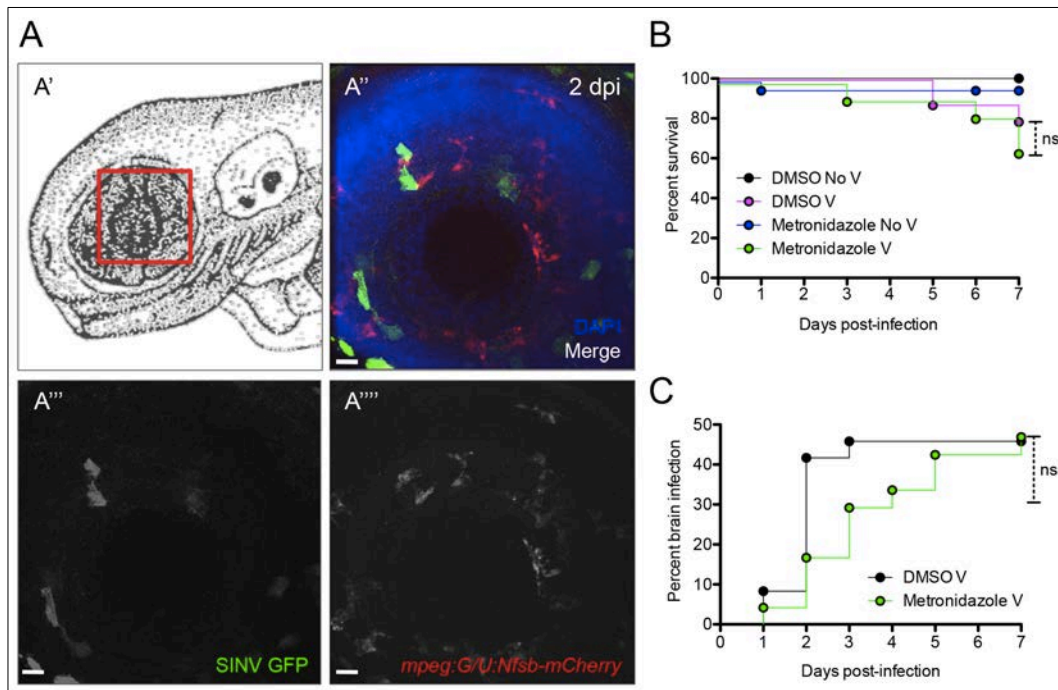


Figure 4. Macrophages are not targeted by SINV. (A) WIHC of SINV-infected *mpeg:G/U:Nfsb-mCherry* larva at 2 dpi. (A') Representative zebrafish head, with inset showing imaged area. (A'') Macrophages in red, SINV-infected cells in green, cell nuclei in blue (DAPI). (A''') SINV-infected cells. (A''') Macrophages. Scalebars: 10 μ m. (B, C) *mpeg:G/U:Nfsb-mCherry* were treated with DMSO (control) or Metronidazole (to deplete macrophages), before injection with SINV. (B) Survival curves and (C) Percentage of brain infected larvae in DMSO-treated and Metronidazole-treated larvae. N = 24 from 2 independent experiments pooled. ns - not significant (Log-rank test). See also Figure S4.

An eye model of infection shows that SINV can be transported via axons to the CNS after infection of peripheral neurons

Several viruses infect and replicate in peripheral nerves (Koyuncu et al., 2013). These are connected to neurons of the CNS and act as springboard for efficient entry and replication in the brain. In the case of SINV infection, we sometimes observed infection of peripheral sensory neurons such as trigeminal ganglion cells prior to infection in the CNS. Early infection of muscle cells followed by appearance of GFP⁺ cells among motor neurons at the corresponding level of the spinal cord was also a common occurrence (Figure 5A). These observations strongly suggest axonal spreading of the virus to the CNS.

To directly test if axonal transport of SINV can occur, we exploited the well-known connectivity of the visual system (Grove, 2008). We injected the

virus in the retina, in an area comprised between the ganglion cell layer and the outer nuclear layer (Figure 1A). Fish injected in the eye did not show an increase in mortality rates and overall disease scores were comparable to those obtained in *iv* inoculated fish (data not shown). Injections in the dense retinal parenchyma is difficult and leakage of inoculum to other tissues probably occurred; however, leakage can be predicted to predominantly result in infection of areas close to the injected eye, while axonal transport should result in infection of the contralateral optic tectum (Figure 5B). All infected larvae displayed GFP in the retina at 1 dpi and 45% showed also infection of the contralateral optic tectum.

Altogether, because we excluded alternative possibilities, these data indicate that infection of peripheral nerves and subsequent axonal transport is the major route of CNS access for SINV (Figure 5).

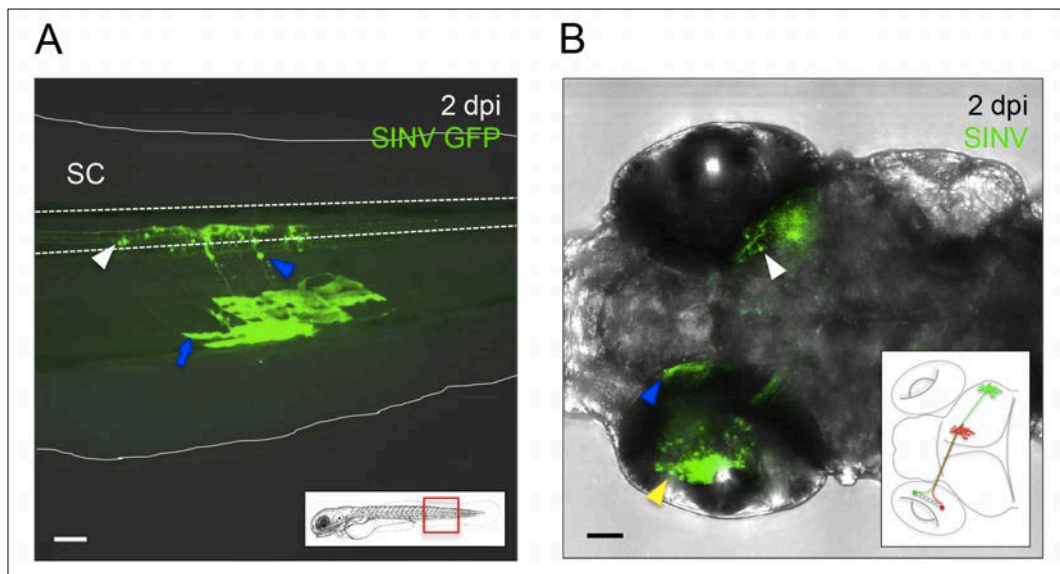


Figure 5. SINV is transported to the CNS via peripheral neurons. (A) Infection of muscle cells and spinal cord neurons in the tail region. WIHC processed *iv*-infected larva at 2 dpi. Lateral view, with inset depicting the imaged area. Dotted lines: dorsal and ventral limits of the spinal cord; plain lines: limits of fins; blue arrow: muscle fiber; blue arrowhead: peripheral neuron; white arrowhead: neuron inside the spinal cord. (B) *In vivo* confocal imaging of SINV-infected larva at 2 dpi, upon injection of the virus in the left eye. Dorsal view, with inset showing a scheme of the projection of the retinal neurons to the optic tectum. White arrowhead: infected cell body in the optic tectum; yellow arrowhead: infected cell in the eye. Probable inoculum leakage indicated by blue arrowhead. Overlay of transmitted light (grayscale) and GFP fluorescence (green). Scalebars: 50 μm.

SINV-infected larvae mount a strong IFN-mediated immune response

In all vertebrates, type I interferons (IFNs) represent the first line of defense and the main cytokines involved in protection against viral infections. The type I IFN response of larval zebrafish is substantially mediated by IFN ϕ 1 and IFN ϕ 3 (Aggad et al., 2009; Palha et al., 2013). qRT-PCR analyses of RNA extracted from SINV infected larvae revealed that the virus induces high mRNA levels of both *ifn ϕ 1* and *ifn ϕ 3* as well as of interferon stimulated genes (ISGs), such as *rsad2* (also known as *viperin* or *vig1*). The peak of expression was observed at 1 dpi and was followed by a slow (*ifn ϕ 1*, *vig1*) or relatively rapid (*ifn ϕ 3*) decrease in mRNA levels over time (Figure 6A–C). Fluorescence microscopy of *ifn ϕ 1:mCherry* transgenic larvae (Palha et al., 2013) revealed two main IFN producing cell types during SINV infection: hepatocytes and motile leukocytes. Many mCherry-positive leukocytes were observed (Figure 6D) as early as 2 dpi and increased steadily over time (data not shown). To determine the relevance of the response, we knocked-down (kd) all type I IFN receptors (IFN-R) through injection of antisense Morpholino Oligonucleotides (MOs) directed against the CRFB1 and CRFB2 subunits (Aggad et al., 2009). The survival rate of IFN-R morphants dropped to about 50% after infection (Figure 6E) and the disease severity increased sharply, with respect to embryos injected with a control MO and later infected with SINV (Figure 6F). Surprisingly however, IFN-R morphants did not develop brain infection at earlier time-points or at higher frequency with respect to controls (Figure 6G). Nevertheless, we observed an extensive increase in the number of infected cells in IFN-R morphants (Figure 6H-I) and more cell types became susceptible to SINV (*e.g.* chondrocytes, fibroblasts – data not shown).

RNA interference (RNAi) is used by plants and many invertebrates as a major antiviral defense mechanism (Kemp and Imler, 2009), and whether an antiviral function for RNAi exists in mammals has been a long-standing debate (Umbach and Cullen, 2009). Thus, we tested whether zebrafish larvae show evidence of a RNAi mediated response against SINV, in parallel with the IFN response, by performing deep sequencing of small RNAs extracted from 1 dpi SINV-infected larvae. Unlike what happens in SINV-infected *Drosophila* (Saleh et al., 2009), no

SINV-specific small RNAs produced through the RNAi machinery could be detected in zebrafish (data not shown). These results show that zebrafish larvae mount a protective IFN response against SINV, which limits infection in the periphery but is not sufficient to prevent neuroinvasion (**Figure 6**).

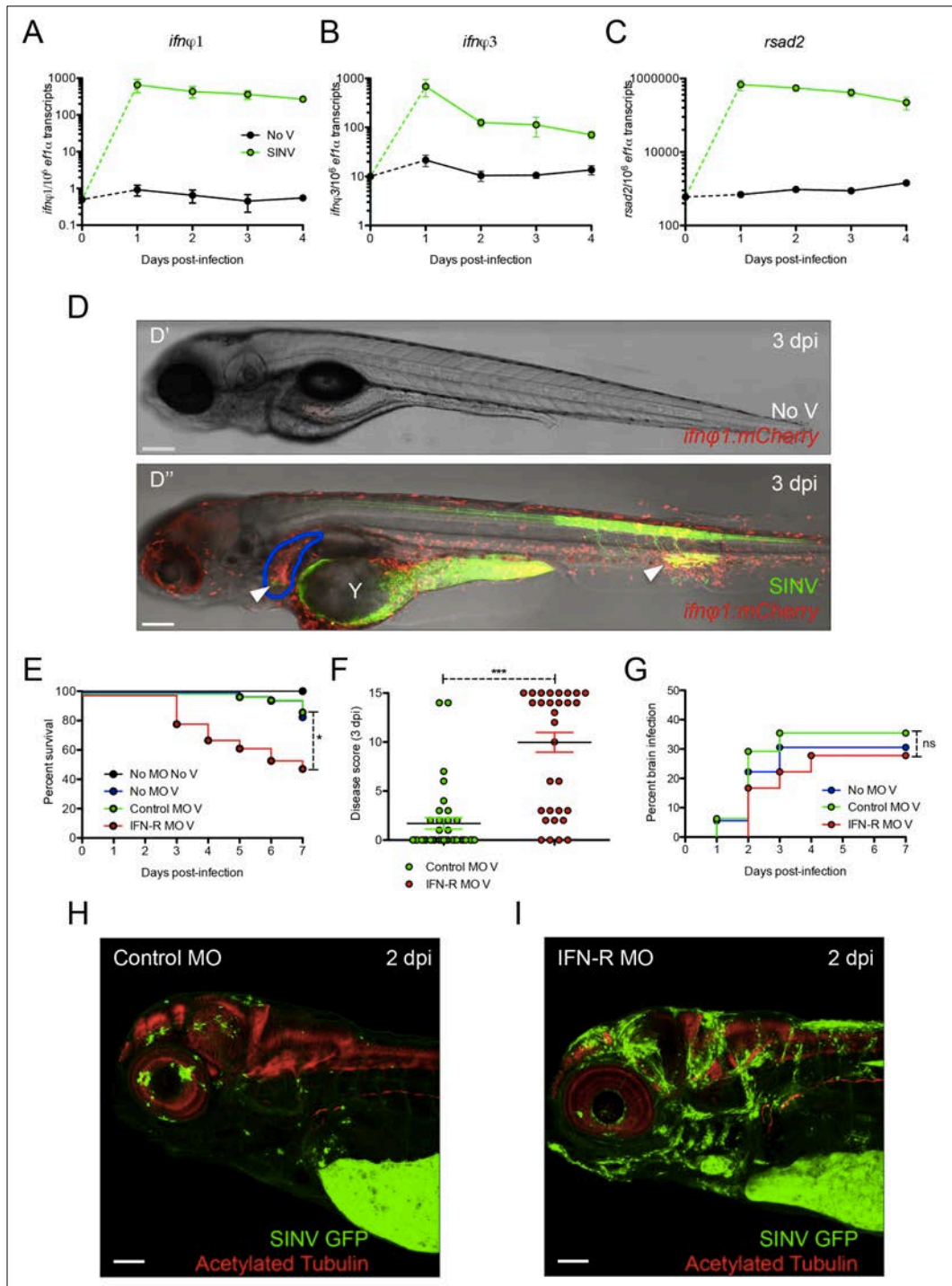


Figure R1.6. A protective IFN-mediated immune response is induced upon SINV-infection. (A–C) Expression of zebrafish type I IFNs (A) *ifn ϕ 1*, (B) *ifn ϕ 3*, and (C) IFN-stimulated gene (A–C) Expression of zebrafish type I IFNs (A) *ifn ϕ 1*, (B) *ifn ϕ 3*, and (C) IFN-stimulated gene *rsad2/viperin*. qRT-PCR, mean \pm s.e.m. of 3 pools of 9 larvae from 1 representative experiment. (D) *In vivo* distribution of IFN-producing cells upon infection of *ifn ϕ 1:mCherry* transgenic larvae at 3 dpi. (D') No V and (D'') SINV-infected larva, iv. White arrowheads: IFN-expressing cells close to infection foci; blue circle: liver; Y: the left part of the yolk was not imaged to reduce acquisition time and results in an incomplete pattern of infection. (E) Survival curves, (F) Disease score, and (E) Percentage of brain infected larvae in Control and IFN-R morphants infected larvae. (E, F) N = 36 from 3 independent experiments pooled. (G) N = 24 from 2 independent experiments pooled. ***P<0.001, *P<0.1, ns - not significant [(E, G) Log-rank; (F) two-tailed unpaired t-test]. (H, I) SINV infection pattern in (H) Control and (I) IFN-R morphants at 2 dpi. Confocal imaging of WIHC processed larvae, GFP in green, acetylated tubulin in red. (D, H, I) Scalebars: 50 μ m.

Supplemental Data Items

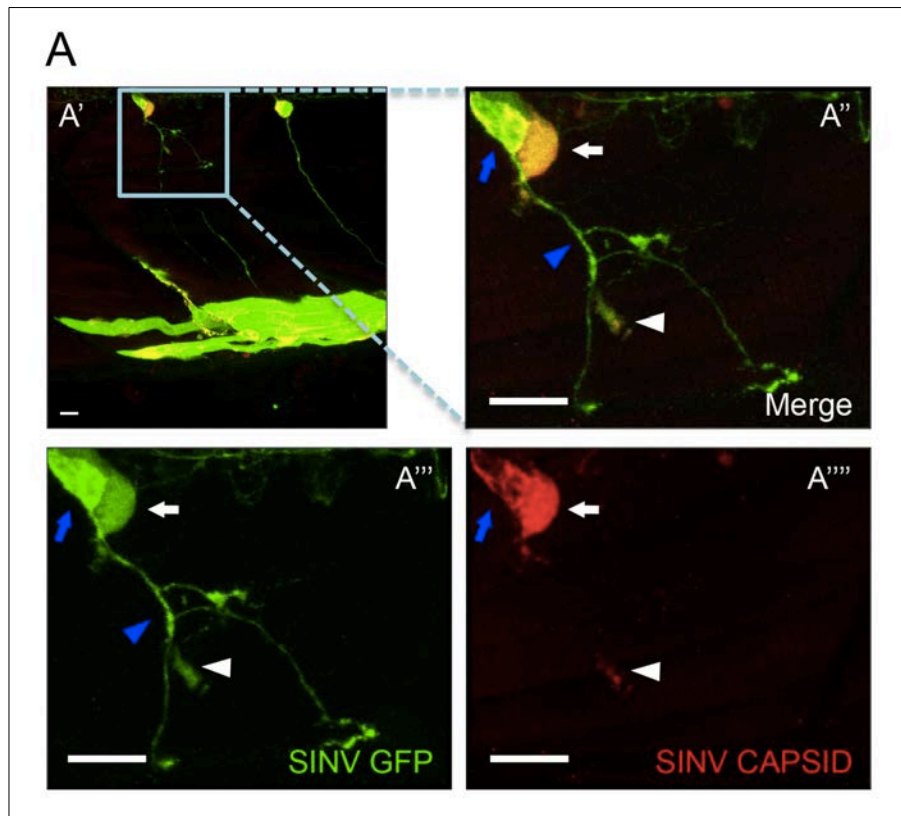


Figure S1. Infection of neurons in the tail region. Related to Figure 1. (A'–A''') Confocal imaging of WIHC processed infected larva. (A'') Infected cells displaying both GFP (green) and viral capsid (red) staining. Infection of the cell body is indicated by arrows; infection of axons is indicated by arrowheads. (A''') GFP staining is present in both cells (blue and white arrows and arrowheads). (A''') Capsid staining is present in the cell body of both cells (blue and white arrows) but only in the axon of one cell (white arrowhead). Scalebars: 10 μ m.

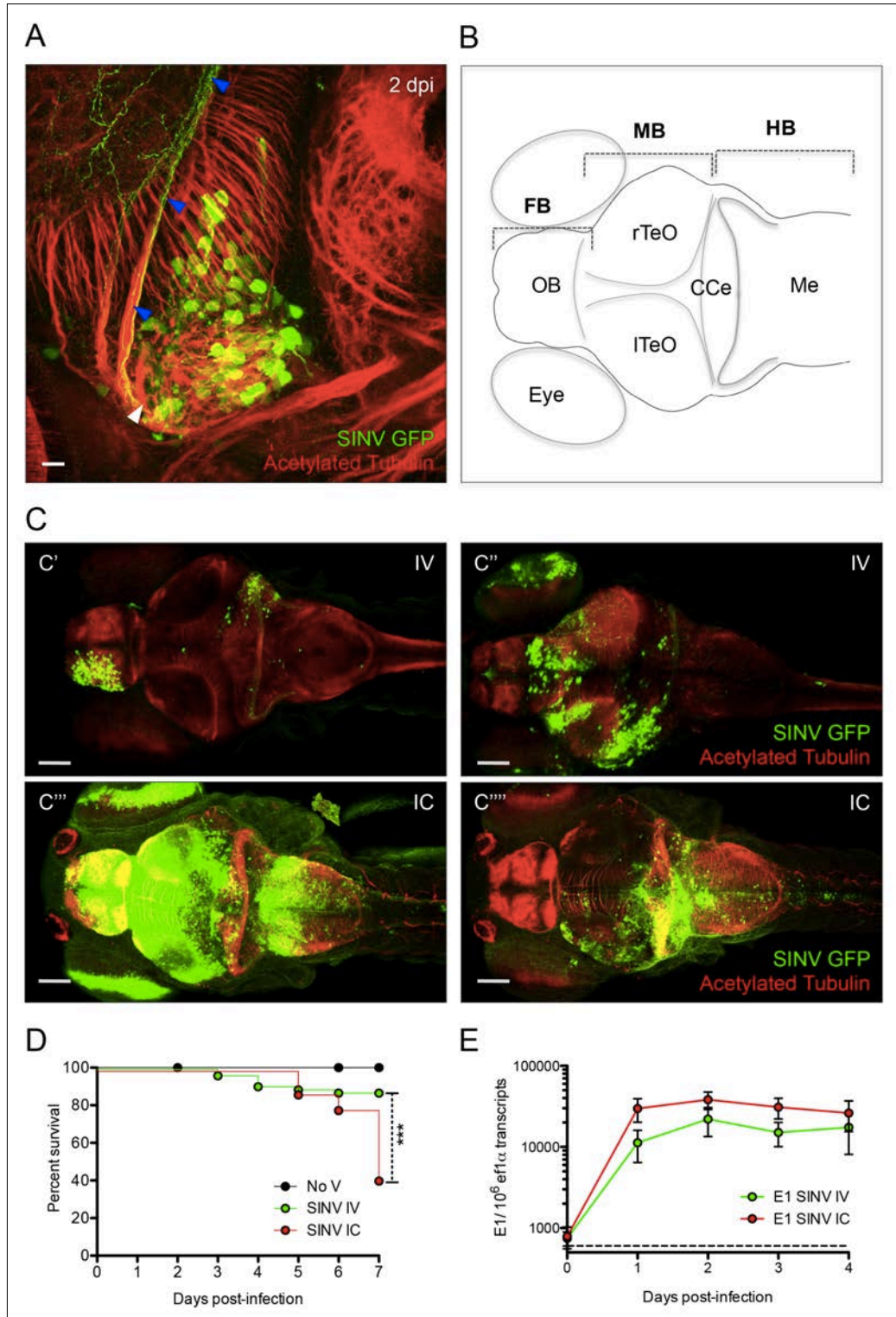


Figure S2. SINV infection in iv and ic injected larvae. Related to Figure 2. (A) Confocal image of WIHC processed SINV-infected larva. GFP staining (SINV-infected cells) in green, acetylated tubulin (axons) in red. Magnification from Figure 2C. White arrowhead: neurons of the trigeminal ganglion. Blue arrowheads: axon from a trigeminal ganglion neuron reaching the optic tectum. (B) Schematic representation of brain areas most commonly infected upon SINV inoculation. Dorsal view of the general organization of the zebrafish brain. OB, olfactory bulb; TeO, optic tectum; CCe, corpus cerebelli; Me, medulla oblongata. (C'–C''') Confocal images of WIHC processed SINV-infected larvae at 2 dpi, showing infection foci in different areas of the brain. GFP staining (SINV-infected cells) in green, acetylated tubulin (axons) in red. (C', C'') Representative iv-injected larvae, showing infection foci in different areas of the brain. (C''', C''') Representative ic-injected larvae, showing widespread infection in the brain. (D, E) Effects on (D) Survival and (E) Viral replication upon iv or ic SINV inoculation. (D) Data pooled from 5 independent experiments. N = 12 per group. ***P<0.001 (Log-rank test). (E) Virus titers in iv and ic infected zebrafish larvae. Data represent mean ± s.e.m. of 5 larvae per time-point, from 2 experiments pooled. Dotted line: threshold level from uninfected control larvae.

