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CONSTRUCTION OF REPLICATION-DEFECTIVE HERPES SIMPLEX VIRAL VECTORS FOR TARGETING THE LHX2 GENE IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

Epilepsy is a chronic disorder affecting about 65 million people worldwide and temporal lobe epilepsy (TLE) is among the most frequent types of intractable epilepsy. In most cases the causes of TLE are unknown but it is believed that it may take place after an initial precipitating injury (IPI) such as brain tumor, ictus, head trauma, meningitis, encephalitis, and febrile seizures during childhood. Despite the development of new antiepileptic drugs (AEDs), about 35% of epileptic patients still suffer from pharmacoresistant seizures, with surgical resection of the epileptic locus as possible last option. In addition, AEDs don't prevent the progression of the disease but they are designed only for treatment of patients with an already established syndrome. Hence, there is an unmet medical need for the prevention of seizures for the patients at high-risk of developing epilepsy. This clarify how urgent is the need to find novel therapeutic concepts to fill this gap.

Epilepsy could develop when the intracerebral balance between excitation and inhibitory neurotransmission is impaired. Experimental findings show that after an epileptogenic insult the brain react to the injury with an enhanced hippocampal neurogenesis as a homotypic response to the neuronal loss in an attempt to restore the pre-existing cellular network. However, this plastic remodeling that the brain goes through is usually aberrant, since the cells that undergo the replacement of degenerating neurons upon severe brain injury are mostly high proliferating reactive astrocytes. This leads to important alterations of brain signals and, consequently, high risk of seizure development.

Starting from this concept, we hypothesize that controlling the neural stem cells fate after an initial precipitating injury could prevent epileptogenesis or at least improve the clinical picture of the patient.

Based on the recent literature, we decided to test the effects of Lhx2 protein overexpression on cells of central nervous system. Lhx2 is a transcription factor that plays a crucial role since early stages in telencephalic patterning but its function is not limited to the early embryonic neuroepithelium: recent evidences have shown a unique role for this protein in the phase of active neurogenesis, when its overexpression may enhances and prolongs the neurogenesis to generate neurons from progenitors that would otherwise give rise to astrocytes.

The recent advances of gene therapy promise innovative and revolutionary new treatments for neurological disorders. Various methods have been developed for gene delivery to target cells. However, gene transfer by viral vectors is thus far the widest used approach. In particular, up today the most efficient systems to achieve a long term transgene expression is based upon retroviral and lentiviral vectors. Unfortunately both these viruses cannot be designed for clinical applications since their infections result in insertion of viral DNA into the host chromosomes at an unpredictable position, a dangerous event which can seriously disturbs cellular genes functions potentially leading to cancer transformation of infected cells. It is then important to set up novel tools to safely deliver genes. Herpes simplex virus-1 (HSV-1) offers unique features that support its development as a great candidate viral vector especially for targeting the nervous system: it is a highly infectious, naturally neurotropic virus able to establish life-long latency in neurons, along with the largest capacity for exogenous DNA cloning. Moreover, it doesn't integrate into the host genome, avoiding any possibility of insertional activation or inactivation of cellular genes. However, some technical problems still need to be overcome, such as the efficient delivery of the vector to target cells, the maintenance and control of foreign gene expression, and the control of unwanted host immune responses.

This thesis describes the development of a highly efficient method for *in vitro* and *in vivo* targeting of the Lhx2 gene using novel replication-defective herpes simplex viral vectors, named JΔβββ4 and JΔNI, opportunely engineered to reduce the innate toxicity of the virus and to allow a good expression of the transgene. HSV-mediated delivery of Lhx2 resulted in highly effective gene overexpression in several cell types *in vitro*, including mouse neuronal and non-neuronal cells, along with reduced or null toxicity and a differential transgene expression, depending on the viral backbones. These vectors have been additionally tested *in vivo* by injection into the hippocampus of naïve rats and of rat models of epilepsy. Ex vivo analyses of injected brains showed good infection pattern from both viruses along with no evident toxicity. Moreover, the hippocampal delivery of Lhx2 by JΔβββ4-based vector was associated with reductions of both astrocyte density and recurring seizures, giving rise to more favorable pathologic features and improved outcomes. Put together, we can finally assess that both the JΔβββ4 and JΔNI-based vectors could represent useful tools for differential purposes: while for in vitro applications the JΔNI vector is the best compromise between transgene expression and low toxicity

effects, for in vivo gene transfer it result almost ineffective. On the other hand, the JΔβββ4 vector displayed an opposite behavior, too toxic for in vitro approaches but much more effective for gene delivery *in vivo*.

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Alle mie nonne,

insostituibili maestre di vita.

1. INTRODUCTION

1.1 EPILEPSY

1.1.1 Historical overview

Epilepsy is a medical disorder old as human existence; few diseases have generated same interests and controversies as epilepsy.

The term epilepsy derives from the Greek verb *epilambanem*, meaning "*to take hold on* or *to seize upon*." The astonishing manifestation of the epileptic seizures inspired in people of prehistoric civilizations the belief of a supernatural punishing event.

Hippocrates, the Father of Medicine, made the first historical records of epilepsy in the fifth century BC referring to epilepsy as the “Sacred Disease”, an attack from demons. Later in the fifteenth century AD, it was believed to be a sign of witchcraft. It wasn't until the late nineteenth century when pioneering studies on human brain showed the true nature of epilepsy as a disease of the central nervous system, which could be identified by electroencephalography (Hughlings-Jackson, 1888; Berger, 1929).

1.1.2 Epidemiology

Epilepsy is the commonest neurological condition that affects people of all ages, race and social class. It is estimated that about 1% of the world’s population is affected by epilepsy and up to 5% may experience just a single unprovoked seizure (Adelöw et al., 2009). Across all ages worldwide, the incidence is approximately 50-100000/year in developed countries and 100-200000/year in developing countries (Theodore et al., 2006; Ngugi et al., 2010)

Incidence and prevalence studies are critical to provide measures of frequency and therefore the burden of disease, and allow for proper planning of services.

The epidemiology of epilepsy is subjected to several factors that vary worldwide as age, sex, comorbidities, overall health, etc. The age distribution of the incidence of epileptic seizures follows a bimodal trend, showing two peaks of frequency in childhood and seniority.