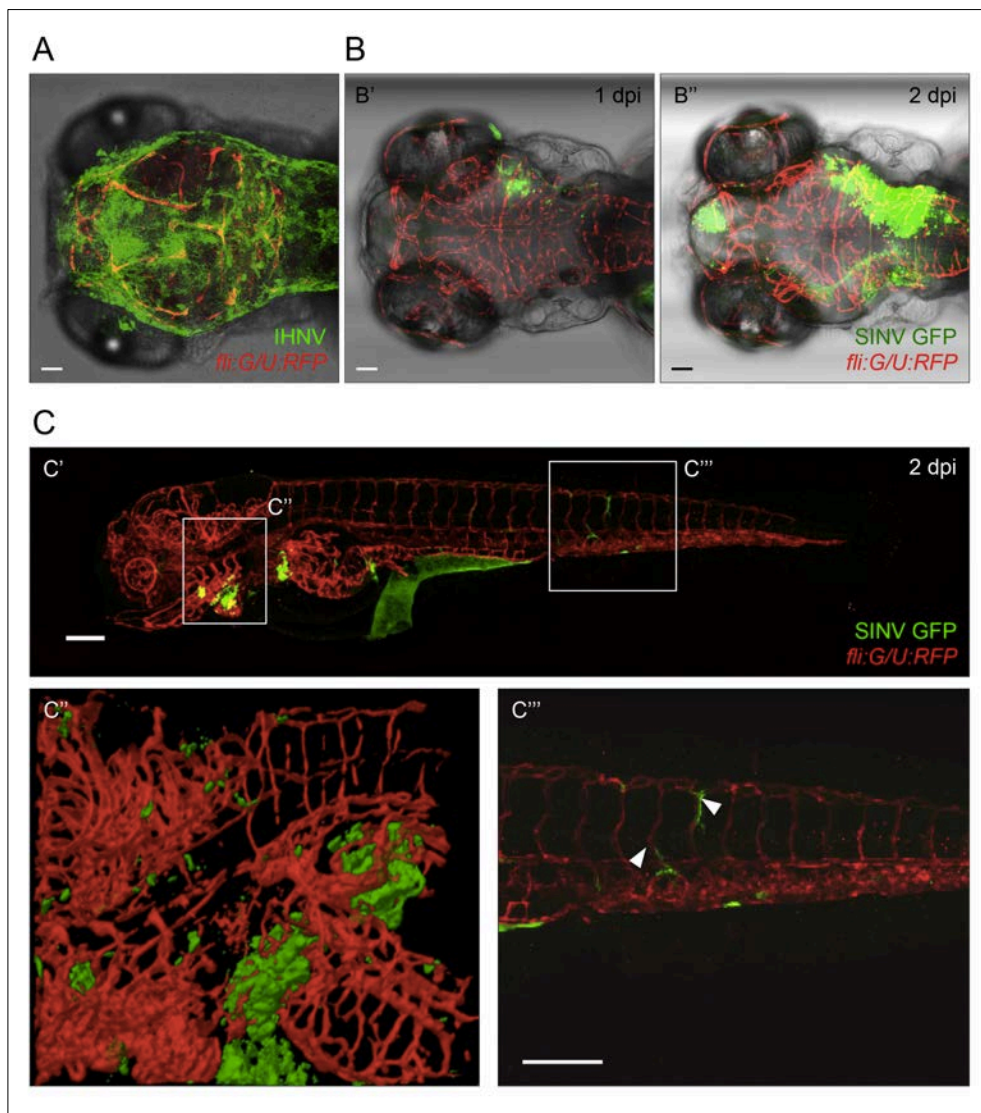


Figure S3. SINV infection in *fli:G/U:RFP* larvae. Related to Figure 3. (A, B) Confocal imaging of (A) fixed and (B) *in vivo* *fli:G/U:RFP* larvae. Merge with transmitted light, vasculature in red, virus in green. (A) WIHC of IHNV-infected larva, at 2 dpi. (B', B'') *In vivo* imaging of the same SINV-infected larva, at (B') 1 dpi and (B'') 2 dpi. Scalebars: 50 μ m. (C'–C'') Confocal imaging of Clarity-treated *fli:G/U:RFP* SINV-infected larva, at 2 dpi. (C') 3D rendering of the infected area. (C'') High magnification of the infected area in the tail region. White arrowheads: infected cells close to the vasculature. Scalebars: 150 μ m.

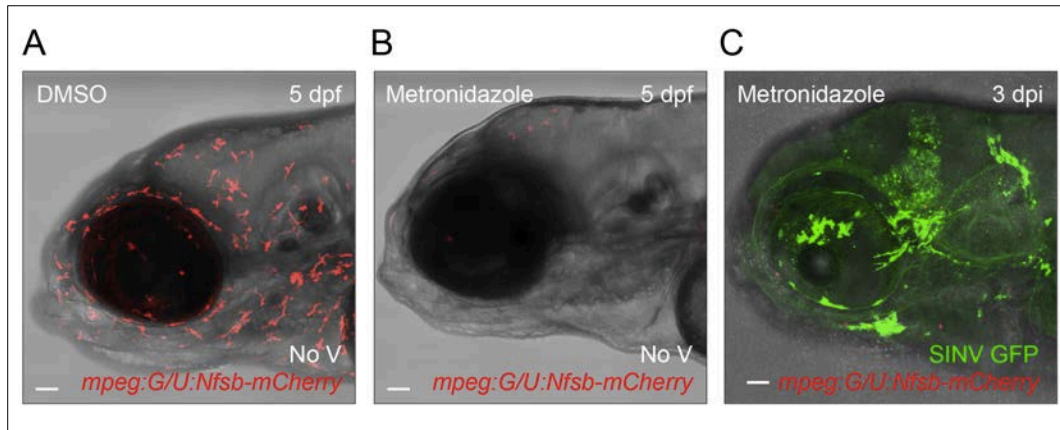


Figure S4. Efficiency of macrophage depletion. Related to Figure 4.

In vivo confocal imaging of 5 dpf larvae (A) DMSO and (B) Metronidazole-treated. Macrophages are shown in red. (C) Confocal imaging of WIHC processed SINV-infected larva at 3 dpi, pretreated with metronidazole. GFP in green, macrophages in red. Scalebars: 50 μ m.

Supplemental Movies:

S1. Movement through Z-stacks of *fli:G/U:RFP* IHNV-infected larva, at 2 dpi. Related to Figure S3A.

S2. Movement through Z-stacks of *fli:G/U:RFP* SINV-infected larva, at 1 dpi. Related to Figure S3B'.

S3. Movement through Z-stacks of *fli:G/U:RFP* SINV-infected larva, at 2 dpi. Related to Figure S3B''.

S4. 3D rendering of *fli:G/U:RFP* SINV-infected larva. Related to Figure S3C.

Discussion

Invasion of the CNS is a key event during viral infections, and often a matter of life and death for the host. It is however quite difficult to observe this phenomenon in mammals, thereby hindering the design of preventive strategies.

In the present study, we used zebrafish as a model to study the infection course of SINV, with a focus on its neuroinvasiveness. We found that the peak of viremia is at 48 hpi and it slowly declines over time. A similar kinetic was obtained for the IFN-mediated immune response. Observed clinical signs included edema, yolk opacification, and blood flow arrest. Thanks to *in vivo* imaging studies, we also assessed the progression of the infection. Infected cells appeared in the periphery as early as 8 hpi, always prior to infected neurons of the peripheral or the central nervous system. Overall, the disease signs and kinetic of SINV infection in zebrafish were quite similar to those previously observed in CHIKV-infected fish (Palha *et al.*, 2013), suggesting that the mechanisms that determine disease onset are conserved between related *Alphaviruses*.

While ic injections resulted in rapid onset of CNS infection in 100% of cases, iv injected larvae displayed a greater variability in terms of time of appearance and progression of CNS infection. This phenomenon was also observed in inbred mice injected with SINV subcutaneously (Cook and Griffin, 2003), and therefore suggests that the variability observed is not due to the higher genetic heterogeneity of zebrafish, which are not fully inbred. However, iv injections better represent the actual entry route of the virus upon bite from an infected mosquito and therefore allowed us to characterize the natural mechanism of SINV entry in the CNS.

Fleming *et al.* showed that the maturation of the BBB in zebrafish larvae is progressive until 10 dpf. At 3 dpf, the time at which larvae were injected, high molecular weight molecules (~900 Da) are already excluded from the CNS (Fleming *et al.*, 2013). We could therefore exclude passive transport of viral particles from the blood. In some cases, viruses have been shown to enter via the choroid plexus (ChP) (*e.g.* HIV, Falangola *et al.*, 1995), the region of the brain where the cerebrospinal fluid is formed. Studies on morphogenesis formation have shown that in zebrafish the ChP develops at the dorsal midline on the fourth

ventricle and shifts towards the ear level at around 3 dpf (García-Lecea et al., 2008). Here we found that commonly infected areas in the brain were rather lateral or distant from the ChP (*e.g.* olfactory bulb, optic tectum, medulla), which strongly suggests that SINV does not enter the brain via prior infection of the forming ChP.

The vasculature was shown to remain intact and infection of endothelial cells of the brain microvessels was never observed. Moreover, ic injections revealed little or no infection of cells outside the CNS, thereby corroborating the observation that SINV does not compromise the integrity of the BBB.

We also showed that the virus does not target macrophages. Macrophage depletion assays induced a slightly higher disease severity in treated fish, as in the CHIKV model, but did not prevent the virus from accessing the CNS. These results show that, even though macrophages may contribute in part to the control of SINV infection, they are not required for the virus to reach the CNS.

The observations we collected from SINV-inoculated larvae therefore strongly support the hypothesis that viral entry in the CNS occurs after infection of peripheral nerves, such as infection of trigeminal ganglion cells prior to infection in the CNS or infection of muscle cells followed by infection of motor neurons. These results are in accordance with the aforementioned study of mice infected with a luciferase-recombinant SINV strain, where viral replication in the nose or the spinal cord was shown to always precede infection in the brain (Cook and Griffin, 2003).

As previously observed in CHIKV-infected larvae, IFN was highly induced with the expression peaking at 24 hpi and slowly declining over time. Moreover, we showed that IFN-R kd induces changes in viral tropism and determines infection of several cell types not normally targeted by the virus in zebrafish. This is in accordance with a previous study conducted in mice by Ryman et al. (2000). Interestingly, while it did result in more infected cells, IFN-R kd did not cause an increase in occurrence of CNS invasion. Overall these data indicate that the IFN-mediated immune response is a major component involved in the protection against SINV infection and that IFNs limit SINV replication efficiency at early stages post-infection in the periphery, yet they do not prevent entry in the CNS. Other more complex immune mechanisms are probably involved in the response

against SINV once the virus reaches the CNS, which are worth investigating in the future.

While IFN was clearly a major defense mechanism of zebrafish larvae against SINV, no evidence for a RNAi-mediated response could be detected. Our simple experiment does not rule out the existence of antiviral RNAi in fish. However, since it has been proposed that the RNAi response is restricted to undifferentiated stem cells in mammals, and therefore more prominent in the early life stages (Maillard et al., 2013), our use of zebrafish larvae reinforces our conclusions. Moreover, a recent study provided compelling evidence for the absence of an antiviral role of RNAi in mammals (Backes et al., 2014). Since antiviral RNAi exists in plants, insects, and nematodes, it is very likely to be ancestral, and thus would have been lost in the lineage leading to mammals. If, as we believe, teleost fish do not use antiviral RNAi, then this loss occurred more than 350 My ago – very possibly when the protein-based antiviral defense appeared in early vertebrates.

Understanding the entry mechanism of a certain pathogen in the CNS has always been impaired by the difficulties in visualizing the progression of the infection. Moreover, a high number of animals and organ/tissue samples are generally needed at different time-points, to avoid missing important cues. The combined use of a recombinant SINV-GFP strain with fluorescent-reporter zebrafish lines, on the contrary, allowed us to gain single-cell resolution details at the whole organism level and with minimal invasiveness for the host. This in turn will be useful in the future for the study of neurotropic viruses and to test new antiviral therapeutics *in vivo*.

Experimental Procedures

Ethical statement

Animal experiments were conducted according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). All protocols were approved by the Ethical Committee for Animal Experimentation of Institut Pasteur and French Ministry of Research and Education (permit #01265.03).

Fish lines and husbandry

Zebrafish embryos were raised according to standard procedures as previously described (Westerfield, 2000; Levraud et al., 2008). Wild-type AB zebrafish were initially obtained from ZIRC (Eugene, OR). The following transgenic and mutant lines were also used: Tg(*elavl3:Gal4*)^{z349} (Akerboom et al., 2012), Tg(*fli1a:Gal4FF*)^{ubs4} (Zygmunt et al., 2011), Tg(*5xUAS:RFP*)^{nkuasrfp1a} (Asakawa et al., 2008), Tg(*mpeg:Gal4FF*)^{g125} (Ellett et al., 2011), Tg(*UAS-Elb:Eco.NfsB-mCherry*)^{c26} (Davison et al., 2007), Tg(*ifnphil:mCherry*)^{ip2} (Palha et al., 2013). Because of silencing issues of UAS-driven transgenes – a rare occurrence with the *UAS:RFP*^{nkuasrfp1a} allele – careful selection of fish expressing the complete brain and vasculature pattern was performed at 3 dpf before inoculation.

Eggs obtained by natural spawning were bleached and raised at 28°C in Volvic source water. Eggs used for imaging purposes were raised in 1-phenyl-2-thiourea/Volvic (Sigma-Aldrich, 0.003% final) from 24 hours post-fertilization (hpf) onwards to prevent melanin pigment formation. At 3 dpf, just before infections, larvae that had not hatched spontaneously were manually dechorionated.

Viruses

SINV-GFP was produced on BHK cells, according to (Hardwick and Levine, 2000). The virus used corresponds to the pTE3'2J-eGFP strain (NC_001547.1), and was obtained by insertion of the eGFP gene under the control of a second

subgenomic promoter (Hahn et al., 1992). Heat adapted IHNV strain 25.70 was produced on EPC cells as described previously (Ludwig et al., 2011).

Infection and disease scores

Injections and handling of larvae were performed as described (Levraud et al., 2008). Briefly, zebrafish larvae aged 70-72 hpf were inoculated by microinjection of $\sim 10^2$ TCID₅₀ viral SINV particles (~ 1 nL of supernatant from infected BHK cells, diluted to 10^8 TCID₅₀/mL). Injections were performed either in the caudal vein/aorta or in the left optic tectum or in the left retina. Larvae were then distributed in individual wells of 24-well culture plates with 1ml water, kept at 28°C and regularly inspected with a stereomicroscope until 7 dpi. Clinical signs of infection were assessed first on aware animals, which were then anesthetized for better observation. Quantitative assessment of the clinical status was based on a precise list of criteria, as previously described (Palha et al., 2013). Briefly, clinical signs were assessed blindly, yielding a disease score ranging from 0 (no disease sign) to 15 (dead or terminally ill). For ethical reasons, all larvae used in the experiments were killed by anesthesia overdose at 7 dpi. IHNV infections were performed as described in (Ludwig et al., 2011). Briefly, larvae were injected iv with 10^2 PFU of IHNV25.70, distributed in individual 24-well plates and incubated at 24°C.

qRT-PCR

RNA extraction, cDNA synthesis and quantitative PCR were performed as previously described (Ludwig et al., 2011); externally quantified standards were included to provide absolute transcript amounts. The following pairs of primers (sense and antisense) were used: *ef1a*: GCTGATCGTTGGAGTCAACA and ACAGACTTGACCTCAGTGGT; *SINV EI*: GACAACATGCAATGCAGAATG and CTAGTCAGCATCATGCTGCA; *ifn ϕ 1* (secreted isoform): TGAGAACTCAAATGTGGACCT and GTCCTCCACCTTTGACTTGT; *ifn ϕ 3*: GAGGATCAGGTTACTGGTGT and GTTCATGATGCATGTGCTGTA; *rsad2*: GCTGAAAGAAGCAGGAATGG and AAACACTGGAAGACCTTCCAA. To normalize cDNA amounts, we used the housekeeping gene *ef1a* transcripts.

Time-lapse in vivo imaging

For *in vivo* time-lapse imaging, 5-10 larvae were anaesthetized with 112 mg/mL tricaine and immobilized in ~1% low melting-point agarose in the center of a 35-mm glass bottom Ibidi® dish, then covered with ~2 mL water containing tricaine. Transmitted light/fluorescence imaging was performed using a Leica SPE inverted confocal microscope equipped with a 10X (NA 0.30) dry objective and 16X (NA 0.5), 40X (NA 1.15), 63X (NA 1.30) oil immersion objectives. Imaging was typically performed at 26°C and Z-stacks with 2 µm steps were taken at least every 10 min. Fish were imaged every day beginning from 1 dpi up to 4-5 dpi with imaging sessions typically lasting 30 min; control uninfected larvae were always included.

Whole-mount immunohistochemistry (WIHC) and imaging of fixed samples

WIHC was performed as described (Svoboda et al., 2001). The following primary antibodies were used: chicken polyclonal to GFP (1:500, Abcam), mouse mAb to acetylated tubulin (1:1000, Sigma), rabbit polyclonal to DsRed (1:500, Clontech), which also labels the mCherry protein, 19B7 mouse mAb antibody specific against IHNV G protein (glycoprotein) (1:500) (Biacchesi et al., 2002), rabbit polyclonal to SINV capsid (1:200) (a kind gift from R. Kuhn). Secondary antibodies used were: Alexa 488-labeled goat anti-chicken (1:500, Invitrogen), Alexa Cy5-labeled goat anti-mouse (1:500, Jackson ImmunoResearch), Cy3-labeled goat anti-rabbit IgG (1:500, Jackson ImmunoResearch). Nuclei were stained for 45 min at room temperature with DAPI/PBT 1:20.000 (Sigma). Fixed embryos were progressively transferred into 80% glycerol before imaging. Images were acquired with the Leica SPE inverted confocal microscope, as described above and Z-stacks of maximum 150 µm with 2 µm steps were taken. Image processing (maximal projections and reconstruction of whole embryos) was carried out with Adobe Photoshop CS6 software.

CLARITY procedure

Zebrafish larvae from 4 dpf to 7 dpf were fixed in freshly prepared ice cold methanol free paraformaldehyde (PFA) 4% (wt/vol) in 0.01 M PBS (pH 7.4) overnight at 4°C. Samples were then infused in a pre-cooled solution of (4°C)

freshly prepared hydrogel monomers (0.01 M PBS, 0.25% VA-044 initiator (wt/vol), 5% dimethyl sulfoxide (vol/vol), 1% PFA (wt/vol), 4% acrylamide (wt/vol) and 0.0025% bis-acrylamide (wt/vol) for 2 days at 4°C. After degassing the samples, the hydrogel polymerization was triggered by replacing atmospheric oxygen with nitrogen in a desiccation chamber for 3 hours at 37°C. Samples were cleaned from superfluous hydrogel and transferred into embedding cassettes for lipid clearing. Passive lipid clearing was performed for 5 days at 40°C in the clearing solution (8% SDS (wt/vol), 0.2 M boric acid, pH adjusted to 8.5) under gentle agitation. Subsequently the samples were thoroughly washed in 0.01 M PBS, tween 0.1% (wt/vol) (PBSt) for 2 days at room temperature with gentle agitation.

Immunostaining of clarified samples

CLARITY processed larvae were incubated in blocking solution (0.01 M PBS, 0.1% tween 20 (vol/vol), 1% TritonX100 (vol/vol), 10% dimethyl sulfoxide (vol/vol), 10% normal goat serum (vol/vol), 0.05 M glycine) overnight at 4°C. Subsequently samples were incubated in staining solution (0.01 M PBS, 0.1% tween 20 (vol/vol), 0.1% Triton X100 (vol/vol), 10% dimethyl sulfoxide (vol/vol), 2% normal goat serum (vol/vol), 0.05% azide (vol/vol)) with primary antibody (chicken anti-GFP, 1:600 – Avès Labs) for 5 days at room temperature under gentle agitation. After four washing steps in PBSt, samples were incubated in staining solution with secondary antibody (goat anti-chicken Alexa Fluor 488, 1:600 – Invitrogen) for 5 days at room temperature. Samples were washed for 2 days in PBSt and stained with 1 µM DiIC18(3) solution (DiI Stain, Molecular Probes).

Imaging in high refractive index solution

A fructose based high refractive index solution (fbHRI) was prepared as follows: 70% fructose (wt/vol), 20% DMSO (wt/vol) in 0.002 M PBS, 0.005% sodium azide (wt/vol). The refractive index of the solution was adjusted to 1.4571 using a refractometer (Kruss). In preparation for imaging, the samples were incubated in 50% (vol/vol) fbHRI for 6 hours and finally incubated in fbHRI for at least 12 hours. For imaging, samples were mounted in 1% (wt/vol) low melting-point

agarose and covered with fbHRI. Whole-mount larvae fluorescence was recorded with a Leica TCS SP8 two photon microscope equipped with a mode locked Ti:Sapphire laser (Chameleon, Coherent) at 770 nm and the Leica HC FLUOTAR L 25x/1.00 IMM motCorr objective.

Macrophage depletion

Metronidazole-mediated depletion was performed as described in Davison et al., 2007. Briefly, Tg(*mpeg:Gal4FF*)^{gl25} fish (Ellett et al., 2011) were crossed to Tg(*UAS-Elb:Eco.NfsB-mCherry*)^{c26} (Davison et al., 2007), to generate double-positive transgenics and single-positive sibling controls. Embryos were placed from 48 hpf to 72 hpf in a 10 mM Metronidazole/0.1% DMSO solution to induce specific depletion of NfsB-mCherry-expressing macrophages. Embryos were then rinsed 3X with embryo water and subsequently injected with the virus.

Morpholino injection

Morpholino antisense oligonucleotides (Gene Tools) were injected into 1-2-cell stage embryos as previously described (Levraud et al., 2008). *crfb1* splice morpholino (CGCCAAGATCATACCTGTAAAGTAA) (2 ng) was injected together with *crfb2* splice morpholino (CTATGAATCCTCACCTAGGGTAAAC) (2 ng), knocking down all type I IFN receptors (Aggad et al., 2009). As control a morpholino with no target (NRP) was used: (GAAAGCATGGCATCTGGATCATCGA) (4 ng).

Statistical analysis

To evaluate difference between means, a two-tailed unpaired t-test or an analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test were used, when appropriate. Normal distributions were analyzed with the Kolmogorov-Smirnov test. Non-Gaussian data were analyzed with a Kruskal-Wallis test followed by Dunn's multiple comparison test. P<0.05 was considered statistically significant (symbols: ***P<0.001; **P<0.01; *P<0.05; ns=not-significant). Survival data were plotted using the Kaplan-Meier estimator and log-rank tests were performed to assess differences between groups. Statistical analyses were performed using Prism 5 software.

Supplemental Information

Supplemental information includes four figures and four movies.

Author Contributions

Conceived and designed the experiments: JPL PB GP CL JSJ MCS PH. Performed the experiments: GP CL JPL NP VB PA EDJ HB. Analyzed the data: GP JPL PB CL LF. Contributed reagents/materials/analysis tools: PA CS. Wrote the paper: GP JPL PB CL.

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Competing Interests Statement

The authors declare no competing interests.

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CHAPTER II. CNS-Specific Immune Responses to SINV infection

The results presented in the previous Chapter relative to the IFN-mediated immune response upon SINV infection have shown that 1. the IFN response, measured at the whole-body level, is readily activated and peaks at 24 hpi, which coincides with the peak of viremia, 2. the main IFN-producing cell populations are hepatocytes and motile leukocytes, most likely neutrophils, and 3. IFN knock-down results in changes in virus tropism, increased disease severity and mortality but no significant increase in brain infection frequency. In accordance with the results from Ryman *et al.*, we have shown that the IFN response is crucial to contain virus spreading in the periphery at early times post-infection (Ryman *et al.*, 2000). Our data also indicated that IFN production is not sufficient to prevent CNS invasion, and even less efficient to stop the progression of the infection in the CNS than in the periphery. Based on these results, we started a more detailed investigation of the immune responses activated in the CNS. In particular, the study of different SINV strains has given us insights into strain-specific modulations of host responses that determine from early time points the outcome of the infection (section 2.1). On the other hand, the further characterization of the *ifn ϕ 1:mCherry* line has helped us shed light into the events occurring at later time points, after CNS invasion (section 2.2). Although preliminary, these data are nonetheless very promising and will continue to be explored in the future.

2.1. Strain-dependent modulation of the IFN signaling pathway

The outcome of an infection heavily depends upon a number of factors, including the genotype of the pathogen as well as the age and immune competence of the host, and is usually decided at the early stages. Rapid activation of the innate antiviral immune response is in fact crucial to block permissivity of uninfected cells to infection. In turn, subversion of the IFN response results in quick and generalized virus spread, even in cells not normally targeted by the virus. The characterization of different virus strains can therefore be helpful to determine

conserved virulence determinants between members of the same virus family as well as their specific interaction with the immune system.

This was one of the reasons that lead us to produce a different SINV recombinant strain, SINV *pTR339-mCherry/2A* (obtained from the original African isolate AR339 and from hereon referred to as SINV2-mCherry), to compare its infectious course with that of our first SINV strain, *pTE3'2J-eGFP* (which we will now rename SINV1-GFP) (Hahn *et al.*, 1992). The other obvious reason was the color complementarity between the two strains.

SINV AR339 was originally isolated from a pool of *Culex pipiens* and *Culex univittatus* mosquitoes collected in Egypt (Taylor *et al.*, 1955). From that strain, a full-length cDNA infectious clone (TR339) was generated (McKnight *et al.*, 1996). In contrast, the recombinant SINV strain TE3'2J was constructed from a chimeric mouse neurovirulent variant of AR339 called TE12 (Lustig *et al.*, 1988). The genomes of the two strains differ by 16 mutations, 5 of which encode aminoacid changes (1 in the 5' UTR, 3 in the ORF encoding NSP2 and NSP3, and 2 in the ORF encoding E2) (Figure R2.1).

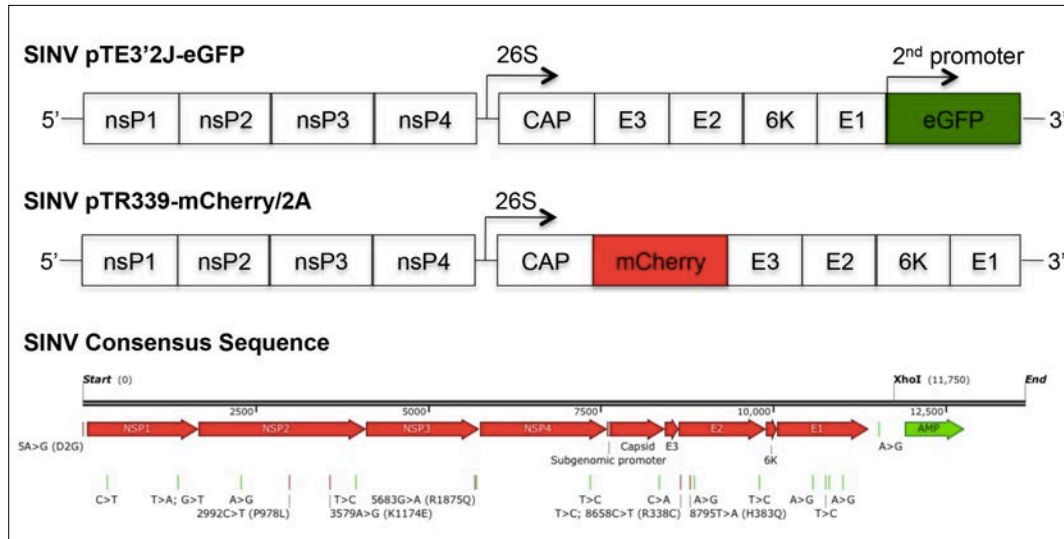


Figure R2.1 Schematic representation of SINV1-GFP and SINV2-mCherry genome organization. Amino acid changes between SINV1-GFP and SINV2-mCherry sequences are indicated in red.

The SINV2-mCherry strain, whose plasmid was kindly donated by Carla Saleh from Pasteur Institute, was produced at INRA according to Hardwick and Levine (2000) under the supervision of Corinne Torhy and Christelle Langevin. Injection of larvae with $\sim 10^2$ TCID₅₀ of SINV2-mCherry (1 nL at a concentration of $\sim 10^8$

TCID₅₀/mL) revealed that the strain was much more virulent than SINV1-GFP. Comparison of the two strains in terms of viral burden and IFN induction at 1 dpi showed that SINV2-mCherry replicates at higher levels (~6 times) while IFN expression was about 2 times lower than that measured in SINV1-GFP infected larvae. Moreover, SINV2-mCherry kills over 60% of injected larvae as opposed to SINV1-GFP, which instead shows ~20% mortality rate at 7 dpi, with a similar inoculum (Figure R2.2).

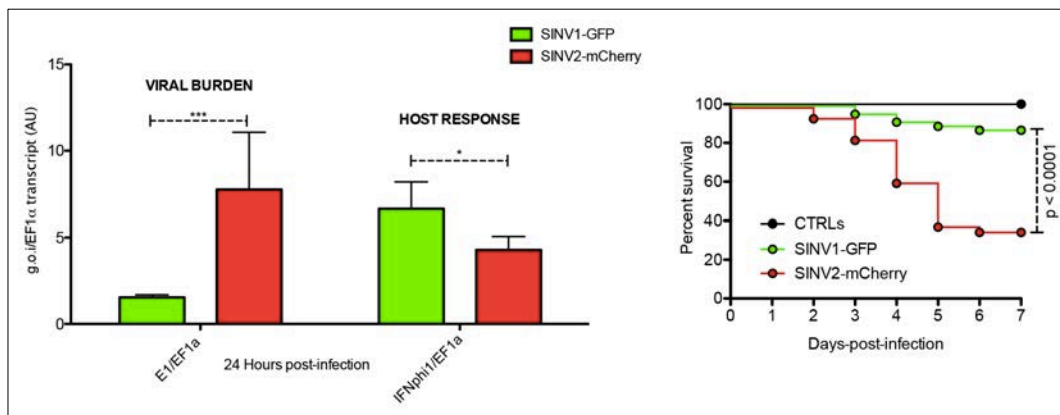


Figure R2.2. Comparison between the two SINV strains, in terms of viral burden, induced host response at 24 hpi, and mortality rate. Left panel, qRT-PCR of *SINV EI*; Middle panel, qRT-PCR of *IFN β 1*. Mean + s.e.m. of 3 larvae from 1 representative experiment. ***P<0.001, *P<0.1; Right panel, survival of infected larvae. Data pooled from 5 independent experiments. N = 12 per group. ***P<0.001 (Log-rank test).

Imaging of fixed samples revealed that SINV1-GFP targets a restricted number of cell types as opposed to SINV2-mCherry, whose infection resembles that observed in IFN-R knock-down morphants (Figure R2.3). Nevertheless, both strains showed infection of neuronal cell types, such as trigeminal ganglion cells (Figure R2.4).

In the case of SINV2-mCherry, however, infection in the CNS was observed in all injected larvae while SINV1-GFP reached the brain in only 40% of cases. To compare the two strains in terms of cell tropism, we performed injections of serial dilutions of SINV2-mCherry and found that 10⁶ TCID₅₀/mL was a concentration phenotypically comparable to that of SINV1-GFP. Finally, co-injection of SINV1-GFP (10⁸ TCID₅₀/mL) and SINV2-mCherry (10⁶ TCID₅₀/mL) showed increased replication of SINV1-GFP, probably helped by IFN expression shutoff induced by SINV2-mCherry (Figure R2.5).

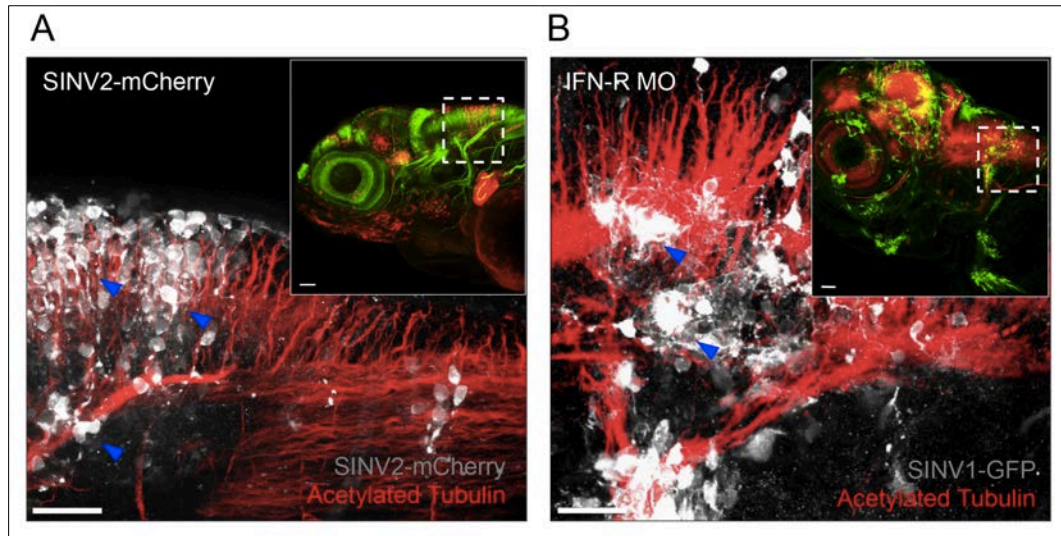


Figure R2.3. Comparison between the effects of SINV2-mCherry and IFN-R knock-down. (A) SINV2-mCherry infected larva. Inset: virus in red, acetylated tubulin in green, with dotted line showing wider panel; high magnification panel: virus in grey, acetylated tubulin in red (pseudocolors). (B) IFN-R morphant infected with SINV1-GFP. Inset: virus in green, acetylated tubulin in red, with dotted line showing wider panel; high magnification panel: virus in grey, acetylated tubulin in red (pseudocolors). Blue arrowheads indicate areas of extensive infection. Scalebars: 25 μm .

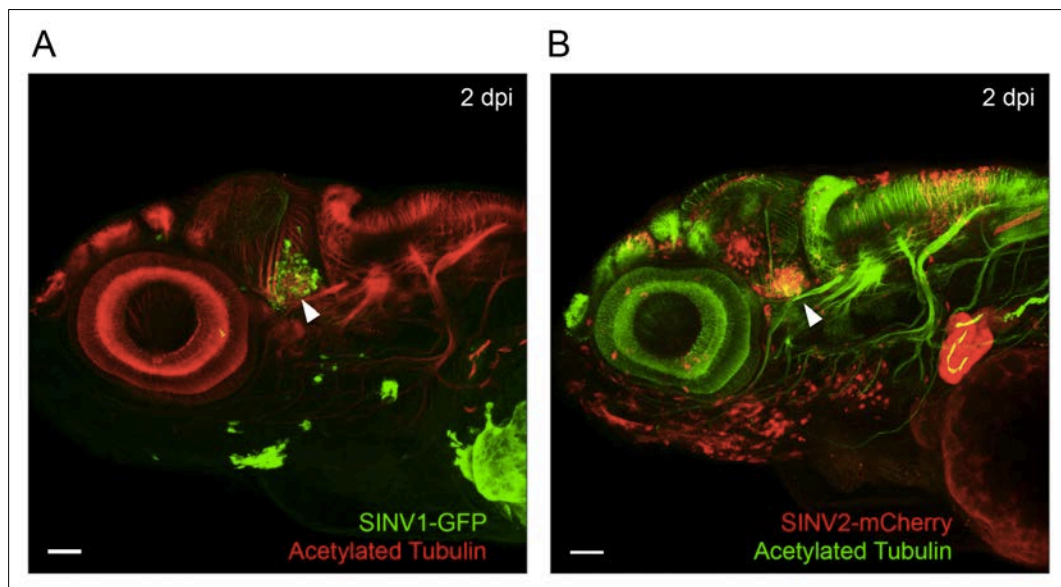


Figure R2.4. Comparison between SINV1-GFP and SINV2-mCherry. (A) SINV1-GFP in green, acetylated tubulin in red. (B) SINV2-mCherry in red, acetylated tubulin in green. White arrowheads indicate common areas of infection (neurons of the trigeminal ganglion). Scalebars: 50 μm .

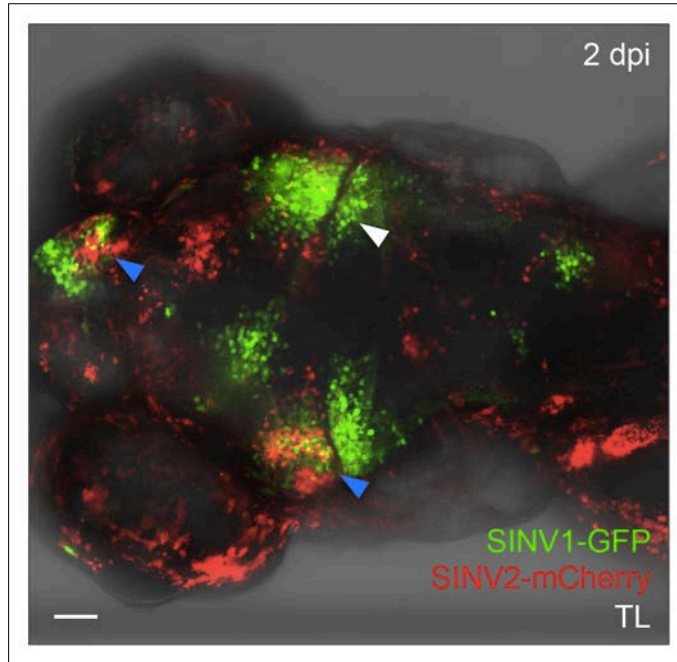


Figure R2.5. *In vivo* confocal imaging of a larva co-infected with SINV1-GFP and SINV2-mCherry. SINV1-GFP in green, SINV2-mCherry in red. White arrowhead indicates extensive infection of SINV1-GFP only; blue arrowheads indicate areas of co-infection. Scalebar: 50 μ m.

2.2. Leukocyte recruitment in the CNS

All experiments described in this and the following sections have only been performed with SINV1-GFP (SINV).

To better characterize the role of IFN-producing cells in the control of SINV-induced CNS infection, we injected *ifn ϕ 1:mCherry* transgenic larvae and performed *in vivo* imaging at daily intervals as previously described. Unlike what was previously reported in CHIKV infection in zebrafish (Palha *et al.*, 2013), IFN-producing motile cells (80% of which were neutrophils in the CHIKV system) were not excluded from the CNS (from which neutrophils are excluded in healthy animals) but rather actively permeating into the brain, even when the infection was relatively contained to one or few infection foci (Figure R2.6).

The morphology and motility of these cells indicated that they were most likely neutrophils; this has been formally verified by injecting SINV in *LyZC:DsRed* transgenic larvae (a red neutrophil reporter line) and observing the same response reported for *ifn ϕ 1:mCherry* transgenic larvae.

IFN-expressing leukocytes were visible in the CNS already at 2 dpi, time at which most larvae begin to show CNS infection, and their number increased over time and later reached a plateau. Moreover, in the case of extended infection, they were shown to “uptake” SINV-infected cells in the brain (Movie R2.1; Movie R2.2; Movie R2.3).

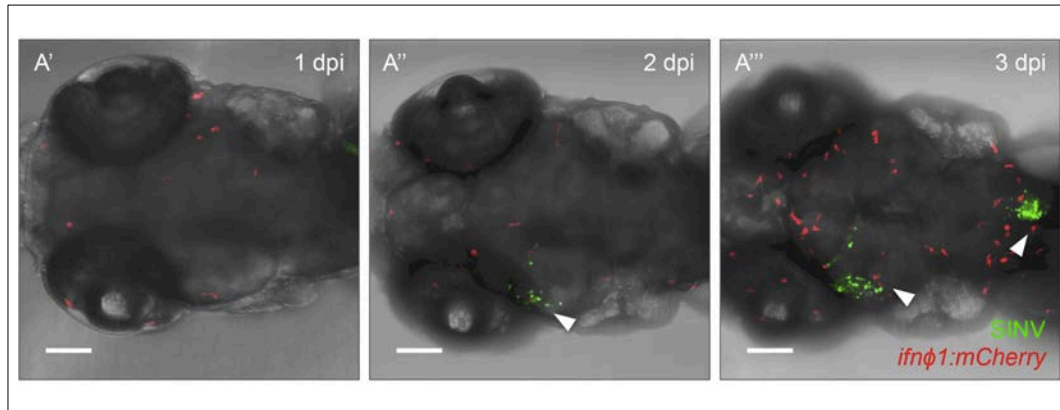


Figure R2.6. Progressive infection and IFN-producing cells recruitment in the CNS, upon SINV inoculation. *In vivo* confocal microscopy of an infected *ifnφ1:mCherry* larva at (A') 1 dpi, (A'') 2 dpi, and (A''') 3 dpi. Virus in green, IFN-producing leukocytes in red. White arrowheads indicate areas of progressive CNS infection. Scalebars: 50 μm.

To determine if microglial macrophages were involved in IFN expression or neutrophil recruitment, we also fixed infected *ifnφ1:mCherry* larvae and stained them to label SINV-infected cells (anti-GFP Antibody (Ab)), IFN-expressing cells (anti-dsRed Ab) and microglia (4C4 Ab). Confocal imaging confirmed the presence of neutrophil-shaped IFN-expressing cells within the brain parenchyma. Remarkably, within some infection foci, infected neurons were also expressing the IFN reporter. We had not observed that in our previous *in vivo* experiments, probably due to the lower laser power used to avoid phototoxicity (Figure R2.7).

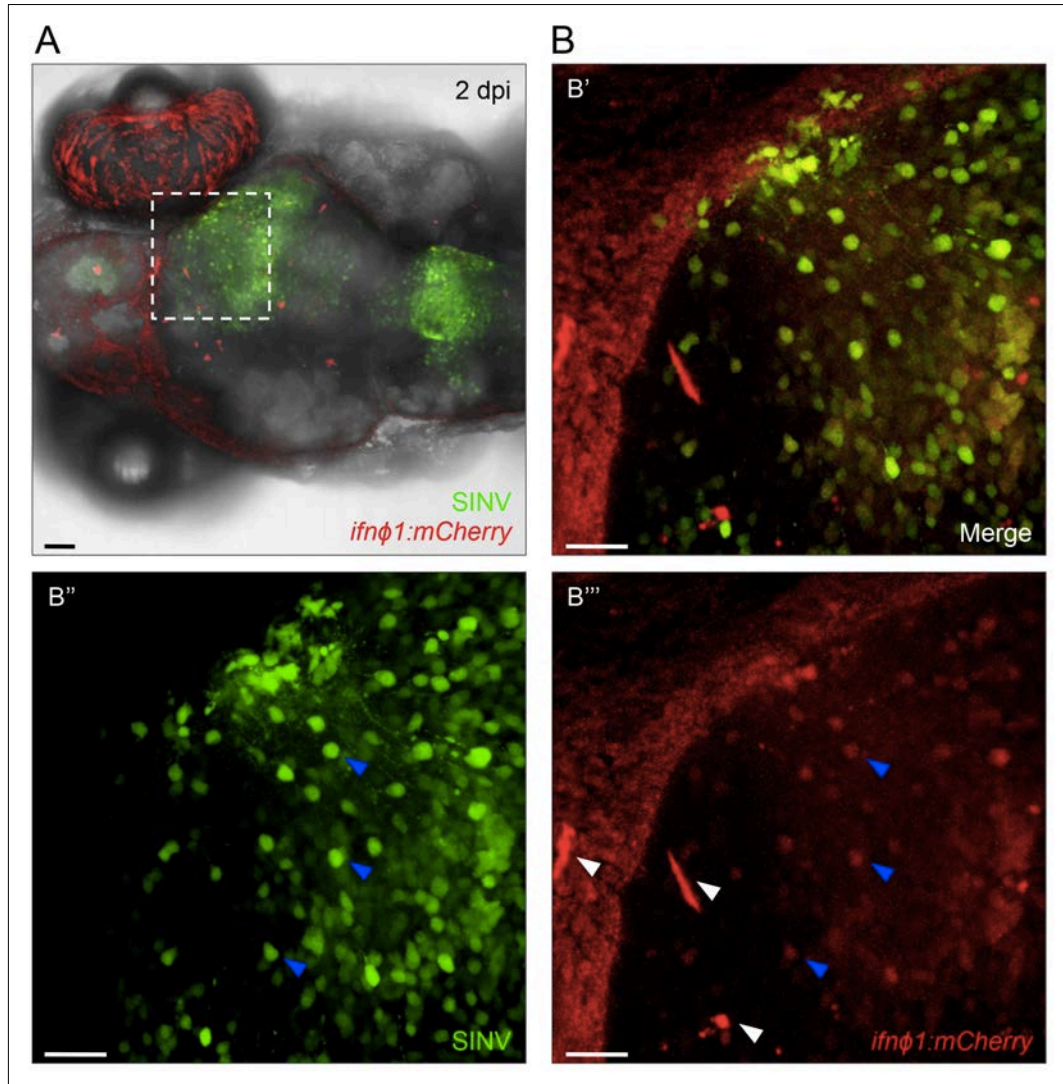


Figure R2.7. IFN expression in the CNS, upon SINV infection. (A–B''') *In vivo* confocal imaging of an infected *ifnφ1:mCherry* larva, at 2 dpi. Virus in green, IFN-producing leukocytes in red, transmitted light in grey. (A') Dorsal view of the head, with dotted line showing high magnification in (B'–B'''). (B') Merge. (B'') SINV-infected neurons. (B''') IFN-expressing cells. Blue arrowheads indicate IFN-expressing SINV-infected neurons, white arrowheads indicate IFN-expressing neutrophils. Scalebars: 25 μm.