1.1.3 Etiology

It is a common misconception that epilepsy is a single homogeneous disease. In reality, epilepsies are highly heterogeneous disorders that can be categorized on the basis of several differentiating factors. Not even the origin of the disease is unique, since it may be acquired or inherent, pediatric or adult onset. There are many possible causes of epilepsy. The etiology of this syndrome is complex, arising from the contribution of multiple genetic and non-genetic factors. It is commonly accepted that epilepsy results from an abnormality in brain wiring and neurotransmitter imbalance. This atypical wiring can result from known brain pathology, traumatic brain injury, brain infections, prolonged febrile seizures, brain tumors, genetic propensity, neural developmental disorders or recreational drugs.

The International League Against Epilepsy (ILAE) has also classified epilepsies by etiology in:

-Familial epilepsies: inherited, these are identified in large families with an epileptic trait segregating in the absence of environmental factors.

-Idiopathic: usually age-dependent, these have a genetic or presumed genetic origin and do not involve underlying structural brain lesions or other signs of neurologic dysfunction. About 500 genes have been estimated to play a role in disease development, either by directly altering protein production or by increasing one's resistance to pharmacological treatments.

-Symptomatic: caused by a specific systemic or environmental factor, without particular neuroanatomic abnormalities.

Additionally, we can include the "Cryptogenic" epilepsies, or rather those without any well identified origin. Even if the number of such cases is diminishing, this category still counts about 40% of adult-onset cases of epilepsy.

1.1.4 Definition

The wide heterogeneity of clinical features regarding epileptic syndromes makes particularly complex to draw up any classification.

Today is well known that epilepsy is not one condition, but a diverse family of disorders occurring when the electrical signals in the brain are disrupted, leading to changes in neuronal activity that give rise to seizures. This medical condition has always been controversial, even regarding its definition.

Several classifications have been proposed since 1970 but any attempts to get a consensus failed until 1997, when the ILAE and the International Bureau for Epilepsy (IBE) defined epilepsy as “a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by neurobiological, cognitive, psychological and social consequences. The definition of epilepsy requires also the occurrence of at least one epileptic seizure” defined as “a transient occurrence of signs and symptoms due to abnormal, excessive or synchronous neuronal activity in brain” (Fisher et al., 2005).

Epilepsy occurs when electrical signals in the brain are disrupted, leading to changes in neuronal activity (neurons may fire up to 6 times as fast as the normal rate of about 80 times a second) that give rise to seizures. A seizure is a synchronized paroxymal event caused by an excessive electrical discharge of central nervous system neurons (Lowenstein et al. 2004), which can cause brief changes in sensations, emotions and behavior and, in worst cases, convulsions, muscle spams and loss of consciousness.

Seizures can also be classified grounding on the evoked physical manifestations, which can range from wild swinging movements of arms and legs (tonic-clonic seizures) to brief losses of awareness appearing as staring spells (absence seizure) and from to the spatial extent of the brain involved at clinical onset: when localized within the brain at the onset of the seizure they are referred to as focal or partial seizures and are usually named by the origin of the epileptic focus as in temporal lobe epilepsy, while those that appear to involve the entire brain at once are called generalized seizures. Finally, seizures can be differentiated based on whether the patient loses consciousness during the seizure event (complex seizure) or not (simple seizure). These categorizations are not mutually exclusive, so one will often describe an epilepsy disorder as complex partial seizures of the frontal lobe, for instance. In 1997, the ILAE has classified human epilepsies by seizure type as self-limited, continuous, or reflex, and as focal or generalized (Engel, 2001). Generalized, self-limited seizures are exemplified by tonic-clonic seizures, which begin with tonic extension of the limbs and trunk, evolve into rhythmic movements (clonus) and terminate spontaneously within a few minutes. Absence seizures also are generalized and self limited, characterized by brief episodes (about 10 sec) of staring and unconsciousness that can occur more than 100 times per day (Panayiotopoulos, 1997). In contrast, focal seizures arise from a focal region of one cerebral hemisphere, and their manifestations depend on the extension of the brain regions involved. Most seizures are self limited, but continuous seizures, also referred as

status epilepticus, sometimes occur. Reflex seizures are rare and are evoked by visual stimuli, somatosensory stimuli, thinking, reading, or tooth brushing. The precipitating stimulus is specific for each patient.

The human classification scheme can be used to classify seizures in other species, since self-limited, generalized tonic-clonic seizures and status epilepticus occur in many species.

1.1.5 Treatments and drugs

Upon diagnosis (EEG monitoring, video monitoring and magnetoencephalogram testing is a typical battery of examinations for diagnosis), the first-line treatment of epilepsy is pharmacological intervention. To date there is no cure for epilepsy and current treatment options only include lifelong drug therapy and, in a number of difficult cases, surgery may be necessary. While the overall goal of antiepileptic drugs is to completely eliminate seizures, this goal has not yet been achieved. Current antiepileptic drugs (AEDs) aim at symptomatically reduce the frequency and the duration of the seizures. Drug treatment of epilepsy began as early as 1857 and mainly involved the use of bromides. Phenobarbital was also a popular choice for epilepsy patients early in history. Since then, a large number of antiepileptic drugs have been developed for the therapy of various forms of epilepsy. No one treatment option works for all types of epilepsy disorders and, therefore, the preferred drug treatment of choice depends on a number of different factors such as the characteristic pharmacokinetic profile of the various AEDs and on the specific biochemical malfunction of the patient (Faught and Pollock, 2001). The most common antiepileptic drugs are summarized in Table 1.

Antiepileptic drugs are divided into a short list of classes according to their mechanisms of action (Howland et al., 2005), which aimed to:

- reduce sodium current (i.e. carbamazepine, lamotrigine, phenytoin)
- reduce excitatory coupling between neurons (i.e. topiramate)
- increase inhibitory coupling between neurons (i.e. clonazepam, divalproic acid, phenobarbital)

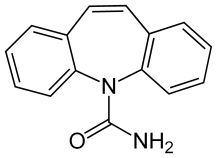
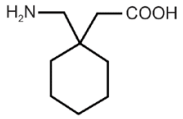
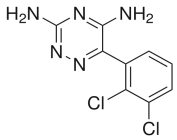
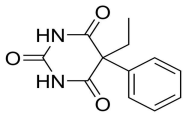
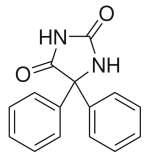
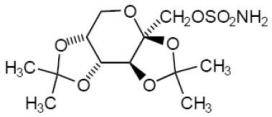
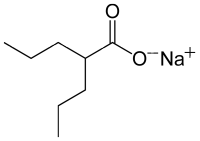
DRUG COMMON NAME	CHEMICAL STRUCTURE	RECEPTOR TARGET	COMMON SIDE EFFECTS
Carbamazepine		Na^+-channel antagonist; possible glutamatergic inhibition	Diplopia, headache, dizziness, nausea, and drug interactions.
Gabapentin		Na^+-channel antagonist; Ca^{2+}-channel antagonist	Ataxia, somnolence and peripheral edema.
Lamotrigine		Na^+-channel antagonist	Headache, nausea, insomnia, dizziness and possible severe, life threatening, skin rashes.
Phenobarbital		$GABA_A$ receptor agonist	Sedation, decreased cognitive function.
Phenytoin		Na^+-channel antagonist; Ca^{2+}-channel antagonist	Sedation, cosmetic changes (gum hypertrophy, hirsutism, acne, and facial coarsening), drowsiness, dysarthria, tremors, cognitive difficulties, and ataxia.
Topiramate		Na^+-channel antagonist; $GABA_A$ receptor agonist	Anorexia, paresthesias, somnolence, impaired concentration, confusion, and abnormal thinking.
Valproate		Na^+-channel antagonist	Hair loss and major weight gain. Rarely it can cause fatal hepatotoxicity and teratogenicity (neural tube defects).

Table 1: *Most common used antiepileptic drugs in clinic.*

These AEDs are clearly intended to reduce the abnormal excessive neural activity of epileptic patients. Their effects on neural synchrony, however, still remain unclear and more than 30% do not respond to current anticonvulsant drugs (Kwan and Brodie, 2000); this percentage has remained unchanged for over twenty years despite the discovery of several new anti-epileptic medications.

This lack of improvement in efficacy can be attributed to the fact that none of the recently discovered medications exhibit a novel mechanism of action. The most commonly prescribed medications are Carbamazepine, Valproate or Phenytoin.

When seizures cannot be controlled pharmacologically, surgery is the most considered alternative. Three broad categories of epilepsy can be treated successfully with surgery: partial seizures, seizures that begin as partial seizures before spreading to the rest of the brain and unilateral multifocal epilepsy with infantile hemiplegia (Bleck, 1987). The most common type of surgery for epilepsy is removal of a seizure focus (often called topectomy or lesionectomy). Usually, the more well defined the seizure focus the better is the post-surgical prognosis (Bleck, 1987). Other types of surgery include loboectomy (i.e. temporal lobe resection, reportedly having up to 70-90% success rate, Brodie & Schachter, 2000), subpial transection (Morrel et al., 1989), callosotomy, severing the bundle of neural connections between the right and left cerebral hemispheres, and similar procedures involving the anterior and posterior commissures (Brodie & Schacter, 2000). The most radical surgical procedure to treat epilepsy is hemispherectomy.

Other treatment strategies of epilepsy include vagus nerve stimulation, transcranial magnetic stimulation and biofeedback.

An additional line of research is currently investigating development of implantable devices that can deliver drugs to specific parts of the brain (Pathan at al., 2009). Furthermore, researchers are looking into altering certain environmental and life-style factors such as diet, which might alleviate seizure disorders (Korsholm & Law, 2013.

Unfortunately many patients (about 20%) continue to experience seizures even after several treatment attempts, highlighting the need of new innovative therapies to improve their threatening condition.

1.1.6 Temporal Lobe Epilepsy

Temporal lobe epilepsy (TLE) is the most common type of epilepsy in adults, and many patients continue to be affected by uncontrolled seizures despite treatment with AEDs (Engel et al., 1997). This condition is associated with specific structural lesions in the hippocampus (which may be surgically resected in medically intractable cases), leading to progressive development of spontaneous recurrent seizures (SRS) from the medial or lateral temporal lobe (so called Medial temporal lobe epilepsy or Lateral temporal lobe epilepsy).

Patients with temporal lobe epilepsy have approximately 2 to 30 seizures per month (French et al., 1993). These seizures may be partial, complex or generalized (depending on the involvement of one or both emispheres) and are unfortunately unpredictable. They start with an aura (King, 1977), during which the patient is still conscious, that is a visceral sensation of nausea, pressure, “butterflies” and/or epigastric rising, followed by a focal motor seizure and loss of consciousness which begins with motor arrest and staring and evolves to oral-alimentary automatisms (e.g., lip smacking, chewing, tooth grinding). Focal motor seizures sometimes progress to generalized tonic-clonic seizures. The mechanisms that terminate seizures and determine whether focal seizures will become generalized are still unknown.

Most patients with temporal lobe epilepsy have a history of brain injury and up to 2/3 of them had febrile seizures. Age at time of the injury is quite variable, but it usually happens during childhood (Mathern et al., 2002). Other precipitating injuries include head trauma, brain infections or hypoxia/ischemia (Mathern et al., 1995). Not all these precipitating events involve seizures at the time: after recovery from the initial precipitating injury, patients usually begin a seizure-free latent period, ranging from months to years, when aberrant changes in structure and physiology of the brain tissue happen before the development of SRS (Mathern, et al., 2002.). After this time, spontaneous, recurrent seizures occur, which typically continue throughout life.

This seizure-free latent period has been matter of great interest. It has been proposed that it is attributable to the time necessary for synaptic reorganization to establish a sufficient degree of recurrent excitation to surpass the seizure threshold. During the latent period, the hippocampus is undoubtedly the brain region mainly affected by unique morphological alterations. The most common lesional abnormality observed in patients with TLE is the Hippocampal Sclerosis (HS) (Babb and Brown; 1987), consisting in a massive loss and widening of the neurons in the hilus of the dentate gyrus and in the CA1 and CA3 layers (Engel, 1989; Ben-Ari and Cossart, 2000), and the mossy fiber sprouting, that is the aberrant growth of granule cell axons into the inner molecular layer of the dentate gyrus (Sutula et al., 1989; Babb et al., 1991; Isokawa et al., 1993). Consequently, a prominent hypothesis states that hippocampal neuronal loss and mossy fiber sprouting play a critical role in the genesis and progression of TLE (Lothman and Bertram, 1993); nevertheless, lesions also may develop in subregions of the amygdala and entorhinal cortex (Bernasconi et al., 2003).