By contrast, microglia did not express IFN; they were either close to the infection foci and with a rounded shape or distant from the infection sites and with a ramified shape (Figure R2.8). They were not observed interacting with IFN-expressing leukocytes. Thus although they probably phagocytose a lot of cellular debris generated at viral foci (hence their rounded shape, which can also indicate they are undergoing apoptosis), they do not seem to be activated or attracted by the infection, nor to contribute to the recruitment of IFN-making neutrophils.

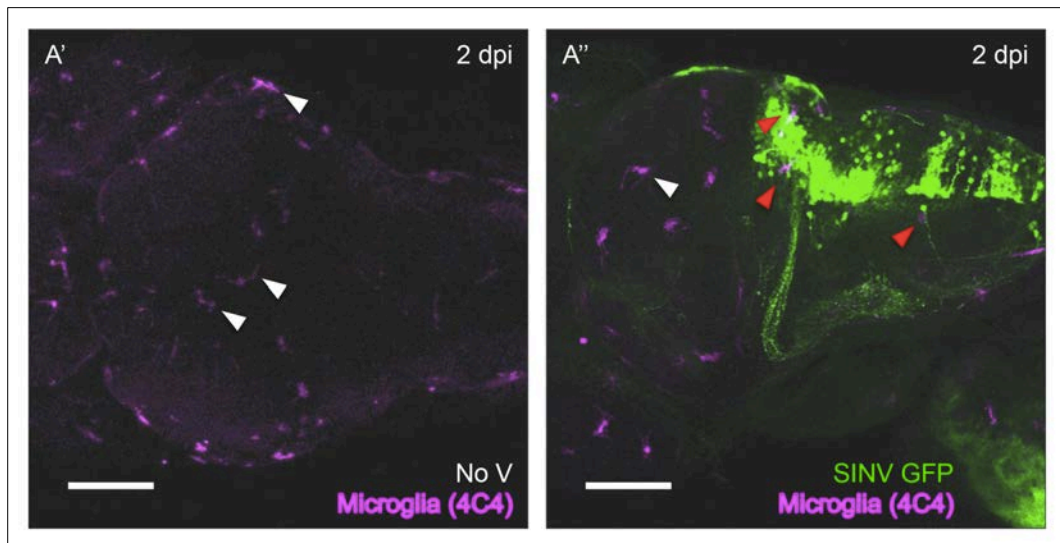


Figure R2.8. State of microglia, upon SINV infection. Confocal microscopy of IHC-processed (A') No V and (A'') SINV-infected larvae, at 2 dpi. GFP in green, microglia in purple. White arrowheads indicate ramified-shaped microglia, red arrowheads indicate rounded-shaped microglia. Scalebars: 100 μ m.

Finally, with the aim to improve the reproducibility and speed of data extraction from our *in vivo* imaging, we set up a collaboration with Prof. Reyes Aldasoro at City University London and Prof. Renshaw from the University of Sheffield, to use Matlab® for automatic image analyses. In fact, they recently developed an open source Matlab® package, *PhagoSight*, to analyse macrophages and neutrophils behavioral changes in response to a wound (Henry *et al.*, 2013). Therefore, I spent two weeks in Sheffield, where I used a simplified version of this algorithm to analyse our data related to leukocyte behavior in SINV infected *LyZC:DsRed* larvae and have confirmed that neutrophils are important for the control of the infection in the CNS, by uptaking infected cells (Figure R2.9).

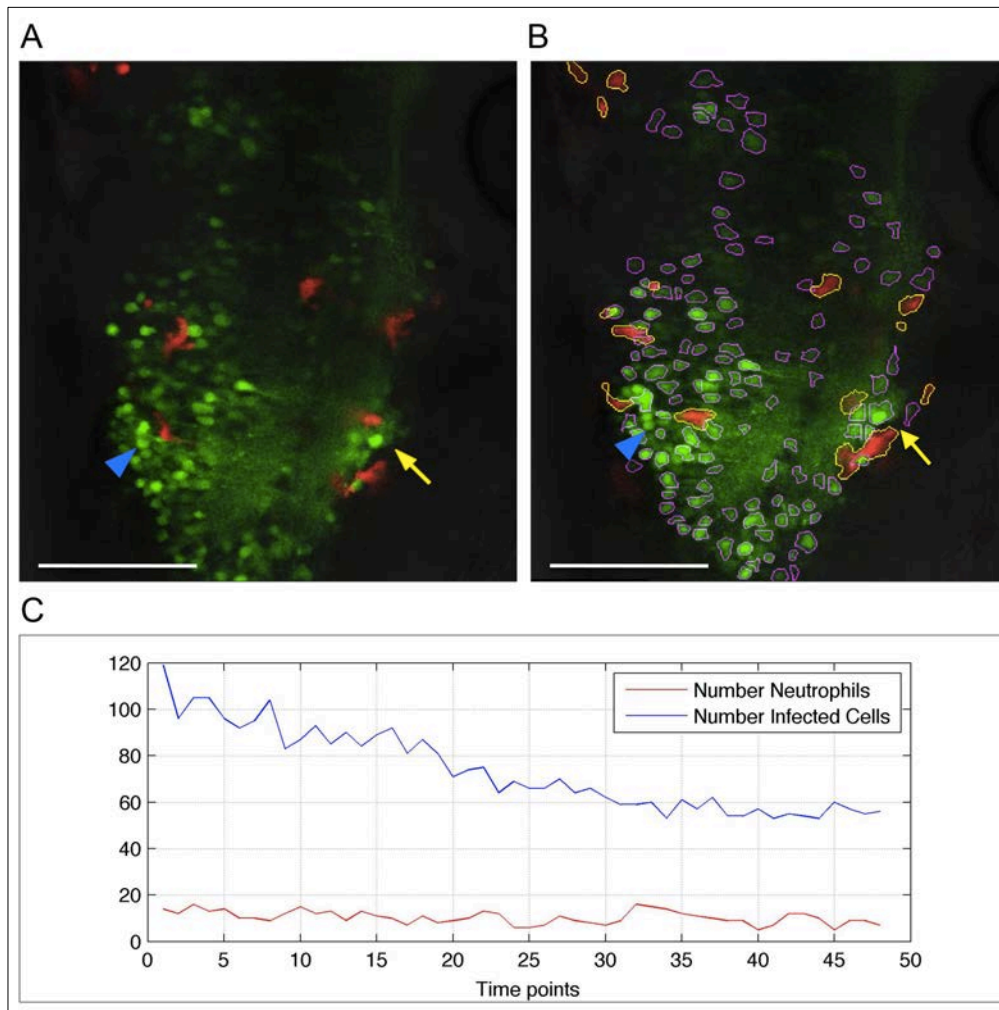


Figure R2.9. Image analysis performed with Matlab®. (A) Raw data. (B) Detection of cells based on fluorescence intensity. Red neutrophils are circled in yellow, green infected cells are circled in purple. (A–B) Yellow arrow indicates a GFP-high cell (included in the quantification), blue arrowhead indicates a low-GFP cell (erroneously excluded from the quantification). (C) Quantification of the number of neutrophils and infected cells over time, based on the analysis performed automatically by the algorithm.

The quantification has shown that within 12 hrs the number of neutrophils close to a highly infected area does not change significantly. However, the number of infected neuronal cells decreases considerably. Additional analyses are needed to relate the number of events in which a neutrophil interacts with an infected cell to that in which an infected cell disappears, thereby proving that neutrophils are needed to clear the infection in the CNS. Despite being incomplete for our purposes, the algorithm shows great potentials and helped reducing considerably the time required to perform such an analysis manually.

CHAPTER III. SINV Spreading and Persistence Mechanisms

Upon injection of SINV into zebrafish larvae, we showed that the virus enters the CNS most likely through infection of peripheral neurons and subsequent retrograde axonal transport (Chapter 1 of the Results). In a few larvae infected intracerebrally, infection of spinal cord neurons and muscle cells following brain infection was also observed. This strongly suggests that the virus can also use the anterograde transport machinery and reach areas distant from the first infection foci. Spreading within the CNS occurred mostly via long distance axonal transport, as evidenced by infection of Mauthner neurons. These are bilaterally symmetric reticulospinal neurons whose axonal projections are distributed from the caudal midbrain to the spinal cord (Zottoli and Faber, 2000).

Unlike what occurs in CHIKV-infected zebrafish larvae (Palha *et al.*, 2013), SINV-infected cells in the CNS did not all survive very long. In accordance with the results obtained in mice from Lewis *et al.* (1996), apoptotic cells were observed in several infected areas and correlated with the increasing number of circulating leukocytes in the brain (Movie R3.1).

To better document viral persistence over neuronal cell death in the CNS, we started setting up a feeding protocol for the long-term maintenance of infected larvae. Larvae can begin to feed between 4-5 dpf, time at which they exit the chorion and the mouth begins to open. Larvae raised in the fish facility are normally kept in groups of 50 or 60 in 2 liter tanks, with 3 rounds of food per day and regular water changes. However, to monitor the progression of the infection in our injected larvae, we are obliged to keep them in separate wells and feed them regularly while avoiding contamination of the water from residual food. Adapting a method previously developed for the feeding of individual axenic larvae (Rendueles *et al.*, 2012), we fed larvae daily with *Tetrahymena thermophila* and followed the progression of SINV infection in the CNS by daily observation at the fluorescent stereomicroscope. In a preliminary experiment, the majority of iv infected larvae succumbed within 4 to 5 dpi and only 20% survive until 21 dpi, end point of the experiment. However, only 8% of total infected larvae also retained infection in the CNS for the whole duration of the experiment. These experiments are so far inconclusive because the low surviving rates are

inconsistent with the results normally obtained with unfed larvae in short-term experiments (within 7 dpi). In all likelihood, there was a problem with the feeding procedure, which will need to be solved before a new test. Nevertheless, they showed that, in some cases, SINV infection can persist for at least three weeks in the CNS.

With the aim to characterize the effect of SINV on survival and response of neuronal cells, we also established a protocol to obtain primary cultures of zebrafish brain cells. This consists in the dissection of 72 hpf zebrafish larvae, to isolate brain samples before enzymatic and mechanical dissociation. Cells are then plated on pre-coated coverslips and cultured in a modified version of Minimum Essential Medium at 24°C for at least one week. To determine the cell composition of our culture, we established primary cell cultures from transgenic lines expressing the GFP or the mCherry fluorescent proteins in neuronal or in glial cells, respectively (Figure R3.1).

We hope that this newly developed tool will be helpful to identify the transport mechanism employed by SINV in neuronal cells, SINV effect on cell survival, role of autophagy, and cell responsiveness to IFN before and after the infection.

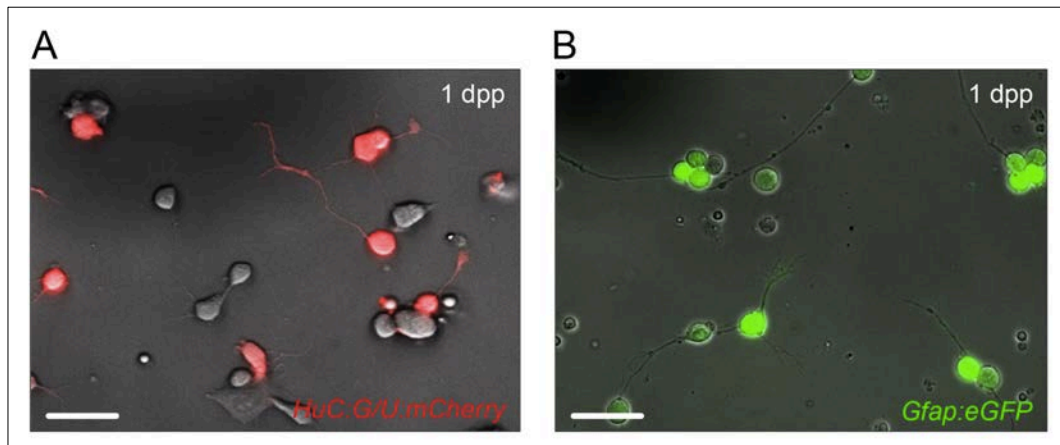


Figure R3.1. Zebrafish primary neural cell cultures. *In vivo* fluorescence microscopy of (A) *Huc:G/U:mCherry*-derived neurons and (B) *Gfap:eGFP*-derived glial cells, 1 day post-plating (dpp). Scalebars: 20 μ m.

CHAPTER IV. Tests on a Potential Broad-Spectrum Antiviral Drug

In the context of a collaboration with the “*Viral populations and Pathogenesis*” Unit headed by Marco Vignuzzi at Pasteur Institute, we also recently started investigating the potential antiviral activity of Difluoromethylornithine (DFMO) against SINV infection *in vivo*.

DFMO is a drug that irreversibly inhibits ornithine decarboxylase 1 (ODC1), a critical enzyme in polyamine biosynthesis (Metcalf *et al.*, 1978). These are small positively-charged molecules derived from arginine, involved in several cellular processes, including cellular proliferation (Gerner and Meyskens, 2004), apoptosis (Schipper *et al.*, 2000), ion channel regulation (Williams *et al.*, 1997), DNA conformation (Thomas *et al.*, 1995), transcription (Frugier *et al.*, 1994; Law *et al.*, 2001), and translation (Mandal *et al.*, 2013) in both mammalian and non-mammalian cells; their exact mechanism of action, however, is generally unclear. In the case of Semliki Forest virus and other RNA viruses, they have also been shown to promote viral RNA synthesis (Tuomi *et al.*, 1982).

The experiments performed *in vitro* by our collaborator Bryan Mounce showed that DFMO has no toxicity on Baby Hamster Kidney-21 (BHK-21) cells and is capable of reducing titers of several different viruses, including Poliovirus, Coxsackievirus B3, Dengue virus and West Nile virus.

Based on these results, we started testing whether DFMO could block viral synthesis and reduce viral titers *in vivo* in zebrafish larvae, while other labs performed few similar tests in mouse and in *Drosophila*.

Initial experiments performed after placing 1-cell stage eggs in DFMO-containing water at different increasing concentrations showed that the compound can be toxic at early stages of development, as the majority of embryos showed developmental defects including a crooked spinal cord, slow blood flow, and edemas.

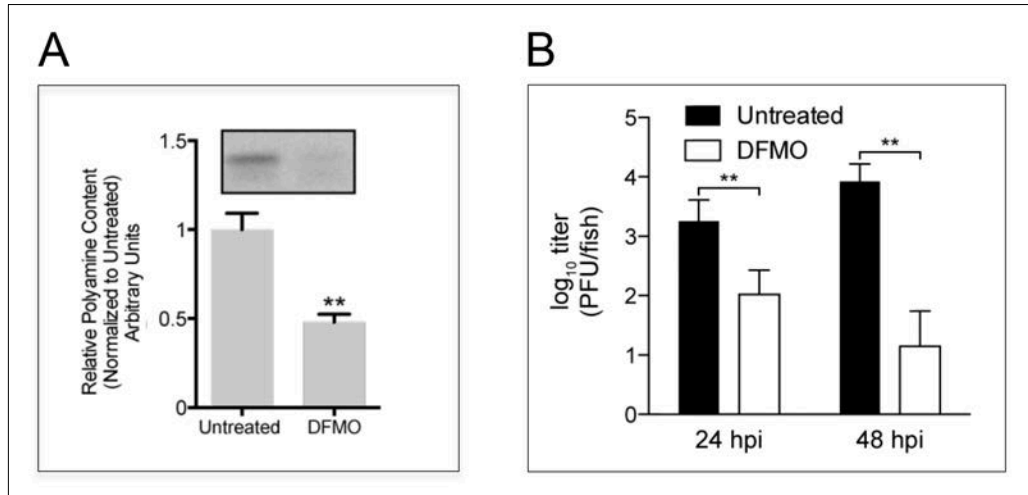


Figure R4.1. Quantification of polyamine depletion and viral replication in control non-treated and DFMO-treated infected larvae. (A) Polyamine quantitation. Inset shows representative chromatogram from each condition. Error bars represent + s.e.m. N = 3 from 1 representative experiment. (B) Viral titers from single larvae homogenates, at 24 and 48 hpi. N = 5 from 1 representative experiment. **P< 0.01.

Therefore, we placed 24 hpf zebrafish embryos in 1 mM DFMO-containing water for three days, and later measured polyamine content from fish homogenates with thin layer chromatography. In this case, the embryos developed normally, and underwent a significant reduction in polyamines quantity, thereby showing that DFMO is active in larvae as well (Figure R4.1A). Three days after treatment, zebrafish were inoculated ic with $\sim 10^2$ TCID50 SINV as previously described. Viral titers enumerated at 1 and 2 dpi were significantly reduced in DFMO-treated zebrafish (Figure R4.1B), showing the potential *in vivo* antiviral activity of DFMO.

This was corroborated by tests in other species, and with other viruses, confirming the potential of DFMO as a broad-range inhibitor of RNA virus replication that could be useful during viral outbreaks. This work is currently being submitted for publication (Mounce *et al.*, submitted).

Future experiments will focus on the determination of the exact mechanism of action of DFMO in the context of suppression/reduction of viral replication in zebrafish infected larvae.

Supplemental Movies:

R2.1: *ifn ϕ 1:mCherry* infected larva at 3 dpi. 1 Z-stack. 30 min time-lapse. Acquired with Leica SP8 inverted confocal microscope.

R2.2: *LyZC:DsRed* infected larva at 2 dpi. 1 Z-stack. 12 hrs time-lapse. Acquired with Leica SPE inverted confocal microscope.

R2.3: *LyZC:DsRed* infected larva at 2 dpi. Maximal Projection. 12 hrs time-lapse. Acquired with Leica SPE inverted confocal microscope.

R3.1: *ifn ϕ 1:mCherry* infected larva at 2 dpi. Maximal Projection. 10 hrs time-lapse. Acquired with Nikon Biostation.

DISCUSSION

DISCUSSION

The major results obtained during this PhD work have led to the characterization of the entry mechanism of Sindbis virus (SINV) into the Central Nervous System (CNS) of zebrafish larvae. We have shown that the virus neither disrupts the Blood-Brain Barrier (BBB) nor infects macrophages to reach the CNS. Instead, our data is consistent with axonal transport to the CNS after infection of peripheral neurons or nerve termini.

In the submitted article, we have identified neurons as an important target of the virus, thanks to *in vivo* analyses of *elavl3:Gal4/5xUAS:RFP* infected larvae (*HuC:G/U:RFP*) and *ex vivo* after co-immunolabeling of infected cells and axonal projections. However, we have not been able so far to determine the precise composition of the cell subtypes infected in the CNS. Infection of our glia-specific transgenic line (*Gfap:eGFP*) (Bernardos and Raymond, 2006) was not possible as it is a GFP-expressing transgenic line, and at the time our SINV strain was only available as a GFP recombinant. Moreover, we have tested several antibodies directed against glial cells (anti-GFAP, anti-Glutamine Synthetase) but have failed to observe a specific staining in the CNS. This could be due to the fact that this cell type is not mature at the time of the infection and therefore does not express a yet usable marker (the same antibodies give a reproducible staining in adult zebrafish). We have encountered the same difficulties when trying to assess the percentage of post-mitotic over undifferentiated neurons, as our antibody directed against neurogenin (a marker of undifferentiated neurons) also resulted in non-specific signal. This represents a drawback for the use of zebrafish with respect to other model organisms, as fewer biochemistry tools have been set up to date. We hope, nevertheless, that the use of the Clarity protocol together with the neuroanatomy expertise provided by the group of Jean-Stéphane Joly in Gif-sur-Yvette will help us better characterize the specific neuronal subtypes targeted by the virus. In addition, we now have the isogenic non-GFP pTE3'2J strain, which can be used in GFP transgenic larvae; the infection cannot be

followed live but, in fixed larvae, infected cells can be detected with our anti-capsid antibody.

At the same time, we have focused our attention on the IFN-mediated immune response both at the whole-organism as well as at the CNS-specific level and have identified a prominent role of IFN at early stages post-infection. However, we have shown that IFN alone is not sufficient to prevent the virus from reaching the CNS, as demonstrated by the knock-down of IFN receptors (IFN-R). The characterization of different SINV variants has also revealed how these can modulate the host response and determine very different outcomes for the infected host (section 1 of this Chapter includes a more detailed discussion). At later times post-infection and when the virus has reached the CNS, we have observed an infiltration of neutrophils probably involved in the clearance of the infection (see section 2 of the present Chapter).

Despite some initial similarities with respect to the kinetic of replication and of the immune response, we observed many differences when comparing the infection course of SINV and Chikungunya virus (CHIKV) in zebrafish larvae. Unlike CHIKV, SINV exhibited a stronger neurotropism and induced recruitment of IFN-producing cells in the CNS upon infection (section 3 of the Discussion). However, the infection course described for SINV with respect to its entry mechanism in the CNS, its neurotropism, and the variability observed in different animals (section 4 of the Discussion) is in accordance with several important studies conducted in mice.

1. Identification of Viral Factors of Neuroinvasion

The results obtained from the study of different SINV variants in Chapter 2 section 2.1 of the Results have shown that the two strains differ from each other in terms of:

- Viral burden, with SINV2-mCherry replicating at higher levels
- Type I Interferon (IFN) response, with SINV1-GFP inducing IFN at higher levels
- Observed mortality rates, with SINV2-mCherry being more lethal

- Cell tropism, with SINV1-GFP infecting fewer cell types
- Neuroinvasiveness, with SINV2-mCherry reaching the CNS in all larvae, while SINV1-GFP only in about half, when inoculated iv at the same concentration

A clear difference between the two variants is their different fluorescent reporter proteins, which have been inserted at different locations in the viral genomes. A previous report by Furuta *et al.* has shown that replication defective SINV exhibits different properties (in terms of labeling capacity and movement within infected neurons) when expressing a palGFP or a palDsRed fluorescent reporter (Furuta *et al.*, 2001). Therefore, in order to make certain that the observed differences are not linked to the insertion of the fluorescent protein, we will compare the infection of SINV1-GFP with that of SINV2-GFP. These experiments will be conducted soon, as SINV2-GFP has already been produced but requires a further concentration step. However, the two insertions encode proteins of comparable size with no plausible relevance to virulence. It seems quite unlikely that they would result in such significant differences; hence, we will assume they do not for the rest of the discussion.

The differences observed between these two strains in terms of viral burden in zebrafish correlate with those observed in mosquitoes. Examination of SINV TR339 propagation (from which SINV2-mCherry was derived) in *Aedes aegypti* mosquitoes showed a greater than 90% midgut infection rate at 7 dpi (Myles *et al.*, 2004). In contrast, SINV TE3'2J (SINV1-GFP progenitor) was shown to infect less than 15% of mosquitoes when analysed similarly (Pierro *et al.*, 2003). This may be purely stochastic, since the virus can be expected to rely on distinct sets of host cellular components for its replication in different species. However, it is interesting to note that a common feature of the zebrafish and mosquito infections is their lower body temperature as compared to mammals. SINV shuttles between insects and birds and must be able to replicate from about 20 to 42°C (68 to 107°F). Each strain probably has an optimal temperature and this suggest it may be closer to 28°C (82°F) for SINV2-mCherry than for SINV1-GFP.

Moreover, the higher viral burden measured in SINV2-mCherry injected larvae could be related to its broadened cell tropism. In turn, this can be associated with

the lower IFN induction observed. SINV2-mCherry may induce host transcription and translation shutoff in infected cells, thereby blocking IFN and/or ISG expression. The disease signs and tropism observed in SINV2-mCherry infected larvae were indeed reminiscent of those observed in IFN-receptor (IFN-R) morphants injected with SINV1-GFP. Thus far we have not tested *in vivo* whether IFN producing cells are indeed infected with SINV2-mCherry and if this, in turn, results in decreased levels of IFN expression. However, the ability to modulate IFN expression has been reported also for several other SINV strains and recombinant mutants. Cruz *et al.* showed that mutations at the P3 cleavage site of SINV AR86 (another SINV isolates) determine a higher induction of IFN with respect to the wild type isolates (Cruz *et al.*, 2010), although this modulation was independent from virus mediated host shutoff. Frolov *et al.* showed the importance of IFN induction early after infection (2-4 hpi), to block virus spreading and indicated mutations in nsP2 as the factors involved in host defense induction (Frolov *et al.*, 2012). Indeed, one mutation resulting in an amino acid change in the NSP2 protein (Pro 978 Leu) is present in SINV2-mCherry as compared to SINV1-GFP.

Interestingly, the higher neuroinvasiveness observed in SINV2-mCherry-infected larvae did not correlate with that measured in SINV1-GFP injected IFN-R morphants. This difference therefore must be linked to specific SINV2-mCherry determinants that are independent from its ability to modulate IFN. These determinants may be absent in SINV1-GFP, thereby explaining its unchanged neuroinvasiveness in IFN-R morphants. SINV neurovirulence has been directly linked to specific amino acid changes in the E1 and E2 genes and mutations present in either of the two can determine differences in the observed neurovirulence (Lustig *et al.*, 1988; Tucker and Griffin, 1991). As a consequence, strains with specific E1 and E2 mutations have been shown to induce significant levels of apoptosis in brains and spinal cord of weanling but also of older mice (Lewis *et al.*, 1996). Comparison of the genomic cDNA sequences of SINV1-GFP and SINV2-mCherry identified two amino acid changes in E2 (Arg 338 Cys; His 383 Gln). In the future we will try to isolate the specific residues that determine SINV2-mCherry neuroinvasiveness by building various SINV1/SINV2 chimeras,

and using *in vivo* imaging of infected zebrafish larvae as readout for the neuroinvasive phenotype.

2. Leukocyte Behavior in Response to SINV Infection in the CNS

The data acquired on the cellular immune response against SINV infection suggest that neutrophils represent the main IFN producing cells, in accordance with the results obtained by Palha *et al.* in CHIKV infected larvae (Palha *et al.*, 2013).

However, they were not excluded from the brain but rather accessing it rapidly, sometimes also in cases in which infection in the CNS was relatively contained. Moreover, they were observed directly interacting with infected cells in the brain. These were unexpected findings since neutrophils, which are strictly excluded from the brain of healthy vertebrates, had not been observed entering the brain of CHIKV-infected zebrafish larvae, despite persistent CNS infection (Palha *et al.*, 2013). Nevertheless, this “neutrophil invasion” phenotype may be quite relevant for the study of viral encephalitis, since neutrophils have been shown to invade the CNS during encephalitis caused by other viruses, such as Herpesviruses (Silverman *et al.*, 1992) or West Nile virus (Davis *et al.*, 2006).

Future experiments will be directed towards the understanding of the exact role played by neutrophils during CNS invasion. In particular, we will deplete them specifically using the metronidazole-mediated depletion system (Davison *et al.*, 2007), to determine their importance in the control/eradication of SINV infected neuronal cell types. It may also be worth testing for possible deleterious side effects of the presence of neutrophils in the CNS, for instance using behavioral testing, some of which exist for zebrafish larvae (Lange *et al.*, 2013), although this would require a setup compatible with the use of infected larvae.

Another aspect that needs to be further addressed is the role of microglia in the control of SINV infection in the CNS. Our preliminary data suggest that microglia are not capable of restricting the infection in the CNS. Very few cells were observed close to infection sites and, according to their rounded shape, they were possibly undergoing apoptosis. These experiments were performed *ex vivo* on

fixed larvae; therefore, a more detailed analysis *in vivo* is necessary. Nevertheless, these observations are in accordance with the results obtained after macrophage depletion in the *mpeg:G/U:Nfsb-mCherry* larvae. We showed in fact that their absence did not result in increased mortality rates, thereby suggesting that this cell population plays a minor role, if any, in controlling SINV infection.

Finally, we will perform comparative *in vivo* analyses on the expression patterns of *ifn ϕ 1* and *ifn ϕ 3*, thanks to the newly generated *ifn ϕ 3:GAL4FF* line. The transgene was generated in the laboratory of George Lutfalla in Montpellier and the line was produced by the AMAGEN transgenesis platform at Gif-sur-Yvette. The line will be crossed to *UAS:GFP* or *UAS:RFP* fish, and the resulting double-transgenic larvae will serve the purpose of characterizing IFN ϕ 3 producing cells in the context of both CHIKV and SINV infection. At the transcriptional level in fact we measured high expression levels of *ifn ϕ 3*, which peaked at 24 hpi (like *ifn ϕ 1*) and then quickly decreased (unlike *ifn ϕ 1*). This new transgenic line will be useful to analyse cell population or tissue specific expression of IFN ϕ 1 and IFN ϕ 3. Our preliminary results indicate that IFN ϕ 1 and IFN ϕ 3 are expressed by non-overlapping cell populations. In the case of IFN ϕ 1, we have been able to observe its expression not only in leukocytes and infected hepatocytes, but also in infected neurons. However, a more detailed quantification is required to determine the relative contributions of these expression patterns. At the same time, we will follow IFN-responding cells with the help of the *MxA:mCherry* line, also developed by George Lutfalla.

3. SINV and CHIKV Infection in Zebrafish

The characterization of the infection course of CHIKV in zebrafish larvae provided two important findings: 1) Several cell types are targeted by the virus, including cells of the brain parenchyma, but only brain cells remain infected for several days. 2) A potent IFN-mediated response is induced. IFN is mostly expressed by neutrophils, though these are excluded from the CNS, and knockdown of IFN receptors results in higher viral loads and dramatic increase in lethality following CHIKV infection (Palha *et al.*, 2013). However, due to its

classification as a BSL3 pathogen, CHIKV could only be studied *ex vivo* on fixed samples or *in vivo* but under very restrictive conditions (BSL2⁺ facility, low-resolution microscopy). Therefore, in order to better characterize the viral infection course in the CNS and the relative immune response but with less security restrictions, we decided to employ the closely related SINV as a model virus with neurotropic activity. The initial characterization of its mortality rate, using the SINV1-GFP strain, and kinetic of infection gave comparable results with respect to those observed in CHIKV-infected larvae. Both viruses replicate efficiently, with replication peaking at 24 hpi, and show relatively low mortality rates (10% for CHIKV and 20% for SINV). The kinetic of the immune response is also comparable between CHIKV and SINV, and follows the kinetic of virus replication. However, while CHIKV targets predominantly muscle cells, fibroblasts, hepatocytes and endothelial cells, SINV rarely infects fibroblasts, it has never been observed in co-localization with cells of the vascular endothelium, and is predominantly neurotropic. Moreover, CHIKV infection of the CNS occurs in almost all infected larvae, but is self-limited to a few cells, while SINV reaches the CNS in ~50% of infected larvae, where it expands. These differences suggest that the two viruses may employ different mechanisms to enter the CNS. While CHIKV could possibly infect endothelial cells of the brain microvasculature, SINV is instead transported via axonal projections of peripheral neurons. However, a detailed characterization of CHIKV mode of entry is required in order to confirm this hypothesis. At the moment, CHIKV mechanism of neuroinfection remains largely ill-characterized, although it has been suggested to occur upon dissemination from the choroid plexus and the meninges in infected mice (Couderc *et al.*, 2008). It would be interesting to test this hypothesis in zebrafish larvae, although it will have to be performed *ex vivo*.

Another important difference between the two viruses lies in their effect on infected neuronal cells. While CHIKV-infected neurons have been shown to survive longer than other infected cell types (suggesting neurons could constitute a previously unknown CHIKV reservoir), SINV induces cell death in infected neurons (likely apoptosis), a phenomenon that was especially obvious in larvae with an extensive CNS infection. This in turn could be related with the observed infiltration of neutrophils in the brain upon SINV infection.

An important point that needs to be further characterized is the effect of IFN-R knock-down in SINV-infected larvae. Over 90% of CHIKV-injected IFN-R morphants succumb from the infection, demonstrating the protective role of IFN. In the case of SINV-injected morphants, on the other hand, only 50% of them die after infection and the number of larvae presenting CNS infection does not increase with respect to control morphants. This is in contrast with the observation that infected neurons produce IFN and require IFN-producing cells to contain the infection in the CNS, which suggests IFN plays a more prominent role than the one observed in IFN-R morphants. However, since morpholino mediated knock-down is transient, it is very possible that its effect can only be felt on peripheral infection, which occurs first, and has worn off by the time IFN is induced in the CNS. This should be re-evaluated when true IFN or IFN-R mutants, obtained using CRISPR or other technique, will have become available. Taken together, these observations suggest that SINV may be more closely related to other markedly neurotropic *Alphaviruses* than to CHIKV, and therefore a more detailed comparative analysis between the two is needed to identify conserved mechanisms of infection.

4. SINV Infection in Mice and Zebrafish

Despite its low pathogenicity in humans, SINV has been studied over several years in the mouse model to characterize virus-induced encephalitis. In particular, it was shown that SINV-induced lethality is dependent on its neurotropism as well as on the age of the infected host (Tucker *et al.*, 1993). Moreover, it was shown that the NSV strain induces high mortality rates in both weanling and adult mice, showing how different strains can result in very different infectivity and lethality. This is also well exemplified by the comparison of our two SINV strains (SINV-1GFP and SINV-2mCherry).

Importantly, even though it was reported that SINV persists in a non-replicative form in the CNS of mice that recovered from acute encephalitis (Levine and Griffin, 1992), it was also shown that SINV can induce apoptosis, and this correlates with its neurovirulence (Lewis *et al.*, 1996). This phenomenon was also

observed in zebrafish larvae, in particular those displaying an extensive infection in the CNS. However, we will need to quantify precisely the level of apoptosis in correlation with the level of CNS infection, also with the help of our newly established zebrafish neuronal cell cultures. SINV-induced death of neurons has been shown to be limited by autophagy in a cell-autonomous manner, in elegant *in vivo* experiments in mice (Orvedahl *et al.*, 2010). This is something that we could also tackle in zebrafish, in collaboration with Emma Colucci-Guyon who developed tools to manipulate autophagy in the context of bacterial infections (Mostowy *et al.*, 2013 and unpublished results).

Although in the majority of studies conducted in mice SINV is inoculated ic, Cook and Griffin have shown how the use of a luciferase-expressing SINV can be used to monitor infection after subcutaneous injection (Cook and Griffin, 2003), and have suggested entry in the CNS via retrograde axonal transport. Importantly, they have also shown how the variability of the infection between different mice is not exclusively dependent on the mouse strain. Different mice of the same strain developed in fact infection in different areas (nose, spinal cord, brain) and at different times post-inoculation. In our study, we have also observed a high variability in terms of number of larvae with CNS infection and patterns of infection in the brain. Therefore, these differences can be attributed to intrinsic characteristics of the virus rather than to the genetic heterogeneity of the zebrafish.

General Perspective: Pride and Prejudice of *In Vivo* Models

“Remember that all models are wrong; the practical question is how wrong do they have to be to not be useful.”

Box, G.E.P., Draper, N.R., (1987). *Empirical Model Building and Response Surfaces*. John Wiley and Sons, New York, NY., p. 74.

According to the British mathematician George E.P. Box (1919-2013), all models are intrinsically wrong, being a simplified representation of an infinitely complex truth. Although perhaps discouraging at first, this concept should instead be the driving force leading researchers into finding the most appropriate model to answer their scientific question, thereby turning something “wrong” into something useful.

Often no non-animal alternative is feasible (*e.g. in vitro* cell cultures, *in silico* modeling); therefore, we must carefully choose the appropriate animal model for the validity of our research. According to Davidson *et al.*, the selection of animal models should be based on several considerations (Davidson *et al.*, 1987):

1. Appropriateness as an analog
2. Genetic uniformity of organisms, where applicable
3. Transferability of information, in particular in the case of disease modeling
4. Generalizability of the results, in particular for pharmacological and toxicological modeling
5. Background knowledge of biological properties
6. Ethical implications, by justifying the use of animal models
7. Ease of and adaptability to experimental manipulation
8. Costs and availability
9. Ecological consequences

With all these criteria in mind, it seems natural that only a limited number of animal models is widely accepted and largely supported by funding agencies. The mouse (*Mus musculus*), the nematode worm (*Caenorhabditis elegans*), the fruitfly (*Drosophila melanogaster*) and the thale cress (*Arabidopsis thaliana*) represent

for many experimental biologists the only acceptable models. Often, submitting a grant proposal using a standard organism does not necessitate explaining the chosen model. In contrast, choosing a less common one requires detailed justifications to convince sceptical and more conservative colleagues.

However, a usually widely accepted organism can turn out to be the wrong one, because of wrong initial assumptions. In the case of drug discovery, we find an increasing number of examples where potential drugs fail to pass Phase II clinical trials due to lack of efficacy or toxicity, despite being proven safe and effective in preclinical models. Inbred mice administered with an experimental treatment for multiple sclerosis, for example, were shown to improve from the induced disease while the same treatment in human patients gave unpredicted and/or adverse responses during Phase II trials (Kappos *et al.*, 2000). It is clear that for certain immunological studies, inbred mice fail at mimicking the appropriate level of complexity and diversity of human genetics.

In other cases, we fail at choosing the appropriate number of animals to detect a significant effect, either because of the costs, of the limited availability of the animals, of errors in experimental setup, or for the pressure of reducing the number of animals used. Although, one of the tasks of the Ethical Committees is to make sure that an appropriate number of animals is used (not too high, but not too low either as to become useless for statistical analyses).

After these considerations, where does the zebrafish fit in the mayhem of accepted and acceptable model organisms? Its optical clarity, ease of genetic manipulations, and small size at the larval stage have always been regarded as major advantages over other vertebrate models. However, when used for drug screenings, we must keep in mind the differences in body size, stage of development and, potentially, in metabolic patterns between fish and humans to find the correct dose response curve.

With these premises in mind, the high number of eggs per clutch, the ease of screening for specific phenotypes, and the small amount of compound needed, make the zebrafish a valuable model to test activity and toxicity of a large number of potential therapeutics, in a short time frame and with reduced costs. Our experiments to validate the antiviral effects of DFMO in zebrafish larvae are a clear example of a fast and relatively cheap *in vivo* drug test that was much more

difficult and limited in mouse due to the elevated costs. Moreover, behavioral studies in the context of compound toxicity investigations are also becoming common practice in zebrafish and could be included in disease modeling studies, to obtain more exhaustive data with respect to the observed clinical signs. Another major advantage consists in the possibility to use algorithms that extract meaningful data from images, which greatly help reducing the time required to screen the efficacy of a compound in hundreds of fish. All these points make the zebrafish a good model for translational research.

In this work, we have shown how the combined use of fluorescent recombinant viral strains and zebrafish transgenic lines allow for the investigation of a virus infection course from early to late times post-infection. At the same time, we have been able to follow the elicited immune response and to correlate it with the levels of infection observed in single larvae. We have shown that the zebrafish and the mouse model can provide comparable information, suggesting we should apply inter-species approaches to validate our respective models of infection. We have shown how zebrafish larvae can be employed in drug screening tests, providing important results at reduced costs and with less time required for the analysis. I hope that with this and future works, we will continue to show how the zebrafish can represent a useful model to understand virus-induced pathogenesis and to find safe and valuable therapeutics.

**MATERIALS
AND
METHODS**

MATERIALS AND METHODS

This section provides a detailed description of the protocol used to produce SINV *pTR339-mCherry/2A* and SINV *pTR339-GFP/2A*, as well as the protocol set up to obtain zebrafish neuronal cells in culture. All other methods have been previously described or included in the “Materials and Methods” section of the submitted article, and therefore will not be described further.

1. Sindbis Virus Production and Titration on BHK cells

The following protocol was kindly provided by Benjamin Obadia and Carla Saleh and has been adapted from Hardwick and Levine (2000). It consists in the linearization, *in vitro* transcription and transfection of SINV plasmids, in order to produce new concentrated virus stocks.

The following reagents are needed:

SINV plasmid (linearizable with XhoI)	
mMessage mMachine SP6 Kit	(Ambion) (AM1340)
GlycoBlue™ coprecipitant	(Ambion) (AM9515)
RNase OUT™ (40 U, μL-1)	(Life Technologies) (10777-019)
Ethanol Absolute (EtOH)	(VWR) (20820.362)
Lipofectamine® 2000	(Life Technologies) (11668-027)
Opti-MEM® (reduced serum medium)	(Life Technologies) (51985-042)
DMEM (high glucose)	(Life Technologies) (61965-026)
Ultra Pure™ Agarose	(Life Technologies) (16500-500)
BHK-21 cell line in DMEM/5% FBS	(ATCC) (CCL-10)
DMEM +GlutaMAX (+Glucose 4.5 g/L, -Pyruvate)	(Life Technologies) (61965-026)
Fetal Bovine Serum (FBS)	(GIBCO) (10270-106)
L-Glutamine (L-Glu, 200 mM)	(Life Technologies) (25030-024)

Penicillin (Pen, 100 U/mL)	(Biovalley) (P0142.0100)
Streptomycin (Strep, 100 µg/mL)	(Biovalley) (S09470)
DMEM (powder) with Glucose (4.5 g/L)	(Sigma-Aldrich) (D7777-1L)
Methyl Cellulose (MC)	(Sigma-Aldrich) (M0512-100G)
Sodium Bicarbonate (MW: 84.01 g/mol)	(Sigma-Aldrich) (S5761-500G)
9.6% Sodium Hypochlorite	(commercial)
Crystal Violet Solution (CV) (2.3% w/v)	(Sigma-Aldrich) (HT90132-1L)
D-PBS/Modified	(HyClone) (SH30028.02)
37% Formaldehyde (MW: 30.03 g/mol)	(Sigma-Aldrich) (15512)
Filter Millex Express Plus (0.22 µm)	(Millipore) (SLGP033NS)
Cell culture flask (162 cm ²)	(Corning) (3151)
6-well plate (9,62 cm ²)	(Corning) (3516)

The protocol is as follows:

Plasmid Preparation

1. Linearize 2 µg of plasmid with XhoI (Fermentas)
2. Purify using phenol-chloroform extraction and ethanol precipitation, and resuspend in 20 µL of ddH₂O
3. Run 1 µL on a 0.7% agarose gel in 1× TAE

In vitro Transcription

1. Mix 1 µg of linearized plasmid (0.5 µg/µL in water) with:
 - a. 10 µL of 2× NTP/Cap,
 - b. 3 µL 20 mM GTP,
 - c. 2 µL of 10× Rxn Buffer,
 - d. 2 µL SP6 Enzyme Mix,
 - e. up to 20 µL with ddH₂O
2. Incubate for 1-2 hrs at 37°C
3. Add 1 µL TURBO DNase and incubate for 15 mn at 37°C

Lithium Chloride Precipitation

1. Add 30 µL ddH₂O, 25 µL LiCl, 1 µL GlycoBlue™, mix gently, and keep at least 1 hr at 20°C

2. Centrifuge at 4000 RCF for 15 mn (at least) at 4°C
3. Carefully remove the supernatant and wash the pellet with 200 µL ice-cold 70% EtOH
4. Centrifuge at 4000 RCF for 5 mn at 4°C
5. Carefully remove ethanol and dissolve the RNA pellet in 11 µL ddH₂O
6. Optionally add 1 µL RNaseOUT™. Store immediately at –80°C or proceed with transfection
7. Run 1 µL on a RNase-free 0.7% agarose gel in 1× TAE

BHK Cells Transfection

1. *One day prior to transfection*, subculture BHK cells in DMEM/5% FBS in a 162-cm² flask. Cells must be at ~80% confluence
 2. In an eppendorf, mix 500 µL pure opti-MEM* with 60 µL lipofectamine, and incubate for 10 mn (sol.A)
 3. In an eppendorf, dilute ~30 µg viral RNA in 500 µL pure opti-MEM, mix with sol.A, and incubate for 20 mn at room temperature (sol. B)
 4. Remove the complete DMEM from cells and eventually rinse once with D-PBS
 5. Mix sol. B in 10 mL pure opti-MEM*, and add directly to the cells
- * *DMEM can be used too*
6. Incubate the cells with the transfection complexes for 2 hrs at 37°C, 5% CO₂
 7. Remove the transfection complexes from the flask and add fresh complete DMEM/5% FBS to the cells.
 8. Incubate for 18 to 24 hrs, or until complete cytopathic effect (CPE) has occurred
 9. Proceed to virus harvest