Even though we know which are the morphological changes in patients with TLE, the

mechanisms of epileptogenesis are still unknown. Many hypotheses of temporal lobe epileptogenesis focus on the hippocampal dentate gyrus, which is thought to serve as a seizure-suppressing filter or gate (Lothman et al., 1991): the dentate gyrus displays dramatic lesions, such as loss of hilar neurons (Margerison and Corsellis. 1966) including excitatory mossy cells (Babb et al., 1984) and inhibitory interneurons (Maglóczk et al., 2000). Excitatory dentate granule cells survive but their inhibition is dramatically worsened due to loss of inhibitory GABAergic interneurons that normally inhibite the granule cells making them hyperexcitable and lowering the seizure threshold (Staley and Mody. 1992).

In addition, loss of mossy cells can also give rise to axon sprouting and synaptogenesis, inducing an aberrant positive-feedback circuit between dentate granule cells that generates seizures (Nadler et al., 1980). Mossy cells are the predominant neurons in the hilus and concentrate their glutamatergic axon terminals in the inner molecular layer of the dentate gyrus where they form excitatory synaptic contacts with granule cells (Wenzel et al., 1997). Since mossy cells are particularly sensitive to a wide range of insults (Buckmaster and Schwartzkroin, 1994), when they die their axon terminals degeneration leaves the postsynaptic sites vacant on the proximal dendrites of granule cells (Nadler et al., 1980), leading to abnormal granule cell axon reorganization (Laurberg and Zimmer, 1981).

Granule cells then ectopically sprout axon collaterals to invade the inner molecular layer, forming synapses that fill the empty synaptic sites. Anatomic evidence from patients with temporal lobe epilepsy showed that granule cell axons reorganize to form a positive-feedback circuit (Zhang and Houser. 1999) and the extent of granule cell axon sprouting correlates with the extent of hilar neuron loss (Babb, et al., 1991).

It would be extremely useful to develop methods to block the aberrant reorganization after an epileptogenic injury. Currently such treatments do not exist: prescribed epilepsy medications are only seizure-suppressing anti-convulsants and not anti-epileptogenic, temporarily treating the symptoms by reducing the probability of seizures, but they do not permanently block or reverse the development of epilepsy (Temkin, 2001). Creating antiepileptogenic treatments is an important goal of epilepsy research.

1.1.7 Animal models of TLE

Fundamental questions regarding cause and pathogenesis of epilepsy still persist. In

spite of the large diffusion, the study of epilepsy cannot be performed on humans for disparate reasons, such as ethical issues, unavailability of controls and high costs of human research. Study of brain tissues obtained with surgery or autopsy can be helpful, but is limited in quantity, quality, and experimental versatility, and control tissue is frequently unavailable. Moreover, since TLE is the most common form of drug-refractory epilepsy, studies on human specimens are usually not very informative and consistent because the clinical history of the affected patients is never the same and the results could be altered by a prolonged pharmacology therapy. The need of animal models to pursue *in vivo* studies that cannot be done in humans is based on the belief that more extensive investigations will provide us a deep knowledge of epilepsy, from the basic mechanisms underlying the epileptogenesis to the consequences of the seizures. Therefore, laboratory animal models are essential to help identify causes of temporal lobe epilepsy and translate such findings into better treatments for patients.

In particular, animal models of seizures or epilepsy serve a variety of purposes, among which:

-Discover novel AEDs.

-Evaluate the possible specific efficacies of the compound against different types of seizures or epilepsy.

-Use of specific models of AED-resistant seizures to investigate whether the novel drug has advantages towards clinically established AEDs for therapy of difficult-to-treat types of seizures or epilepsies.

-Characterize the preclinical efficacy of novel compounds during chronic administration, to evaluate for instance whether drug efficacy changes during prolonged treatment and if epileptogenesis alters the adverse effect potential of a given drug.

-Estimate effective plasma concentrations of new AEDs for first clinical trials.

Since epilepsy is such a complex pathology, the attempts to accurately model all the human aspects of every single disease subtype not always succeeded. Hence, some experimental approaches can reproduce only some of the manifestation of epilepsy, allowing only the investigation on that symptom and not on the whole complex picture of a complete model. In any case, this gives the chance to study an aspect of the disease.

Several animal models have been developed by application of chemical, electrical or damaging insults on a healthy brain to recapitulate the changes in human patients with TLE. Among them, electrical kindling of the limbic regions, systemic injections of convulsants such as kainic acid (KA) or pilocarpine (Pilo), and genetic models in which

human ion channel mutations are expressed, or important regulatory genes for interneuron development have been knocked out. Febrile seizures and traumatic brain injury are additional seizure models that mimic early onset or acquired epilepsy in adult rodents. Electrical kindling creates abnormal brain electrical activities directly by providing high frequency electrical stimulation of the hippocampus and its afferent pathways (Loscher et al., 1998). KA is a glutamatergic neuron agonist and excitotoxic that is specific for AMPA/kainate receptors. When injected systemically or focally into the brain, it can cause excitotoxicity in the CA3 region (Neema et al., 2005; Carpentino et al., 2008). While rats subjected to KA-induced SE will develop spontaneous epileptic seizures, many strains of mice do not.

Pilocarpine is a parasympathomimetic alkaloid that binds to M1 muscarinic receptors, altering Ca^{2+} and K^{+} currents (Segal, 1988). The increased concentration of intracellular Ca^{2+} allows the release of glutamate from presynaptic termini that, in turn, provokes the SE. Once activated, seizures are subsequently maintained by activation of NMDA receptors. Glutamate promotes the entrance of Na^{+} and Ca^{2+} into the cells by interaction with on AMPA/KA receptors, removing the Mg^{2+} which blockades the NMDA receptor. Increased Ca^{2+} concentration into the postsynaptic cells induces excitotoxic effects and cell death. The dose of alkaloid necessary to evoke the SE ranges from 300 to 400 mg/Kg; this treatment evoke powerful limbic seizures by activating cholinergic neurons in the entorhinal cortex. Pilocapine induces seizures within the limbic circuit but is not directly neurotoxic. However, it has been shown to induce leakiness in the blood-brain barrier and the influx of albumin into the brain may be a cause of neuronal degeneration and astrogliosis in this model (Marchi et al., 2010). Due to the excessive excitation to the dentate gyrus, hilar inhibitory interneurons, whose function is to keep dentate gyrus activity under control, degenerate and lead to further excitation of the limbic circuit (Baraban et al., 2009). Moreover, after injection of pilocarpine have been detected high levels of serum IL-1β, known to cause sudden rapid changes in excitability of both inhibitory and excitatory neurons (Plata-Salamán and Ffrench-Mullen, 1992; Yang et al., 2005).

Pilocarpine model is considered one of the best models for severe human TLE. After administration of Pilo, rodents exhibit stereotypical behaviors that can be categorized into 6 classes according to Racine scale (Racine, 1972), recently revised (Veliskova et al., 2006). As schematized, it uses numbers from 1 to 6 to define seizure classes: the first three represent partial seizures, the last three the generalized ones. SRSs begin as

partial seizures and become secondary generalized (Table 2).

Classes	***Features***	
1	Staring and mouth clonus	***Partial***
2	Automatisms	
3	Monolateral forelimb clonus	
4	Bilateral forelimb clonus	***Generalized***
5	Bilateral forelimb clonus with rearing and falling	
6	Tonic-clonic seizures	

Table 2: *Classes of epileptic seizures according to Racine's scale classification (Racine, 1972)*

Some rodents develop prolonged severe seizures, named status epilepticus (SE), characterized by continuous head bobbing and body shake. SE can last from 2 hrs to 12 hrs. Following SE rodents have a latent seizure-free period, in which they then enter post-ictal coma, lasting 1-2 days, before showing spontaneous recurrent seizures (SRSs). The length of the latent period varies between animals and techniques utilized to detect seizures. By using video-EEG monitoring, a study in mice showed that the latent period is two weeks on average, spanning from 4 to 42 days after SE (Cavalheiro, 1997). A more recent study established the mean latent period in rats between 5 and 17 days, averaging one week (Goffin et al., 2007). During the seizure-free phase, brain networks rearrangements occur (Pitkänen and Sutula, 2002). Following the latent period, the recurrence of seizures become quite regular, usually clustered in a cycle peaking every 5-8 days or more with an higher frequency during the day (Arida et al., 1999). In 90% of the cases, EEG trace is characterized by a cerebral activity that starts from the hippocampus and spreads to the neocortex, usually lasting less than 60 seconds (Cavalheiro et al., 1991). Despite SE is routinely pharmacologically stopped by administration of anticonvulsant drugs such as diazepam and ketamine, only a percentage of animals (ranging from 60 to 70%) survive the treatment (Cavalheiro et al., 1991; Liu et al., 1994). During the very first days following Pilo-induced SE, treated animals might experience some occasional, self-limiting generalized seizures for 1-3 days (Mazzuferi et al., 2010).

1.2 NEUROGENESIS AND ASTROGLIOSIS

1.2.1 Neurogenesis

Neurogenesis is the process that gives rise to functional neurons from precursors. Although experimental studies put in evidence for many decades that neurogenesis occurs in the adult mammalian brain (Altman, 1962), the concept has become more widely accepted only upon introduction of the thymidine analog bromodeoxyuridine (BrdU) in these studies (Christie and Cameron, 2006; Gage, 2002). BrdU is incorporated into DNA during synthesis in the S-phase of the cell cycle, allowing the identification of cells within the cell cycle or their postmitotic progeny. The administration of BrdU, mostly conducted in rats or mice, have been extended also to humans, showing evidence of ongoing neurogenesis also in the brain of aged subjects (Eriksson et al., 1998). These results pointed out the attention on the opportunities offered from the modulation of neurogenesis on a clinical perspective view, from the repairing of neuronal loss due to aging, pathologies or injuries to the cell fate control of neural progenitors (Kozorovitskiy and Gould, 2003).

Neurogenesis takes place mostly during embryonic and perinatal stages of mammalian life. However, new neurons are born throughout life (at least under physiological conditions) into restricted two neurogenic regions: the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone of the dentate gyrus (DG) of the hippocampus (Gage, 2000). In humans and rodents, the hippocampus is located in the temporal lobes. Principal cells are confined to compacted layers called the dentate gyrus, composed of granule cells, and the CA regions composed of pyramidal neurons.

Even if neural stem cells (NSCs) both originate from SVZ and DG, distinct progenies arise from these two niches: cells generated in the SVZ migrate toward the olfactory bulb to differentiate into distinct types of olfactory neurons [with the majority being GABAergic or dopaminergic neurons and only a few glutamatergic cells (Nissant and Pallotto, 2011)] whereas in the DG, newborn neurons don't migrate long distances and differentiate into one neuronal subtype, excitatory glutamatergic granule neurons (Young et al., 2011). It's still unknown if this fate divergence is intrinsically predetermined or is due to external signals. Gene expression and protein profiles of NSCs isolated from distinct subregions

within the neurogenic areas indicate strong similarities among NSCs within (e.g. isolated from dorsal and ventral SVZ) and between neurogenic regions (e.g. comparing SVZ and DG). Such profiles could then be compared with the profiles of their progeny at different maturation stages. However, this analysis is incomplete because we still lack of reliable markers that are specific for different stages of neuronal differentiation; the absence of such markers continues to represent one of the major limitations in the field of adult neurogenesis (Suhonen et al., 1996).