Virus Harvest

1. When CPE is observed, transfer medium from the flask to a 15-mL conical polypropylene tube
2. Centrifuge at 1000 RCF for 10 mn at 4°C to remove cell debris
3. Transfer virus-containing supernatant into eppendorf tubes, and store at –80°C
4. Titer on BHK cells. In case the titer is too low for the specific need, the virus can be concentrated adding a centrifugation step. In this case, an Amicon®

Ultra-15 with a 10K (MW) cutoff should be used. Centrifugation should be performed at 4000 RCF for 15-20 mn at 4°C

The following solutions are needed for the plaque assay, in order to determine the virus stock titer:

DMEM/5% FBS: 500 mL DMEM, 25 mL FBS, 5 mL L-Glutamine, 5 mL PenStrep

DMEM (2×): DMEM (powder), 3.7 g Sodium Bicarbonate, up to 460 mL ddH₂O, filter (.22µm) and add 20 mL FBS, 10 mL of 100× L-Glutamine, 10 mL of 100× PenStrep. Store for less than 2 months at 4°C

1.6% MC: 1.6 g of MC, up to 100 mL with H₂O. Autoclave in 100 mL aliquots, store at 4°C. A 100 mL bottle is suitable for fifteen 6-well plates

DMEM/0.8% MC: 6 mL of 1.6% MC in 6 mL of 2× DMEM (per 6-well plate)

0.23% CV Solution: 180 mL H₂O, 45 mL 100% Ethanol, 25 mL 2.3% CV Solution stock

4% formaldehyde: 650 µL 37% formaldehyde, up to 6 mL with D-PBS (per 6-well plate)

2.6% bleach: 270 mL 9.6% sodium hypochlorite, up to 1L with H₂O

Cells and Virus Dilutions

1. *On the day prior to the plaque assay*, split BHK-21 cells into 6-well plate at a concentration of $0.7-1 \times 10^6$ cells per well in 2 mL of 1× DMEM such that cells will be 70-90% confluent on the next day. A 162 cm² flask gives $\sim 32 \times 10^6$ BHK cells. Incubate overnight at 37°C, 5% CO₂
2. Thaw the virus to be titered on ice (~30 mn) and prepare serial virus dilutions in 1.5 mL eppendorf tubes
3. Recover the plate, observe cells, pour medium out of wells, and rinse once with 2 mL D-PBS
4. Infect each well with 250 µL of chosen virus dilution (or DMEM w/o FBS as a negative control)
5. Incubate the plaque for 1 hr at 37°C, agitating every 10 mn

Plaque Assay

1. While incubating, prepare DMEM/1% MC mixture (3 mL per well). Mix by adding MC to DMEM as MC is very viscous. Plan more, as a part of the mixture is lost in the pipette
2. Remove the inoculum from each well, add 3 mL of DMEM/1% MC
3. Incubate 2-3 days at 37°C, 5% CO₂

Revealing

1. Add 1 mL 4% formaldehyde to cover wells, and incubate at least 30 mn
2. Remove gently the DMEM/MC/formaldehyde solution, and gently rinse with H₂O
3. Cover each well with 0.23% CV solution for 5 mn to stain viable cells
4. Empty out the 0.23% CV solution into a bleach-containing sink, and gently rinse with H₂O
5. Let dry, count plaques, and calculate titer in plaque forming units per milliliter (PFU/mL)

2. Zebrafish Neuronal Cell Cultures

The following protocol was developed at INRA in the group 'Virologie et Immunologie Moléculaire', under the invaluable guidance of Pierre Boudinot and Christelle Langevin. It has been adapted from Chen et al. (2013).

It consists in the dissociation of dissected zebrafish embryos, to obtain a suspension enriched in neurons and glial cells that is then plated in complete medium on a suitable substrate. Preliminary experiments have shown that cells derived this way can be kept viable for 10-12 days and can be used for various purposes (viral infection studies, gene expression studies, drug screening tests). Several zebrafish lines have been used, including: wild-type (WT) AB, *HuC:eGFP (Tg(elavl3:EGFP)^{knu3})* with green neurons, *HuC:GAL4* crossed to *UAS:NfsB-mCherry (Tg(elavl3.1:GAL4-VP16)^{hd5})* crossed to *Tg(UAS-E1b:Eco.NfsB-mCherry)^{c264})* with red neurons, *GFAP:eGFP (Tg(gfap:EGFP)^{mi2001})* with green glial cells.

The protocol is as follows:

1. *Four days prior to the dissociation*, set up fish for spawning
2. The following day, collect the eggs and wash them with regular/Volvic water
3. Sort the fertilized eggs and discard the non-fertilized ones
4. Wash again with regular/Volvic water and discard excess water. Add 30 μ L of 5.25% bleach in 50 mL of regular/Volvic water; gently rotate the falcon; do not leave the embryos in bleach for more than 5 minutes
5. Wash 2x with regular/Volvic water
6. Transfer the embryos in a petri dish with regular/Volvic water with methylene blue (4 mg/mL – 70 μ L/L of water) and incubate them at 28.5°C until the day of the dissociation

On the day prior to the dissociation:

The following reagents and solutions are needed:

Ethanol Absolute (EtOH)	(VWR) (20820.362)
Tricaine (25x)	(SIGMA) (E10521)
D-PBS/Modified	(HyClone) (SH30028.02)
Poly-D-lysine (PDL, 1 mg/mL)	(SIGMA) (P7280-5MG)
α -D-glucose (MW: 180.16 g/mol)	(Prolabo) (24 370.294)
Difco™ trypsin 250 (100 g)	(BD) (215240)
DNase (10000 U)	(Roche) (10 776 785 001)
MEM (500 mL)	(Eurobio) (CXXBHK00-01)
Fetal bovine serum (FBS)	(GIBCO) (10270-106)
L-glutamine (L-Glu, 200 mM)	(PAA) (M11-004)
Tryptose (100 mL)	(SIGMA) (T8159)
Penicillin (Pen, 100 U/mL)	(Biovalley) (P0142.0100)
Streptomycin (Strep, 100 μ g/mL)	(Biovalley) (S09470)
Pasteur pipette (5 mL, sterile)	(VWR) (612-1686)
Cell strainer filters (0.40 μ m)	(BD Falcon) (352340)
Cell strainer filters (0.20 μ m)	(Minisart) (16534)
Ibidi μ -Dish ^{35 mm, high}	(Biovalley) (81156)

Ibidi μ -Slide^{8 well}

(Biovalley) (80826)

Ibidi Hi-Q⁴

(Biovalley) (MZI00040)

25 μ g/mL PDL: 25 μ L PDL 1 mg/mL, 975 μ L D-PBS; PDL is fundamental to increase the adherence of cells to the support

4% D-glucose: 2 g in 50 mL of D-PBS; dissolve carefully and filter the solution with a 0.20 μ m cell strainer filter; store at 4°C

EPC/10% FBS: 450 mL MEM, 50 mL FBS, 5 mL L-Glu, 5 mL Tryptose, 500 μ L PenStrep; if the culture is needed for viral infection studies, the virus-containing medium must be EPC/2 % FBS

2. Add a solution of sterile PDL/PBS (25 μ g/mL) in each well of the available incubation plate, such as for example, a glass-covered Ibidi dish and incubate ~1 hr at 37°C

The volume is dependent on the Ibidi dish being used (1 mL/dish for the Ibidi μ -Dish^{35 mm, high} and the Ibidi Hi-Q⁴, 150 μ L/well for the Ibidi μ -Slide^{8 well})

3. Remove PDL and wash 3x with PBS

4. Remove PBS and store the dishes at room temperature

On the day of the dissociation:

The following passages must be performed under a laminar flow hood, to avoid contaminations of the cell culture

Head Dissociation

1. Place ~100-150 72 hpf zebrafish embryos, still in their chorions, in EtOH 70% (5-10 sec)

2. Quickly remove the embryos, using a sterile pipette and wash them 2x with sterile water with methylene blue

3. Dechorionate the embryos and anesthetize them with tricaine (1x)

4. Remove the head from the rest of the body, using fine forceps and a small cutter

Tissue Dissociation and Cell Cultures

1. Transfer the heads in a 1.5 mL eppendorf tube, let them sediment and remove the supernatant
 2. Add 500 μ L of trypsin, mix gently and incubate for 15 min at 37°C
 3. Add 100 μ L of FBS to block the trypsin, mix gently and remove the supernatant, after sedimentation
 4. Add 1 mL of EPC medium to the eppendorf tube and transfer the content in a 15 mL falcon tube already containing 4 mL of EPC medium mixed with DNase (100 μ L). *DNase helps tissue dissociation but it is not essential*
 5. Grind the tissues with a 5 mL pipette (do not grind more than 10 times); add a 1 mL tip on the P5 pipette and repeat the grinding. *At this step, the suspension should appear homogenous*
 6. Filter the suspension through a 0.40 μ m cell strainer
 7. Spin down the cells at 90 RCF for 5 min without brakes (at room temperature)
 8. Remove the supernatant and wash the cells once in EPC medium (~4 mL)
 9. Spin down the cells at 90 RCF for 5 min without brakes
 10. Add 2 mL of EPC medium, resuspend the cells and measure the cell density, using a Bürker chamber. *The cell density should be comprised between 5×10^5 and 1×10^6 cells/mL; if a concentration step is needed, proceed again with passage 9. and resuspend the cells in the appropriate volume of EPC medium*
 11. Plate the cells on coated Ibidi dishes, adding half of the final volume of medium+cells/well (depending on the Ibidi dish being used) and incubate them at 24°C
 12. After 1 h (when cells have adhered), add the other half of the medium
- The medium should be changed every 2-3 days, depending on the state of the culture. *It is advisable to change each time only half of the medium, in order to maintain cells viability*

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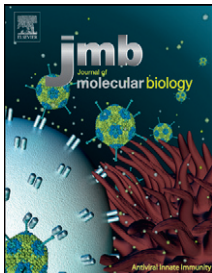
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ANNEX

Annex 1: Antiviral Innate Immune Responses in Fish



The Antiviral Innate Immune Response in Fish: Evolution and Conservation of the IFN System

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Abstract

Innate immunity constitutes the first line of the host defense after pathogen invasion. Viruses trigger the expression of interferons (IFNs). These master antiviral cytokines induce in turn a large number of interferon-stimulated genes, which possess diverse effector and regulatory functions. The IFN system is conserved in all tetrapods as well as in fishes, but not in tunicates or in the lancelet, suggesting that it originated in early vertebrates. Viral diseases are an important concern of fish aquaculture, which is why fish viruses and antiviral responses have been studied mostly in species of commercial value, such as salmonids. More recently, there has been an interest in the use of more tractable model fish species, notably the zebrafish. Progress in genomics now makes it possible to get a relatively complete image of the genes involved in innate antiviral responses in fish. In this review, by comparing the IFN system between teleosts and mammals, we will focus on its evolution in vertebrates.

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Introduction

Teleosts, the largest and best-known clade of ray-finned fish, constitute a highly successful and diverse group, including half of vertebrate species. Their line and ours diverged about 450 million years ago. Several species within this group, both commercial species and model organisms, have been studied to some depth by immunologists, and many details of their antiviral defenses are now known. Although fish genomes have a complex history of whole genome duplications (WGDs) and contractions, the remarkable conservation of the interferon (IFN) system underlines the critical importance of innate antiviral immunity in vertebrates.

Part 1. Architecture of Innate Immune Response in Fish: IFN ϕ , Receptors, General Structure of Pathways

Fish IFNs

Extensive studies performed in mammals in various contexts of viral infection demonstrated the importance of IFNs in antiviral responses. The name of this group of cytokines originates in their ability to “interfere” with the viral progression, as first described in 1957 by Isaacs and Lindenmann [1]. IFNs belong to class II helical cytokine family and, in mammals, can be divided into three different groups

based on biological and structural features as well as receptor usage [2]: mammalian IFNs have been classified as type I (α , β , ω , ϵ , and κ), type II (γ), and type III (λ) IFNs. Actually, only type I and type III IFNs (often grouped under the label “virus-induced IFNs”) are truly specialized as innate antiviral cytokines; IFN γ is rather a regulatory cytokine of innate and adaptive immunity, mostly active against intracellular bacteria.

IFN-like antiviral activity has been reported in fish 40 years ago [3,4]. However, teleost IFN genes could not be identified before the development of fish genomics [5–8]. These virus-induced fish IFNs were clearly responsible for a strong inducible activity against a range of viruses [5–7]. Although some fish species (e.g., fugu or medaka) appear to possess one single virus-induced IFN gene, the number of identified genes grew rapidly in other fish species. There are four virus-induced IFN genes in zebrafish (aka IFN ϕ) [9,10], a number unlikely to change much considering the quality reached by the zebrafish genome assembly. Salmonids, however, have many more IFN genes; the current record is 11 genes in Atlantic salmon [11]. Two main subsets could be distinguished among fish virus-induced IFNs, corresponding to the number of cysteine (C) residues predicted to be engaged in

disulfide bridges: two for IFNs of group I and four for IFNs of group II [9,11], as was later confirmed by three-dimensional crystallography [12]. The 4C configuration is found in all tetrapod type I IFNs, with the exception of mammalian IFN β , which has only one disulfide bridge. However, the cysteine pair of IFN β is different from the one of fish group I IFNs, and one should emphasize that the two groups of fish IFNs do not correspond to the alpha/beta subdivision of mammalian type I IFNs, which occurred after the divergence of avian and mammalian lineages.

Two different isoforms of some fish IFN transcripts, resulting from the usage of alternative promoters, show different levels of induction: upon viral infection, a short transcript encoding a protein with a signal peptide is induced in addition to a constitutively expressed isoform, which lacks signal peptide [13]. This particularity has been observed in a number of fish species, but not for all their IFN genes [14–16]. No function of the presumably non-secreted IFN isoform, unique to teleosts as far as we know, has been reported.

Importantly, the two groups of IFNs were found to signal via two different receptors in zebrafish (Fig. 1) [10]. IFN ϕ s of the first group (IFN ϕ 1 and ϕ 4) bind to the cytokine receptor family B (CRFB)1–CRFB5

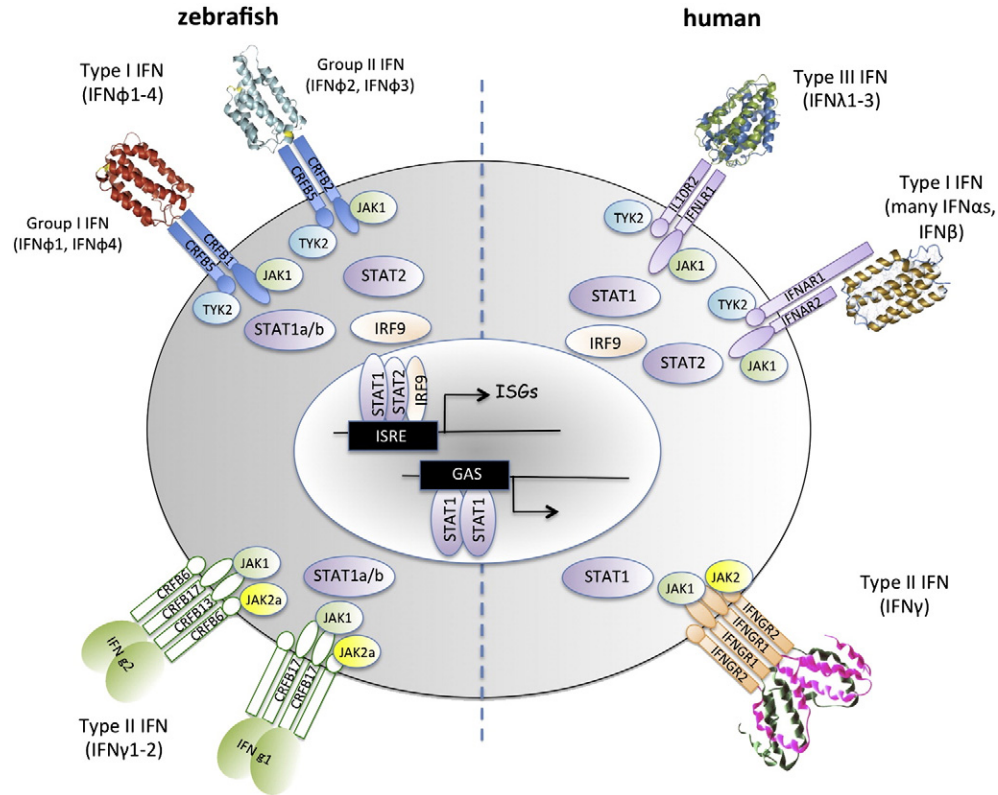


Fig. 1. Schematic representation of zebrafish IFNs and their receptors. Tridimensional representations of IFNs are from the Protein Data Bank (accession numbers: 3PIV, zebrafish IFN ϕ 1; 3PIW, zebrafish IFN ϕ 2; 3HHC, human IFN λ 3; 1AU1, human IFN β ; 1HIG, human IFN γ).

complex while the CRFB5 chain is associated to CRFB2 to form the receptor for group II (IFN ϕ 2 and ϕ 3) [13,10]. Interestingly, both zebrafish IFN ϕ 4 and salmon IFN δ —which are possible orthologues—seem to have lost antiviral activity and might be on their way becoming pseudogenes. Alternatively, they may even play a decoy role for other IFNs.

Do the two groups of fish virus-induced fish IFNs play distinct or redundant roles? By injecting recombinant IFNs in adult zebrafish and challenging them with different pathogens, Lopez-Munoz *et al.* found that both types would protect against a virus, but only the group I IFN would also protect against a bacteria [17]; they also observed an induction of distinct gene subsets. However, it is difficult to reach a firm conclusion from this study, because unfiltered culture supernatants were used as sources of recombinant IFNs, and because the slow kinetics of induction of most downstream genes (including the IFN themselves) suggests indirect effects. Most other studies found quantitative but not clearly qualitative differences between the responses induced by the different IFNs (e.g., Ref. [18]), although this remains to be analyzed in depth. Nevertheless, the distinct receptors for the two IFN groups raise the possibility of different target tissues; in addition, important differences in expression patterns of the different fish IFNs have been demonstrated. The spatial differences of IFN and interferon-stimulated gene (ISG) expression will be reviewed in later sections.

Classification of virus-induced fish IFN genes, relative to mammalian IFNs, has been controversial for some time. Molecular phylogenies were uncertain because the low overall similarity (<25%) between mammalian and fish proteins resulted in uncertain software-generated alignments. It was thus not possible to claim with certainty that fish virus-induced IFNs were closer to mammalian type I or type III IFNs (or co-orthologous to both groups as a set of paralogues), although some sequence features, such as the CAWE sequence at the beginning of the C-terminal helix, were noted by some as characteristic of type I IFNs [9,11,19]. By contrast, fish IFN genes are composed of five exons and four introns [11,19], as are mammalian type III IFN genes, while mammalian type I IFN genes contain a single exon; additionally, when receptors for IFNs were identified in zebrafish, their domain organization had features of the receptor of human IFN λ rather than type I IFN receptor, which has a uniquely large extracellular region in one chain (Fig. 1) [13]. However, the first argument was soon dismissed when frogs were found to have both type I and type III IFNs, all with five-exon structures, indicating that single-exon type I IFN genes were the result of a retrotransposition event in the amniote lineage, not an ancestral feature [20]. Finally, crystal structures revealed a characteristic type I IFN architecture for both groups of IFN ϕ s with a straight

F helix, as opposed to the remaining class II cytokines, including IFN- λ , where helix F is bent [12].

Based on these considerations, different names have been proposed for fish IFNs: type I IFNs, virus-induced IFNs, IFN λ , or even simply IFNs. Following Stein *et al.* [21], zebrafish IFNs are now called IFN ϕ (ϕ for fish). While it is now demonstrated that fish virus-induced IFNs are structurally type I IFNs, a consensus about a consistent nomenclature for these cytokines has still to be reached. The current zebrafish nomenclature avoids orthology assumptions but does not clearly distinguish group I and group II IFNs. The current nomenclature for salmonid IFNs, which groups the genes into four subgroups, IFNa, IFNb, IFNc, and IFNd [11,22], has the same issue (group 1 includes IFNas and IFNds; group 2 includes IFNbs and IFNcs) with the caveat that unaware readers could wrongly assume that IFNas are orthologous to mammalian IFN α s, and IFNbs to IFN β . A self-explanatory nomenclature reflecting the phylogenetic relationships between IFN genes remains to be established.

Fish also possess clear orthologues of mammalian type II IFNs (γ), with many fish species having two type II *ifn* genes (*ifn γ 1* and *ifn γ 2*) [15,23–25]. In zebrafish, IFN γ 1 and IFN γ 2 bind to distinct receptors: the IFN γ 2 receptor includes Crfb6 together with CRFB13 and CRFB17, while the IFN γ 1 receptor does not comprise CRFB6 or CRFB13 but includes CRFB17 (Fig. 1) [26]. Genes encoding a trout receptor of IFN γ have also been identified [27]. Infection studies show that IFN γ signaling is involved in resistance against bacterial infections in the zebrafish embryo, with a proper level required for the fish to clear high doses of *Escherichia coli* or low doses of the fish pathogen *Yersinia ruckeri* [24]. However, a potent antiviral activity of IFN γ was also demonstrated in Atlantic salmon against infectious pancreatic necrosis virus (IPNV) and infectious salmon anemia virus (ISAV), which may partly depend on the coexpression of type I IFN [28]. However, fish IFN γ are not always induced by viral infections under conditions where type I IFNs are [26], indicating that in fish as well as in mammals, IFN γ are probably not specialized antiviral cytokines; they will therefore not be discussed further.

Virus sensors in fish and their signaling pathways

In mammals, viral infection is rapidly detected by specialized PRRs (pattern recognition receptors) such as RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs). These cellular sensors of invading pathogens are directly involved in the activation of the IFN system.