To assess the role of extrinsic cues towards fate determination, several studies involving transplantation of NSCs from one region into another have been made, showing as newborn cells may change their "natural" fate when grafted in a different region, indicating a strong influence of extrinsic cues on fate determination (Suhonen et al., 1996). But these results are controversial since other experiments put in evidence how the fate of NSC progeny is critically affected by intrinsic cues (i.e. NSCs isolated along the dorsoventral axis of the SVZ generate cells retaining their original site-specific behavior even after heterotopic transplantation (Merkle et al., 2007). Certainly additional studies are required to analyze the instructive role of the microenvironment and the cell autonomous vs. cell non-autonomous determination of NSCs.

We can distinguish three layers in the dentate gyrus: the molecular layer is the closest to the cortex, mainly formed by dendrites and axons; just below there is a structure forming a characteristic "U" shaped structure named granule cell layer, formed by the bodies of the granule neurons; underneath the granule cell layer lays the hilus, composed of the axons of the granule cells and interneurons. Adult newborn neurons of the hippocampus develop from stem cells located in the subgranular zone lining the granule cell layer (Kriegstein & Alvarez-Buylla, 2009). Here the multipotent neural stem cells undergo intermittent cycles of division that originate proliferating precursor cells with a progressive limited renewal potential, which then differentiate into various lineages.

The primary multipotent stem cells of the neurogenic niche, called type 1 cells (or radial glia cells), are slowly dividing but they have an unlimited self-renewal capacity and are positive for some neural stem cell markers such as Nestin or Sox2 (Seri et al., 2001). They project a single process that crosses the granule cell layer and ramifies into the molecular layer. Upon division, type 1 cells differentiate into type 2 cells (or non-radial precursor cells), which are more proliferative intermediate neuronal progenitor cells with only short processes (Suh *et* al., 2007*)*. We can consider type 2 cells as the transition phase between multipotency and lineage specialization. Actually, these cells are further

subdivide into type 2a cells, mitotic and multipotent cells which still express some neural stem cell markers, and type 2b cells, lineage-committed proliferative neuronal precursors which start to express immature neuronal markers (i.e. doublecortin) (Steiner et al., 2006). Type 2b cells then lead to type 3 cells (or neuroblasts), which also can proliferate but don't express anymore any stem cell markers and give rise to mature neurons (Filippov et al., 2003; Fukuda et al., 2003; Kempermann et al., 2004; Zhao et al., 2008). Every single stages is finely modulated by signals within the niche: recent experimental evidences have enlightened the critical importance of key molecules involved in the regulation of neurogenesis both during embryonic development and in adult brain. For example, signaling mediated by Bone morphogenetic protein (BMP) and Notch is essential to regulate the balance between quiescent and proliferative neural stem cells, as well as specific growth factors (i.e. Fibroblast Growth Factors or Brain Derived neurotrophic factor) may also play a pivotal role in regulating NSC behavior in the adult brain (Palmer et al., 1995; Ables et al., 2010; Lugert et al., 2010; Mira et al., 2010, Paradiso et al., 2009). However, the mechanism to which these pathways act together and integrate into a common endpoint is still unknown.

Similar to embryonic and early postnatal development, adult-born neurons are generated in excess: about half of them fail to survive and to integrate into pre-existing neural networks. The continuous production and elimination of cells in the DG vouch for the dynamic ability of the brain to remodel discrete networks throughout the entire lifespan (Aasebø et al., 2011). New neurons are specifically selected for integration, a process that seems to be activity-dependent (Kempermann et al., 1997; Tashiro et al., 2006). However, we still do not know which types of signals are necessary and sufficient to enhance the survival and integration of new neurons. Similarly, it remains unclear why new neurons show an increased excitability compared with mature neurons.

In the rat, neurogenesis occurs in the subgranular zone, that lies within the first 50–100 μm of the granule cell layer, and new neurons are thought to derive from radial glia which in turn divide into more differentiated cells that ultimately become dentate gyrus granule cells (Seri et al., 2004). Newly born granule cells can functionally integrate into hippocampal circuitry (Jessberger and Kempermann, 2003), resembling other granule cells both for morphology and for electrophysiological properties (van Praag et al., 2002).

Even though promising new data show that newborn neurons generated into the DG can integrate into the hilus and CA3 region of hippocampus (Toni et al., 2007), further experiments will be essential to understand the cellular mechanisms that underlie the

function of new neurons in adult brain behavior.

1.2.3 Neurogenesis and epilepsy

Even though there is to date no proof for the relevance of adult neurogenesis in brain diseases, the finding that NSCs persist in the adult brain and constantly generate new neurons may represent a novel therapeutic target in a number of neuropsychiatric and neurological disorders. Furthermore, NSCs may not only be disease-relevant or a therapeutic target in the two neurogenic areas: under certain conditions (such as ischemic stroke), it has been shown that new neurons are generated within normally non-neurogenic areas such as the striatum (Arvidsson et al., 2002).

The proliferation and integration of newborn cells in adult brain is highly sensitive to environmental stimuli (Kempermann et al., 1997) and, in particular, can be strongly affected by physiopathological events. For instance, neurogenesis drastically increases upon induced epileptic seizures in rodents (Parent et al., 1997; Scharfman et al., 2000). However, increasing evidence suggests that aberrant neurogenesis might concur for abnormalities both in human and experimental model of TLE, such as mossy fiber sprouting, dentate gyrus cells (DGCs) layer dispersion, and the appearance of DGCs in ectopic locations or with abnormal hilar basal dendrites (Kron et al., 2010). In contrast, other work suggests that newborn DGCs that integrate normally during epileptogenesis may serve a compensatory role to restore inhibition (Jakubs et al., 2006).

Experimental evidences on animal model of epilepsy showed that the most affected neural stem cell population by kainic acid-evoked seizures are cell type 1, 2a and 3; the administration of proconvulsant drugs wreaks drastic effects on adult neurogenesis and on morphology and localization of the newborn cells, with dispersion of granule cell layer (Steiner et al., 2008, Jessberger et al., 2005; Parent and Murphy, 2008; Scharfman et al., 2000, Steiner et al., 2008).

Interestingly, some attempts addressed to induce neuron production in neocortical areas or to reprogram glial cells into neurons have been successful carried out (Magavi et al., 2000; Heinrich et al., 2010).

Regarding epilepsy, the increased generation of new functional neurons to replace the neuronal loss due to epileptic seizures may exert a therapeutic effect, even though the achievement of this goal appears much more difficult than previously anticipated (Scharfman, 2004).