Three RLRs, that is, RNA helicases containing canonical DExD/H motifs, have been identified to date in humans: retinoic acid-inducible gene I (RIG-I, also

known as DDX58), melanoma differentiation-associated gene 5 (MDA5, or IFIH1), and laboratory of genetics and physiology 2 (LGP2, or DHX58). *In silico* analyses led to the identification of RLRs described in many teleost fish including zebrafish, Atlantic salmon, grass carp, Japanese flounder, rainbow trout, and fathead minnow [22,29–36]. These sequences are highly conserved between mammalian and fish orthologues [37]. LGP2 and MDA5 seem to be conserved in all fish species, while RIG-I has been retrieved only in some groups including salmonids and cyprinids [38]. Like their mammalian counterparts, expression of RLRs is modulated upon viral infection [29,31,32,36,39,40] and IFN stimulation through poly(I:C) treatment [33] or by ubiquitin-like ISG15 [41], which also modulates RIG-I activity [42]. Interestingly, LGP2 appears to be a positive activator of the IFN pathway in fish. Sequence analysis suggests a fair conservation of signaling pathways downstream of RLR (Fig. 2), with a critical role of for the mitochondrial antiviral signaling protein (MAVS, also known as CARDIF, VISA, or IPS-1) [22,29,34,43,44]. Association of MAVS with TRAF [tumor necrosis factor (TNF) receptor-associated factor] 3 and activation of the pathway by TBK1 (TANK binding kinase 1) via

phosphorylation of IFN regulatory factor (IRF)3/7 transcriptional factors have also been shown in fish [44,45]. Nuclear translocation of these factors induced the transcription of different cytokines including IFN genes. The adaptor STING (aka “mediator of IRF3 activation” or MITA, ERIS, and MYPS), a transmembrane protein located in the endoplasmic reticulum, links signaling between MAVS and downstream cytosolic kinase TBK1 [46,47]. In mammals, STING is also involved in the induction of IFN β by DNA viruses, connecting cytosolic DNA sensing to TBK1 and IRF3 activation [48]. STING has been identified in fish and plays an important role in the RLR/IRF3-dependent signaling [39,49]. The pathways induced by DNA viruses are still poorly known in fish, and the importance of STING in this signaling remains to be established. Interestingly, the DNA sensors AIM2 and IFI6-16 seem to be missing in fish.

A diverse TLR repertoire has been found in fish [50,51]. Some TLRs have been described only in lower vertebrates including TLR14 and TLR23 [50]; TLR18, TLR19, and TLR20 [52]; TLR21 and TLR22 [53]; TLR24 [54]; and TLR25 and TLR26 [55]. TLRs, which are involved in the recognition of double-stranded RNA (dsRNA) (TLR3) or single-stranded

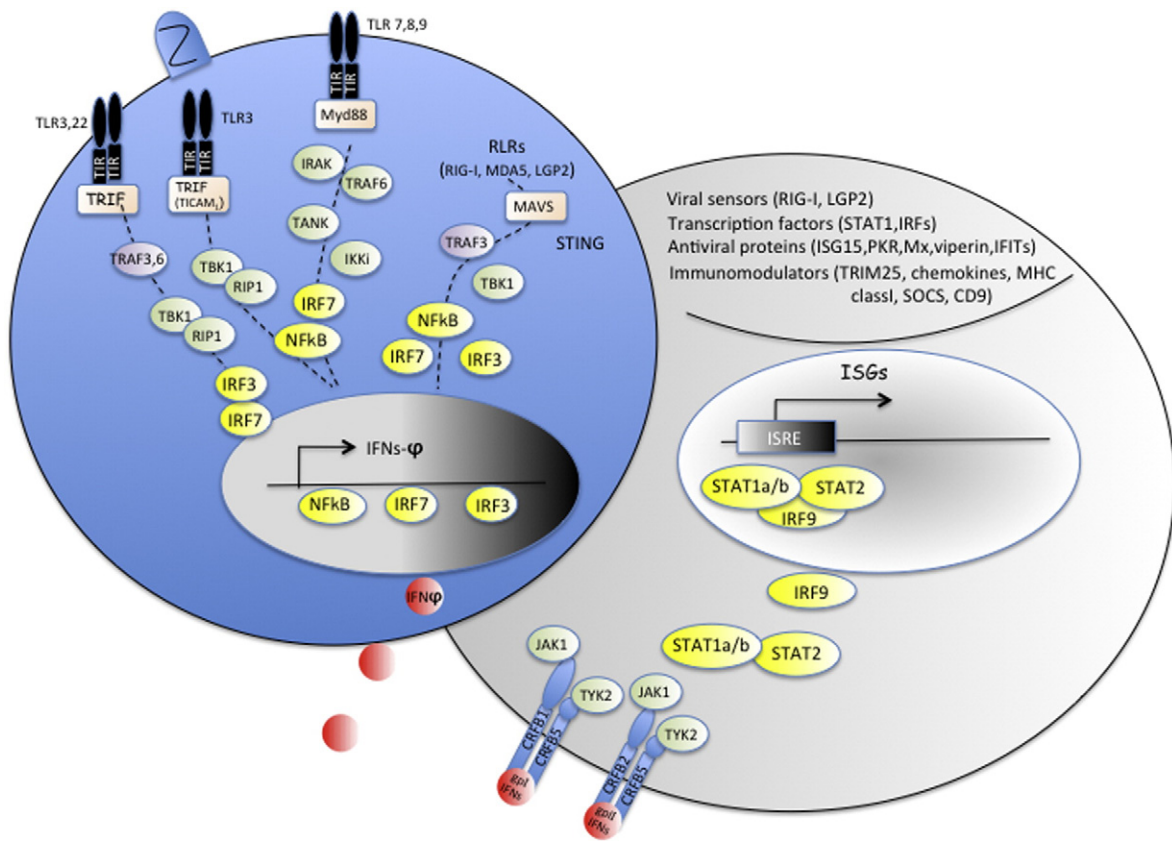


Fig. 2. Schematic representation of IFN signaling pathways in fish. Adaptor molecules are represented in orange, kinases are in green, TRAFs are in purple, transcription factors are in yellow, and IFNs are in red.

RNA (TLR7 and 8) in mammals, have good orthologues in fish [52,53,56]. Both structural and functional evidence indicate that these TLR are also involved in virus sensing in fish: all critical residues for binding to dsRNA are conserved in fish TLR3 [55], and RTG-2 rainbow trout cells transfected with TLR3 showed increased IFN response after poly(I:C) stimulation [57]. Similarly, the leucine-rich repeats of TLR7 are remarkably conserved between mammals and fish [55,58], and a known ligand of TLR7 and TLR8 (R-848) induces a typical IFN response in salmonid leukocytes [18,59]. Additionally, among fish-specific TLRs, TLR22 is responsive to virus infections, poly(I:C), and dsRNA [57,60]. Fugu TLR22 recognizes long-sized dsRNA on the cell surface, while TLR3 binds short-sized dsRNA in the endoplasmic reticulum [57], which may represent a dual pathway for RNA virus sensing in fish.

Upon ligand binding, TLRs dimerize and their intracytoplasmic TIR (Toll-interleukin 1 receptor) domains recruit adaptor molecules through homotypic TIR/TIR interactions. In mammals, most TLRs signal through the Myd88 adaptor, which recruits interleukin-1R-associated kinase (IRAK) (Fig. 2). This protein then associates with TRAF6, subsequently involving TANK (TRAF family member-associated NfκB activator kinase) and IKKi (inhibitor of NfκB kinase) inducing NfκB nuclear translocation and type I IFN gene transcription. In contrast, TLR3 (specific for dsRNA) signaling occurs independently of Myd88 through the recruitment of TRIF (TIR domain containing adaptor inducing IFNβ, also known as TICAM-1 or Myd88-3), leading to TRAF3 signaling cascade, IRF3 phosphorylation preceding nuclear translocation, and recognition of IFN-stimulated responses elements on type I IFN promoters. Viral infection alternatively activates IRF7 via TLR7–9 in a TRAF6-dependent manner [61]. Although TLR families show distinct features among vertebrates, the components of signaling pathways are well conserved as suggested by the presence of kinase and adaptor molecule orthologues in zebrafish and pufferfish [21]. Myd88 and other TIR adaptors were identified in zebrafish [56], and morpholino approaches as well as infectious models demonstrated the functionality of Myd88 in the establishment of TLR-mediated immune response [62]. Further studies confirmed these observations using different stimulations [poly(I:C), flagelin, or chemical treatments] [63,64]. Since then, *myd88* has been identified in many fish species [64–68]. Zebrafish TRIF similarly triggered activation of type I IFN. The TRIF-dependent TLR pathway converges with the RLR pathway by activating the TBK1 kinase, which is conserved in fish as mentioned above. However, the TICAM1 signaling pathway observed in zebrafish is apparently independent of IRF3 and IRF7 and does not require interaction with TRAF6 [69]. Also, a gene coding for the IRAK2 kinase is missing from the genome of pufferfish,

zebrafish, medaka, and stickleback [21], while an IRAK1 orthologue is present and can trigger innate immune response [70].

Thus, IFN-inducing signaling pathways are overall fairly well conserved between fish and mammals. Regarding the sensors, RLRs are also remarkably well conserved, while the fish TLR repertoire include a variety of receptors absent in mammals—some of which, at least, contribute to viral detection—in addition to well-conserved ones such as TLR3 and TLR7.

Conserved signaling pathways downstream of IFN receptors

In mammals, IFN binding to their membrane receptors leads to the activation of the JAK-STAT signaling pathway (Fig. 1). Type I IFN association to its receptor triggers recruitment and binding of the kinases TYK2 and JAK1 to IFNAR1 and IFNAR2, respectively. Subsequently, these kinases promote the phosphorylation of STAT1 and STAT2 proteins preceding their oligomerization. Conjugation of cytoplasmic IRF9 to the STAT1/2 oligomers generates the complex ISGF3 (IFN-stimulated gene factor), which induces the transcription of ISGs after binding nuclear IFN-stimulated responses elements on their promoter. In fish, the *stat1* gene has been described in many species [67,71–73]; the zebrafish genome encodes two different paralogues, *stat1a* and *stat1b* [21]. Functional studies highlighted their role in the regulation of the type I IFN pathway in different species [67,71,73]. However, the respective roles of the different STAT1s in IFN pathway regulation remain unclear in zebrafish. Kinases JAK1 and TyK2 as well as STAT2 and IRF9 are also present in fish genomes [21]. Aggad *et al.* proposed that TYK2 would be associated to CRFB5, while JAK1 would be associated to CRFB1 and 2, thus leading to the activation of the IFN signaling pathway and to *viperin* transcription (Fig. 2) [10].

In contrast, type II IFNs signal after binding to IFNGR1–2 by recruiting JAK1 and JAK2; these kinases promote phosphorylation of STAT1 homodimer, which directly translocates to the nucleus and bind a GAS element (IFN gamma-activated site), thus mediating up-regulation of a broad repertoire of genes, partly overlapping with the type I IFN-mediated response. In zebrafish, IFN-γ1 and IFN-γ2 bind distinct receptors (CRFB6–CRFB13 and CRFB17 for IFN-γ2 and CRFB17, plus unidentified chains, for IFN-γ1) with conserved binding regions of JAK1 and 2 kinases [26]. Two JAK2 kinases are expressed in this species (JAK2a and b), and only JAK2a has been involved in IFNγ signaling using constitutively active mutants (Figs. 1 and 2) [26]. Future studies will be required to determine which of the two STAT1 paralogues constitutes the active protein involved in the signaling pathway of type I and type II IFNs.

Part 2. ISGs and Their Diverse Evolutionary Patterns

Type I IFNs do not possess antiviral activity *per se* but interfere with viral infection through induction of a vast repertoire of ISGs via the JAK/STAT pathway. A few hundred ISGs have been identified in human [74,75], with a rich diversity of molecular functions. Some ISGs exert a direct antiviral activity such as MX, VIPERIN/VIG1, ISG15, PKR, and TRIM5. However, the connection of most ISGs to antiviral mechanisms, and even their role in the biology of the cell, remain unknown.

While ISGs are intrinsically located downstream of IFN in the antiviral pathways induced by viral infections, a number of them are able to up-regulate type I IFNs and are therefore involved in positive feedback regulatory loops (e.g., *trim25*, *rigl*, *stat1*, *irf7*, and *viperin/vig1* [76–79], while some also feedback negatively on IFN signaling (e.g., *socs1* and *2*). Furthermore, the recognition of viral compounds by cellular sensors can up-regulate some ISGs directly, that is, independently of IFN induction; such bypass has been shown for example for Mx [80,81] and for viperin in human and fish [82,83]. Hence, while IFN definitely plays a central role in the innate antiviral response, a complex and redundant network of regulatory loops and bypass mechanisms is also involved, which makes the whole system more resistant to subversion by viruses.

Orthologues of human ISGs involved in IFN amplification have often been retrieved as ISGs in fish, which may indicate that they belong to the primordial IFN pathway: for example, *trim25*, *rigl*, *stat1*, *irf7*, and *viperin/vig1* are conserved in teleost fish and are induced by type I IFN in these organisms [84]. In fish, this list includes also *irf3* [45,85], which is not an ISG in mammals. Although their induction pathways are partly unknown, IFN-independent induction has been observed for some of them. Whether regulatory loops of signaling pathways for type I IFN and ISGs induction are ancestral, or have been shaped independently during fish *versus* tetrapod evolution, remains to be clarified.

The evolution of teleost fish was marked by an early WGD event, followed by a gene loss phase, and as a consequence, the fish genomes sequenced to date do not contain more genes than humans, but paralogous pairs that arose from this WGD are frequent [86]. To further complicate things, additional WGD episodes occurred in some branches among teleosts—for example, in salmonids—while other fish underwent strong genome contraction, such as the tetraodon/fugu family. Of note, zebrafish has a relatively large genome with many highly expanded gene families, compared to other fish model species [87]. Since genes involved in effector mechanisms of immunity tend to diversify to escape subversion by pathogens, one might expect that fish would have

retained many ISG duplicates and would possess larger repertoires of ISGs.

In fact, this hypothesis is still difficult to validate, since the diversity of fish ISGs is not fully defined. A few typical ISGs were first identified using primers or probes targeting conserved sequences such as Mx [88–90] and genes of the MHC class I presentation pathway [91]. Then, PCR-based approaches for differential display of transcripts (differential display PCR, subtractive suppressive hybridization, etc.) led to the discovery of genes with high induction level; for example, *viperin/vig1* and 20 other viral hemorrhagic septicemia virus (VHSV)-induced genes (*vig*) including *isg15* and two chemokines were identified in rainbow trout leukocytes by DDPCR and SSH [83,84,92]. *cd9* and *isg15* were found induced by the rhabdovirus infectious hematopoietic necrosis virus (IHNV) in Atlantic salmon with the same methods [93,94], which were applied to many fish species. In grass carp (*Carassius carassius*), subtractive approaches showed that an *irf-like* [95], *jak1* and *stat1*, two Mx [96], two *isg15* [96,97], and a number of genes encoding tetratricopeptide-containing proteins [96] are up-regulated by the grass carp hemorrhage virus. In Atlantic cod (*Gadus morhua*), SSH screening after poly(I:C) stimulation identified a number of genes including those encoding ISG15; IRF-1, IRF-7, and IRF-10; MHC class I; VIPERIN/VIG1; and the ATP-dependent helicase LGP2 [98]. In the sea bass (*Dicentrarchus labrax*), brain nodavirus-infected tissue was analyzed and C-type lectins, pentraxin, and an anti-inflammatory galectin were found [99,100]. A more comprehensive representation of the fish transcriptional response to viral infection came only with genome and EST high-throughput sequencing, opening the way to the microarray technology. Microarray analyses were applied to characterize the response induced by different viruses [64,101–105], IFN inducers [106,107], or recombinant IFN itself [108]. These transcriptome analyses from multiple cell types and tissues suggested that a “core” set of 50–100 genes is typically induced [109]. To get a more comprehensive repertoire of ISG in a whole fish, we recently characterized the response of the zebrafish larva to the Chikungunya virus (CHIKV), a virus that induces a powerful type I IFN response [110]. A set of highly induced ISGs was found, which is also typically retrieved in human [75,111]: *rsad2*, *CD9*, *isg12*, *isg15*, *ifit* and *ifi44* family members, *stat1*, *trim25*, *socs1*, *irf1*, and *irf7*. This gene set was concordant with the major list of fish ISGs predicted from different tissues of other species (see above, reviewed in Ref. [109]). A list of zebrafish orthologues of human ISGs was similar to the repertoire of genes up-regulated by CHIKV infection, which also further confirmed the size of this core set [110].

The above-mentioned analysis of the zebrafish orthologues of all human ISGs also revealed some

important mammalian ISGs that are almost certainly lacking an orthologue in the zebrafish genome [110]. Zebrafish (and apparently all teleosts) lacks the APOBEC3, OAS, IFI16, and CLEC4 families altogether. Among other notable absent genes, one may cite *bst2/tetherin*; several *trim* such as *trim5*, *trim22*, or *pml/trim19*; and *isg20*.

A significant antiviral activity was demonstrated in fish for several of the ISGs. For example, overexpression of a Japanese flounder PKR homologue increased eIF-2 phosphorylation and inhibited the replication of the *Scophthalmus maximus* rhabdovirus [112]; MX proteins blocked the birnavirus IPNV [113], but not the rhabdovirus IHNV [89]; fish ubiquitin-like ISG15 shares with its mammalian homologues the anchor LRGG motifs and interacts with cellular and viral proteins [114], and an ISGylation-dependent activity of the zebrafish ISG15 was recently demonstrated against different RNA and DNA viruses [41]. A cytokine-like activity was also reported for the ISG15 secreted form in the tongue sole [115], as previously for mammals [116].

Altogether, these observations indicate that a number of essential ISGs were already important players of the IFN-mediated antiviral response rather early in the vertebrate history, at least in the common ancestor of tetrapods and fish. It starts to be possible to assess the extent of functional conservation of this core gene set, not only by direct comparison of the functions of individual genes but also using global comparative analyses. For example, some ISGs are typically induced more than others. Do human ISGs and their zebrafish homologues show similar response patterns? Figure 3A shows a tentative correlation of the response of zebrafish larva to CHIKV with the response of human liver to IFN α [117] and illustrates that orthologues of strongly induced human ISGs tend to be strongly induced by CHIKV infection in zebrafish as well.

Genes involved in immune responses typically show high rates of evolution due to selection pressures exerted by pathogen subversion. Under this rule, ISGs should show a similar trend, and we should observe a negative correlation between ISG sequence similarity in fish and human and their induction level. The relationship between induction rate and sequence similarity/conservation is obviously complex, and these two parameters are not merely correlated (Fig. 3B). However, the global pattern may suggest a loose negative correlation, and outliers such as *rsad2/viperin*, which are highly conserved and well induced by IFN, constitute interesting exceptions.

Many ISGs are members of gene families, with different evolutionary dynamics of expansion/diversification during the evolution of tetrapods *versus* that of fish. Among families containing ISGs, two different patterns were observed: families that differentiated in parallel in tetrapods and fishes from a single common ancestor gene (“young” families) and families

that had already diversified in the common ancestor to fishes and mammals (“old” families) [110]. Young families (such as MX or IFIT) would likely bind viral components and quickly diversify under strong selection pressure. On the contrary, old, stabilized families typically contain regulatory factors or signal transduction components (i.e., IRFs, STATs, and SOCS) and constitute key molecules in the conserved antiviral machinery.

To illustrate how comparative analysis of human and fish transcriptional responses might suggest important new genes to be targeted in future studies, we will focus on the subset of human ISGs that have a one-to-one orthologue in zebrafish, because they are the easiest to test experimentally, for example, by morpholino knockdown assays. This list includes 178 human genes [110]. Strikingly, among these ISGs, 140 (80%) are not annotated as having a potential role in antiviral defense in the current Ensembl GO classification. Some of those genes surely play important, but for the moment overlooked, roles in antiviral responses. Good candidates for further research would be ancestral ISGs, identifiable within this list by having a zebrafish orthologue induced by IFN. At least four genes fulfill this criterion based on the microarray analysis of the response to CHIKV: *cmpk2*, *phf11*, *upp2*, and *ftsjd2*. The kinase CMPK2 participates in dUTP and dCTP synthesis in mitochondria and may play a role in monocyte differentiation, PHF11 is a positive regulator of Th1-type cytokine gene expression, UPP2 is involved in nucleoside synthesis, and FTSJD2 mediates mRNA cap1 2'-O-ribose methylation to the 5'-cap structure of mRNAs—a feature that, remarkably, distinguishes host mRNAs from some viral mRNAs [118]. More genes shall be added to this list in the future as RNA-seq analysis and improved stimulation protocols will yield a more exhaustive list of zebrafish ISGs.

Part 3. IFN-Producing Cells

The current paradigm for type I IFN production in mammals is that all cell types are able to produce IFN β upon sensing a virus, and in addition, some specialized sentinel cells such as plasmacytoid dendritic cells can produce very high levels of IFN α . The specialized cells have a different array of sensing molecules (e.g., TLR7) and are poised for rapid IFN expression by constitutive expression of some signal-transducing molecules that need to be induced in other cell types (e.g., IRF7). Is the situation similar in fish?