As discussed before, the dentate gyrus is the primary site in the temporal lobe where the majority of neurogenesis is thought to occur in normal adult brain, and the temporal lobe is the most epileptogenic region of the brain. The proliferation rate of neuronal progenitor cells is not steady but is modifiable by environmental and pathological conditions. In particular, neuronal activity exerts a strong influence on proliferation rate: prolonged neuronal depolarizations or repetitive discharge significantly increases the neurogenesis rate in the dentate gyrus, highlighting the strict relation between seizure activity and increased neurogenesis (Bengzon et al., 1997). Indeed, a bilateral increase of neurogenesis has been demonstrated in rodents following status epilepticus evoked by administration of chemoconvulsivant such as pilocarpine (Parent et al., 1997) or kainic acid (Gray and Sundstrom, 1998), but even after amygdala kindling (Scott et al., 1998) or electroconvulsive shock (Madsen et al., 2000). Seizures can also modify the survival of new neurons in a severity-dependent manner (Ekdahl et al., 2001).

1.2.4 Astrocytes and epilepsy

Astrocytes have a prominent role to ensure the central nervous system to work properly by regulation of critical transmitter-signaling pathways including c–aminobutyric acid (GABA), adenosine, and glutamate through regulation of extracellular neurotransmitter levels responsible for fast excitatory and inhibitory signaling in the central nervous system. Consequently, these cells have the potential to modulate synaptic transmission, neuronal excitability, and the generation of ictal discharges. Failing to maintain the right extracellular concentration of these amino acids may results in ruinous effects on neuronal survival and functionality (Rothstein et al., 1996). Although it is commonly accepted that the main cause of epilepsy is an over-excitation of a brain specific neuronal population, the primary involvement of astrocytes in causation of seizure activity becomes undeniable. Astrocytosis and microglial activation are indeed well described features of temporal lobe epilepsy (TLE), confirmed by several studies that suggest their decisive contribution to epileptogenesis (Briellmann et al., 2002; Vessal et al., 2005; Kang et al., 2006; Binder et al., 2006). Upon an epileptogenic insult occur, hypertrophied astrocytes in the dentate gyrus form an ectopic glial scaffold that promotes the aberrant growth of basal dendrites into the hilus (Shapiro et al., 2006). These basal dendrites are targeted for synaptogenesis by mossy fibers (Ribak et al., 2000) and contribute to a recurrent excitatory circuit that may facilitate seizures (Austin and Buckmaster, 2004).

Astrocytes are also involved in metabolism and regulation of neurotransmitter levels. Synaptically released glutamate is normally taken up by glial cells and converted in non-toxic glutamine, essential for neurons as a renewable source of neurotransmitter (Meldrum et al., 1999), by glial-specific enzyme glutamine synthetase (Rothstein et al., 1996). As found in the hippocampus of patients with mesial temporal sclerosis, the expression of the astrocyte-specific enzyme glutamine synthetase (GS) is strongly decreased, leading to a misregulation of glutamate/glutamine rate. GS converts glutamate to glutamine, an essential amino acid supplied to neurons as a renewable source of neurotransmitter (such as the chief inhibitory neurotransmitter GABA). Brain-slice studies showed that selective reactive astrocytosis and the loss of GS leads to a reduction of synaptic inhibition (Ortinski et al., 2010), raising the possibility that astrocytes can contribute significantly to the genesis of epilepsy. Astrocyte and microglial activation are well-described features of temporal lobe epilepsy (TLE), and studies have suggested that glial cells may contribute to epileptogenesis (Briellmann et al., 2002; Vessal et al., 2005; Kang et al., 2006; Binder et al., 2006). Other studies have shown that after seizures, hypertrophied astrocytes in the dentate gyrus form an ectopic glial scaffold that promotes the aberrant growth of basal dendrites into the hilus (Shapiro et al., 2006). These basal dendrites are targeted for synaptogenesis by mossy fibers (Ribak et al., 2000) and contribute to a recurrent excitatory circuit that may facilitate seizures (Austin and Buckmaster, 2004).

Postmortem studies of patients with temporal lobe epilepsy showed that loss of glutamine synthetase is accompanied by reactive astrocytosis with alteration of protein expression that eventually leads to a reduced synaptic inhibition and increased spread of excitation (Ortinski et al., 2010). When taken together with the observation that astrocytes release chemical transmitters, the idea that glial cells might contribute to the generation of seizures has got a foothold (Wetherington et al., 2008). There are no unique mechanisms for the contribution of astrocytes to epileptogenesis, since they can trigger the neuronal hyperactivity in previously normal neurons or just promote the epileptic discharge in abnormal neurons or just fail to arrest neuronal hyperactivity (Castiglioni et al., 1990). Significant increases of astrocytosis have been detected in several brain foci related with seizure generation, leading to the concept that gliotic scar formation is a key feature of human epilepsy and, therefore, suggesting a prominent role for glia in epileptogenesis (Harris, 1975; Mazzuferri et al., 2010; Bovolenta et al., 2010).

In addition to exerting inhibitory influences indirectly through the synthesis of neuronal

GABA, astrocytes regulate the extracellular concentration of adenosine, a powerful endogenous anticonvulsant. An increase in the expression of adenosine kinase, the enzyme involved in the conversion of adenosine to AMP, has been registered in presence of reactive astrocytes (Boison, 2008); thereupon, a reduction in this endogenous anti-seizure is strictly related to reactive astrocytosis.

Moreover, astrocytes are involved in modulation of neuronal N-methyl-D-aspartate (NMDA) receptors expression, contributing to their excitation through the release of glutamate and D-serine. Astrocytic Ca^{2+} signals, which are dampened by some anticonvulsants, stimulate the release of glial glutamate, leading to neuronal excitation (Tian et al., 2005). Considering the decrease in adenosine- and GABA-dependent inhibition that happen during reactive astrocytosis, becomes easy to conceive how these events combination might trigger epileptic seizures.

1.3 HERPES VIRUSES

1.3.1 Herpersviridae classification

The *Herpesviridae* family is a group of enveloped viruses highly dispersed in most animal species.