A few studies have addressed the tissue-specific differences in expression of fish type I IFNs and sometimes identified the cell types involved. Zou *et al.* [9] found important differences between leukocytes and fibroblasts upon poly(I:C) stimulation *in vitro*: thus, head kidney cells would express all IFNs tested, while RTG-2 fibroblasts would express the group I IFNs

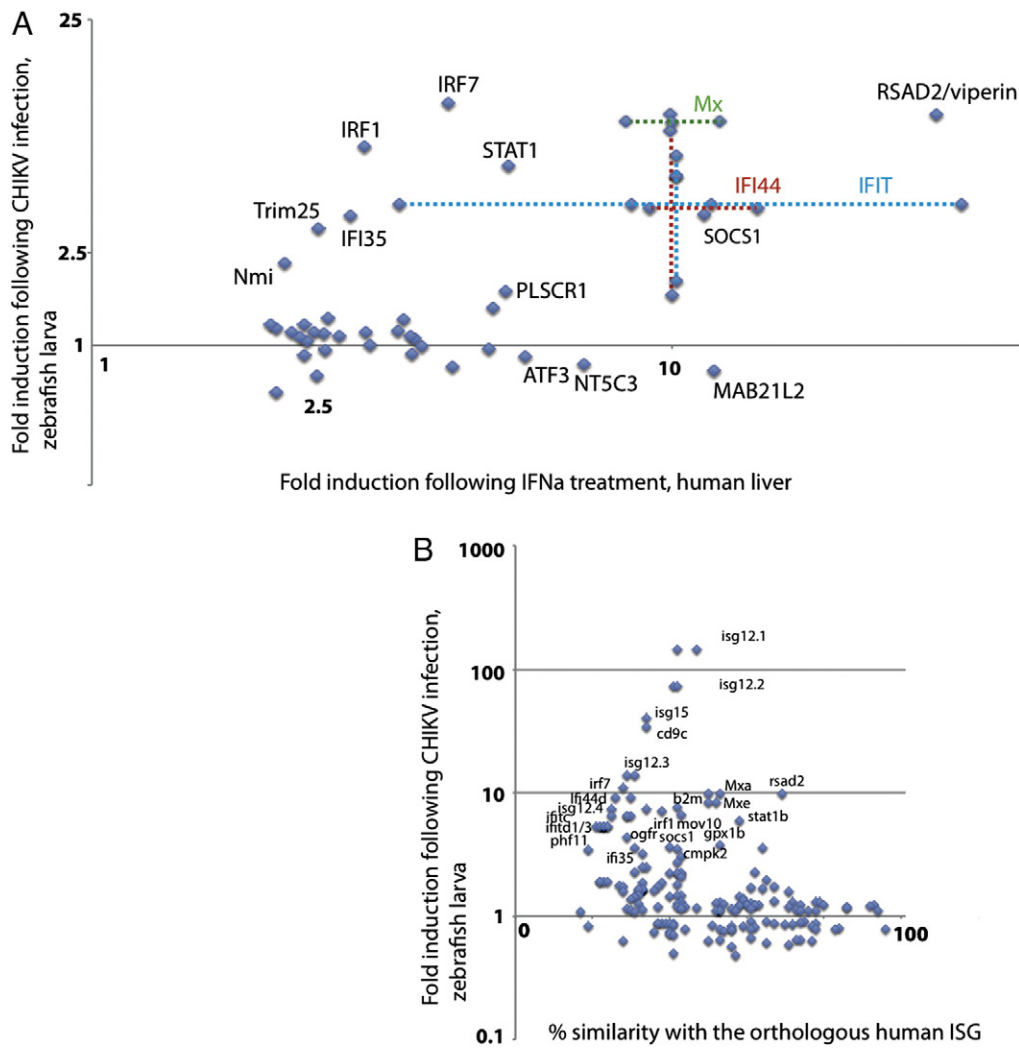


Fig. 3. Assessment of the conservation of ISGs: comparison of induction levels and sequence similarity between human ISGs and their zebrafish orthologues. (A) Induction levels of human ISGs (liver biopsy cells treated for 4 h with IFN α , from Sarasin-Filipowicz *et al.* [117], GEO accession GSE11190) compared with induction levels of their zebrafish orthologues (larvae infected for 48 h with the strong IFN-inducing CHIKV, GEO accession GSE47057). When homologous genes from human and zebrafish were not linked by a one-to-one orthology relationship, they were linked by a colored dotted line and set at the geometric average of the fold changes values of the other species. In these cases, the name of the gene family is indicated in the corresponding color. (B) Level of induction by CHIKV of zebrafish genes orthologous to human ISGs [same data set as for (a)], compared with their degree of similarity with their human orthologues (retrieved from the Ensembl database).

(IFN1 and IFN2) but not the group II IFN (IFN3). *Ex vivo* analysis of tissues from infected trout suggested a similar picture, with IFN3 being expressed in lymphoid tissue (kidney and spleen) but much less in liver [9]. In Atlantic salmon, Sun *et al.* [11] also found a much more restricted expression of IFN subtype by fibroblast-like TO cells, where only IFN α (a group I IFN) was induced more than twofold, while head kidney leukocytes would also express the group II IFN β and IFN γ [11]. In these cells, poly(I:C) would induce IFN α and IFN γ , while S-27609 (a TLR7 agonist) would preferentially induce IFN β . Similar outcomes were found *in vivo* at early time points after

poly(I:C) or S-27609, but the pattern changed strongly after a few days, likely as a result of complex feedback loops [11]. More recently, Svingerud *et al.* published a study that largely confirmed these findings (using R848, a TLR7/8 agonist, instead of S-27609) and added much spatial information, notably by performing *in situ* hybridization on tissue slices [18]. Quite remarkably, in all tissues, expression of all tested IFNs was restricted to a minority of cells. IFN α and IFN γ were sometimes coexpressed by the same cell in poly(I:C)-injected animals, while IFN β and IFN γ could be coexpressed after R848 injection. Cell types that could be identified as expressing IFNs were

endothelial cells and gill pillar cells for IFN α and gill pillar cells for IFN γ . No IFN β -expressing cell could be positively identified, but the data suggest that they were distinct from IFN α -expressing cells. IgM-positive B cells did not express any IFN; neither did melanomacrophages [18].

More recently, this question has been addressed in zebrafish using IFN-reporter transgenes. In larvae, among the four zebrafish *ifn* genes, only *ifn ϕ 1* (a group I *ifn*) and *ifn ϕ 3* (a group II *ifn*) are considered to play a role, because *ifn ϕ 2* is expressed only at the adult stage and *ifn ϕ 4* does not seem to exert a significant antiviral effect [10]. An *ifn ϕ 1* reporter transgene has been recently reported [119] and analyzed in the context of CHIKV infection, which induces a strong IFN response. The transgene was mainly expressed in two cell populations: neutrophils and hepatocytes—a pattern entirely consistent with expression of the endogenous *ifn ϕ 1* gene as seen by *in situ* hybridization, although the transgene expression was somewhat delayed [119]. The pathways inducing *ifn ϕ 1* in these two populations are not yet unraveled but are likely to be different since hepatocytes were a target of CHIKV while neutrophils were not infected. A small macrophage-like population also expressed the transgene. Depletion studies demonstrated that neutrophils, but neither hepatocytes nor macrophages, were critical to control the infection. Interestingly, in control, uninfected fish, a small population of neutrophils (10–30 cells/larva) express the transgene at a weak level [119]. An *IFN ϕ 3* reporter line has also been generated (V. Briolat, N.P., G. Lutfalla, and J.-P.L., unpublished results). The pattern of expression of this transgene during CHIKV infection is very different from that of the *ifn ϕ 1* reporter and includes fibroblasts, endothelial cells, hepatocytes, and muscle fibers, all cell types that may be infected by CHIKV; however, expression of the transgene was only observed in virus capsid-negative cells (N.P., unpublished results).

As a general conclusion, fish IFNs generally appear to be expressed by discrete, scattered cell populations with little overlap between IFN subtypes. Some IFNs are expressed in an “IFN β ” pattern, by fibroblasts and other tissue cells that may be direct targets of the viruses, while others are expressed in an “IFN α ” fashion by more specialized immune cells. Surprisingly, however, while group II IFNs are those that are preferentially expressed by hematopoietic cells in salmonids, the reverse seems true in zebrafish: group I is preferentially expressed by neutrophils.

There is so far no evidence for a cell type similar to plasmacytoid dendritic cells in fish, but these studies are still in their infancy. Neutrophils seem to play such a role in zebrafish larvae, which came as a surprise. It remains to be tested whether neutrophils are also major IFN-producing cells in adult zebrafish, in other fish species, and possibly during some viral infections in tetrapods.

Part 4. Kinetics of the Different IFN Responses in Fish

Early studies in fish cell lines described a quick and early production of IFN-like activity after viral infection or incubation with UV inactivated viruses [4, 120]. IFN production following a virus infection was also demonstrated *in vivo* in rainbow trout, with higher amount on day 1 post-VHSV infection and declines to background level by day 14 post-infection [3]. In keeping with this, in carp injected with 10^7 pfu of virulent spring viremia of carp virus, the IFN-like activity peaked as early as days 1 and 2, started to decline at day 3, and had disappeared by day 14 [121].

In the 1990s, the kinetics of the antiviral response was studied in further detail using (semi)Q RT-PCR to assess expression of ISG transcripts. After the first fish type I IFN genes were cloned in the 2000s, the kinetics of the IFN mRNA itself could be measured in various infection contexts. Different types of kinetics were obtained, a few of which will be illustrated. McBeath *et al.* compared the kinetics of type I IFN in Atlantic salmon after infection by ISAV and IPNV [122]. Type I IFN and Mx expression peaked twice on days 3 and 6 after IPNV infection and declined progressively. This biphasic response might rely on a positive feedback loop depending on IRF induction by the first burst of IFN production as described in mammals [123]; however, the mechanisms underlying the biphasic salmon IFN response to IPNV remain unknown. In contrast to this kinetics, a later, monophasic type I IFN response occurred after ISAV infection; IFN shortly peaked on day 5 or 6, while Mx peaked on day 6, declined to day 9, and remained expressed until day 30 post-infection. These differences likely reflected that these viruses use different mechanisms for dealing with the host response. Early up-regulation of IFN and ISG like Mx by the IPNV probably contributed to the good survival recorded after this infection. In contrast, high mortality and late response were observed after ISAV infection, which could be due to viral anti-IFN mechanisms [124]. Transcriptome profiling of the response induced by recombinant IFN in macrophage-like SHK1 cells showed that Mx and other ISGs were induced after 6 h of incubation and peaked at 24 h [108], supporting other observations reported for different tissues (e.g., trout kidney leukocytes in Ref. [84]).

However, these studies do not reflect the whole complexity of the type I IFN response since (1) most of the first QPCR and array systems did not take into account the IFN alternative transcripts discovered in zebrafish and in other species; hence, measures of IFN up-regulation integrate both secreted and non-secreted isoforms, which provides a partial view of the kinetics of the effective response; (2) fish genome and EST sequences revealed many

type I IFN genes, especially in salmonids; (3) IFN γ s may also contribute to the induction of some ISGs [28].

It is difficult to compare kinetics of IFN gene induction by two different viruses; not only is there a large range of antiviral mechanisms potentially at play (as discussed later), but viral burden (and thus signal) is likely to be different in both cases; comparing induction of different genes in the same context is more informative. For instance, in the zebrafish CHIKV infection model, expression of *ifn ϕ 1* was sustained, while *ifn ϕ 3* expression was more transient [119]. This likely reflects the different pathways (and cell types, as discussed above) involved in their induction, consistently with results of luciferase assays suggesting the variable contribution of IRF3 and/or IRF7 to activate the promoters of the various zebrafish IFNs [49].

Part 5. Tissue-Specific Responses

Expression of IFNs is induced upon detection of viruses and is thus expected to be fairly organ specific, depending on the tropism of the particular virus considered. By contrast, since type I IFN receptors are ubiquitously expressed in mammals and IFNs diffuse via the blood, ISGs would be expressed in a more uniform fashion. However, recent findings have shown this idea to be simplistic. For instance, type III IFNs induce the same set of ISGs than type I IFNs, but their receptor is expressed in a tissue-restricted fashion, allowing for targeted induction of ISGs, notably in epithelia exposed to outer environment such as the gut [125]. In addition, even upon systemic type I IFN administration, ISG expression has been found to be highly variable from tissue to tissue [126]. Do we find a similar situation in fish?

As mentioned above, fish also possess two groups of virus-induced IFNs that signal via two distinct receptors [10]. Although both groups are phylogenetically related to mammalian type I (rather than type III) IFNs [12], it has been proposed that the group I/group II and type I/type III dichotomies may have evolved in a convergent manner in teleosts and tetrapods, respectively [10]. A potential selective advantage of the dichotomy would be that a response restricted to external tissues may deal with most viruses with few of the side effects associated with a full-blown IFN response, which would be triggered only upon the most severe viral infections. Unfortunately, there are as yet no data published regarding the tissue-specific expression of the receptors for the two groups of IFNs. Both receptors share the CRFB5 chain, which is expressed ubiquitously at a relatively high level, but the weak expression of the specific CRFB1 and CRFB2 chains precluded their detection by whole-mount *in situ* hybridization in zebrafish embryos [13].

We also recently used whole-mount *in situ* hybridization to establish the expression pattern of four ISGs (*isg15*, *rsad2/viperin*, *isg12.1*, and *irf7*) in zebrafish larvae, notably in the CHIKV infection model, which results in a very strong endogenous IFN expression [110]. Basal levels of expression were below detection level, but upon infection, strongly tissue-dependent induction was observed, with an overall pattern of expression in liver, gut, and blood vessels, with some gene-specific differences (e.g., *viperin* was comparatively less induced in the gut while *isg12.1* was less induced in the liver). A rather similar, if weaker, pattern was observed after IHNV infection [110] or after intravenous injection of recombinant zebrafish IFNs (J.-P.L., unpublished results), suggesting that it mostly reflects the differential susceptibility of organs to circulating IFNs.

It is still unclear whether this pattern seen in zebrafish larvae can be generalized, as tissue variability in ISG expression has been addressed in relatively few studies. Lymphoid organs constitute the site for the activation of a proper immune response and, therefore, the majority of the studies present in literature focus their attention on the specific responses activated in those tissues. Responses have also sometimes been analyzed in some tissues for which viruses were known to have a preferred tropism. The following paragraphs focus on such studies.

One of the gateways of viral entry and replication in fish is fin bases, for example, for novirhabdoviruses [127]. In response to lethal VHSV infection of Pacific herring (*Clupea pallasii*), *Mx*, *psmb9*, and an *MHC class I* gene were found to be induced both in the spleen and in the fin bases, with a moderately stronger induction in the spleen attributed to the higher viral burden in this organ [128]. Transcriptomic and proteomic studies performed in adult zebrafish during VHSV infection have shown that a number of infection-related genes/proteins are overexpressed in the fins but not in other organs. Among these are complement components, interleukin genes, *hmgb1* protein, *mst1*, and *cd36* [129]. This does not seem to reflect a typical ISG response, and indeed *ifn ϕ 1* transcripts were not identified in this study, possibly because the low temperature required for VHSV replication was suboptimal for induction of a response in zebrafish. Infection of rainbow trout fin bases with VHSV, on the other hand, determines the up-regulation of the chemokines CK10 and CK12, as opposed to those overexpressed in the gills (CK1, CK3, CK9, and CK11). These expression variations may be due to a different permissivity of the tissues (fins or gills) to viral replication [130].

Several fish viruses are also known to have a tropism for the heart. Fish alphaviruses and, more recently, members of the *Totiviridae* family (e.g., piscine myocarditis virus) are associated with cardiac and/or skeletal myopathies. In particular,

alphaviruses, such as salmonid alphavirus subtype-1, are capable of causing acute heart lesions with necrotic foci and hypertrophy of the cardiac muscle. Unlike adult fish, smolts can replace damaged cardiomyocytes by cell division and may, therefore, be subjected to a decreased pathogenesis and impact [131]. Recently, the determinants of resistance of two strains of Atlantic salmon to salmonid alphavirus have been investigated, comparing responses in heart, kidney, and gills (a possible port of virus entry). The two strains displayed significantly different basal expressions of *ifna1* and ISGs (*Mx*, *viperin*, and *cxcl10*); however, the induction by viral infection was comparable in the three organs [132]. Similar results were obtained from Atlantic salmon infected with piscine myocarditis virus [133].

Several fish viruses also have a preferred tropism for the central nervous system. One of the most serious viral diseases affecting marine fish is represented by nodavirus encephalopathy. The central nervous system and the eye constitute the specific targets for nodavirus replication, leading to mass mortality in larvae and juvenile fish. Numerous studies have, therefore, been conducted to determine the immune responses activated in the brain tissue upon infection, but comparison with other tissues remain scarce. Infection of zebrafish larvae with nervous necrosis virus (NNV), for example, leads to mortality rates higher than 95%. This has been linked to the lack of IFN and *Mx* expression, not detectable in the larval stage but expressed by infected adults [104]. A thorough transcriptomic analysis conducted in Atlantic cod (*G. morhua*) has revealed that NNV infection affects mainly neural processes and their regulation and cellular differentiation (down-regulated genes). Many ISGs were found to be induced in the brain, but expression in other tissues was not reported [104]. NNV infection in turbot (*S. maximus*) is followed by overexpression of *Mx*, *irf-1*, and *tnf- α* [134]. Finally, in European sea bass (*D. labrax*), two different *x* genes (*MxA* and *MxB*) were differentially expressed during NNV infection. While *MxA* is highly up-regulated in the brain, *MxB* expression does not differ substantially from controls, thereby suggesting that the former is the predominant isoform and that *MxB* may play a different and independent functional role [135].

Part 6. Subversion Mechanisms by Viruses in Fish

The complexity of antiviral signaling pathways reflects the dynamic interactions between viruses and their hosts and has been shaped by the highly diverse strategies developed by these pathogens to evade antiviral immunity. In mammals, a vast number of strategies have been discovered, targeting immunity

(pattern recognition receptors, IFN signaling, MHC class I presentation, cytokine or chemokine networks, etc.) as well as basic mechanisms of virus–host interactions (autophagy, cell cycle, protein synthesis, etc.).

Such mechanisms are certainly used by fish viruses as well, but remain poorly described. Subversion of host immune response has been mainly studied for novirhabdoviruses, birnaviruses, and orthomyxoviruses.

Novirhabdoviruses are negative-sense single-stranded RNA viruses infecting fishes. They have a small genome encoding four structural proteins (N, P, M, and G) plus a polymerase (L), like other rhabdoviruses, and one specific nonstructural protein (NV), which is a good candidate for subversion of immune pathways. Recombinant IHN and VHS viruses lacking NV were able to replicate in cell culture, although the growth of the IHNV- Δ NV was severely impaired [136–138]. The importance of NV protein for pathogenicity was also strongly suggested by *in vivo* challenges with mutant viruses that caused only 20% mortality, whereas the wild-type control virus causes 100% mortality [136–138]. Although the sequence of the NV protein is not highly similar between novirhabdoviruses, the attenuated phenotype of VHSV- Δ NV can be rescued by re-introduction of NV from IHNV and vice versa [137,139], suggesting that the function of NV during infection is conserved. In fact, cells infected by NV-deletion mutants express higher levels of type I IFN transcripts, suggesting that NV is used to evade the innate antiviral immune response [140]. Moreover, growth of IHNV- Δ NV was inhibited by poly(I:C) treatment at 24 h post-infection, while the wild-type virus was not blocked. The overexpression of VHSV NV protein also reduced the TNF α -mediated activation of NF κ B, which likely contributes to its impact on the innate response [141].

“Multitask” properties are known for M and P proteins of prototypical rhabdoviruses infecting higher vertebrates, rabies virus (RV), and vesicular stomatitis virus (VSV) [142]. RV was shown to diminish IFN β induction through the viral protein P, which blocked IRF3 phosphorylation [143]. The P protein of RV also inhibited IFN downstream signaling by blocking the nuclear import of STAT1 [144] and has an impact on viral transcription and nucleocapsid formation. In fish, such mechanisms have not been reported yet, but the P protein of IHNV (as well as NV) is targeted by ISG15, which may represent a cell countermeasure [41]. Indeed, overexpression of ISG15 in EPC cells is sufficient to trigger antiviral activity against novirhabdoviruses (IHNV, VHSV), birnavirus (IPNV), or iridovirus (EHNV). ISGylation, which targets cellular proteins such as TRIM25 and viral proteins such as the P and NV of IHNV, is required for viral inhibition: the ISG15_{LRAA} mutant (incapable of functional ISGylation)

does not afford any protection. Subversion of IFN induction has also been demonstrated for fish birnaviruses and orthomyxoviruses. The proteins VP4 and VP5 of the birnavirus IPNV had antagonistic properties towards an IFN reporter [145]; however, *in vivo* comparison of IPNV field isolates with different levels of pathogenicity did not clearly confirm the importance of an intact VP5 protein for virulence [146]. Similarly, two ISAV proteins encoded by the genomic segments 7 and 8—respectively named s7ORF1 and s8ORF2—are involved in the modulation of the IFN signaling [124,147]. While s7ORF1 expression is restricted to the cytoplasm [147], s8ORF2 possesses two NLS signals responsible for nuclear expression and binds both dsRNA and polyA RNA [124]. The IFN antagonistic activity of s7ORF1 was shown by Mx-Luc reporter assay or RT QPCR on Mx and IFN upon poly(I:C) treatment [147]. Another study determined that s7ORF1 and s8ORF2 expression down-regulates the activity of a type I IFN promoter upon poly(I:C) exposure [124].

Large DNA viruses often possess genes blocking IFN pathways or inhibiting ISG function. For example, the ranavirus RCV-Z (*Rana catesbeiana* virus Z), a pathogen of fish and frogs, circumvents host-induced transcriptional shutoff and apoptosis by expressing a pseudosubstrate for PKR [148]. Other fish iridoviruses and herpesviruses can also possess such “mimicry” genes: for example, the koi herpesvirus encodes an IL-10 homologue [149], the Singapore grouper iridovirus encodes IgSF members, and another fish iridovirus encodes a B7-like sequence [150].

Viruses also dysregulate a number of basic cellular functions, which they use for their own replication and to block intrinsic antiviral mechanisms. For instance, IHNV has an acute life cycle during which it causes global blockage of cellular transcription, very similarly to the well-studied VSV [151,152]. The M protein of VSV, in addition to repressing cellular transcription, was shown to inhibit nuclear trafficking of RNA and proteins, thereby also inhibiting antiviral responses [153]. Both VSV and IHNV elicit cell rounding, probably by interfering with cytoskeletal dynamics [151,154]. Shutoff of basic cellular machinery eventually leads to apoptosis. Programmed cell death being also one of the host's antiviral strategies, many viruses developed strategies to delay apoptosis and complete their infection cycles. In fish, VHSV was able to block experimentally induced apoptosis in EPC cells in an NV-dependent manner [139].

Conclusion

Antiviral immunity has been studied only in a few fish species, either aquaculture fishes or model species. Fish are vertebrates and share with humans and mice most of the key antiviral pathways.

However, fishes had a long and complex genome history and developed a specific adaptation to the aquatic environment (and to its pathogens). Hence, the fish antiviral immunity represents an alternative version of what could evolve upon highly selective pressures of host–virus interactions, from the ancestral system present in the early vertebrates. Comparison of mammalian and fish innate antiviral mechanisms will be certainly beneficial to distinguish the core system, which is resilient to the subversive selective pressures exerted by the viral world, from the specialized systems that emerged during the evolution of each branch in response to particular viral strategies. In addition, the imaging possibilities offered by model fish species such as the zebrafish will be instrumental, in the future, to unravel the spatiotemporal dynamics of these core antiviral responses shared by all vertebrates.

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Abbreviations used:

IFN interferon; WGD whole genome duplication; CRFB cytokine receptor family B; IPNV infectious pancreatic necrosis virus; ISAV infectious salmon anemia virus; RLR RIG-I-like receptor; TLR Toll-like receptor; RIG-I retinoic acid-inducible gene I; LGP2 laboratory of genetics and physiology 2; TNF tumor necrosis factor; TRAF TNF receptor-associated factor; TBK1 TANK binding kinase 1;

IRF IFN regulatory factor; dsRNA double-stranded RNA; TIR Toll-interleukin 1 receptor; IRAK interleukin-1R-associated kinase; VHSV viral hemorrhagic septicemia virus; IHNV infectious hematopoietic necrosis virus; CHIKV Chikungunya virus; NNV nervous necrosis virus; RV rabies virus; VSV vesicular stomatitis virus

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