Members of this family with human tropism are classified in 8 different subtypes:

Herpes simplex virus Type-1 (HSV-1), Herpes simplex virus type 2 (HSV-2), Varicella zoster virus (VZV of HHV-3), Epstein-Barr virus (EBV or HHV-4), Human cytomegalovirus (HCMV or HHV-5), Human herpesvirus 6 (HHV-6), Human herpesvirus 7 (HHV-7), Human herpesvirus 8 (HHV-8), Kaposi's sarcoma-associated herpesvirus (KSHV) (P. E. Pellett and B. Roizman, 2007).

All these subtypes share distinct biological properties: firstly, their genomes encode a large group of enzymes which participate in nucleic acid metabolism (such as thymidine kinase), DNA synthesis (i.e. DNA polymerase and helicase) and protein modification (i.e. protein kinase); secondly, viral DNA replication and capsid assembly take place in the nucleus, whereas the tegument association and envelope acquisition occur in the cytoplasm, as viruses exit the host cell; finally, infectious viruses can either go through a lytic lifecycle accompanied by destruction of the host cell or establish a latent state in the host cell. The latent genome can be reactivated by various stimuli, entering the lytic life cycle and causing disease upon reactivation. Humans are readily infected by these viruses and often are positive for five or more of these viruses in their lifetime. On the basis of host-cell range, length of replication cycle, cell type where latency is established and genomic analysis, Herpesviruses have been classified into three subfamilies: Alphaherpesviruses, Betaherpesviruses and Gammaherpesviruses (table 3).

FAMILY	NAME	GROWTH CYCLE AND CYTOPATHOLOGY	LATENT INFECTION
Alpha-herpesviruses	HSV-1 HSV-2 VZV	Short, Cytolytic	Neurons
Beta-herpesviruses	CMV HHV-6 HHV-7	Long, Cytomegalic Long, Lymphoproliferative	Glands, Kidneys, Lymphoid tissue
Gamma-herpesviruses	EBV HHV-8	Variable, Lymphoproliferative	Lymphoid tissue

Table 3: *Herpesviridiae family classification*

Members of the Alphaherpesvirus subfamily are characterized by a broad host range, relatively short lytic lifecycle, rapid spread in culture, and efficient destruction of infected cells. In addition, they encode a similar set of homologous genes arranged in similar order and can efficiently invade the peripheral nervous system moving from the infected epithelial cells to infect neurons resident in the dorsal root ganglion or cervical ganglion via retrograde transport of viral capsids (Frampton et al., 2005). The virus will persists in a latent state in the nervous systems of the host for a lifetime where the viral genome remains in an epichromosomal state associated with histones without integrating into the host genome (Steiner and Kennedy, 1993).

The Betaherpevirinae have a narrow host range and long replication cycle in infected cells. They are able to establish latency in secretory glands, lymphoreticular cells, kidneys and other tissues.

The Gammaherpevirinae generally replicate in lymphoblastoid cells, especially in T or B lymphocytes, and also establish latency in lymphoid tissues.

1.3.2 HSV-1 overview

Viral particles mediate the transfer of the viral genome and accessory proteins from an infected host cell through the production of progeny virus or from cell-to-cell spread to a noninfected host cell. Since viruses with a DNA genome must find a way to get the DNA to the nucleus, viruses use a basic strategy in which the infected cell assists the virus. To infect a target cell, a virus particle proceeds through a multistep entry process, tightly regulated in time and space. In general, this mechanism occurs via a universal set of steps involving attachment, stable binding, and fusion.

The HSV virion is designed to protect the viral genome from adverse conditions in the extracellular environment and to permit cell invasion so that the viral genome can be released to the cell nucleus to efficiently express its genes.

The Herpes Simplex Virus Type-1 virion is composed of four main elements: a linear double-stranded DNA encompassed in an electron-opaque core, an icosahedral capsid, a large proteinacous space referred as the tegument, and an envelope with glycoprotein spikes at the outermost layer of the virion (Fig.1).

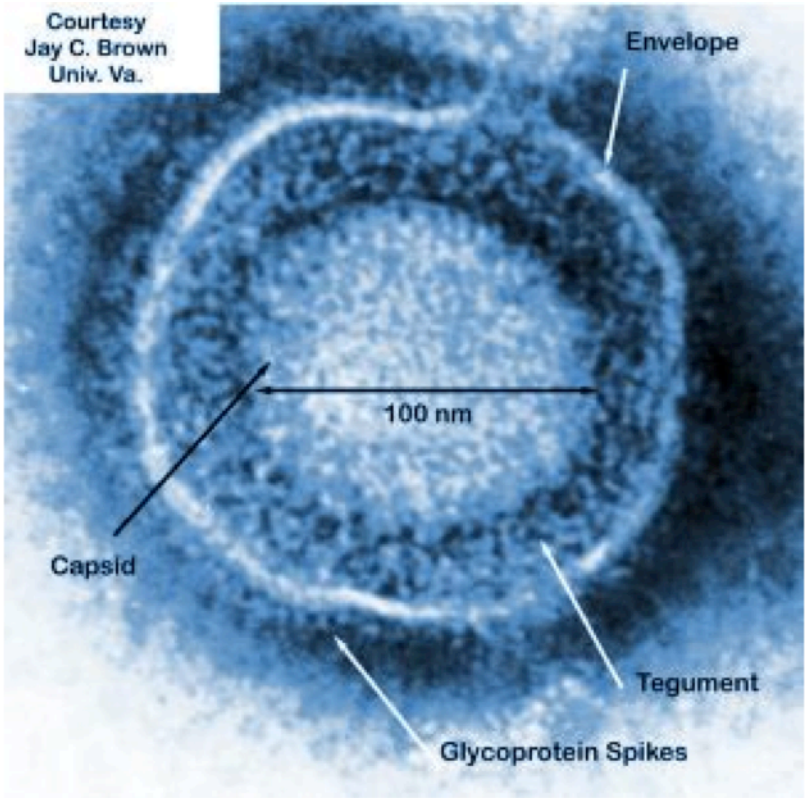


Fig. 1: Electron microscroscopy picture of HSV-1 virus

1.3.3 HSV-1 DNA genome

The genome of HSV-1 in a mature virion is a large linear and double stranded DNA (152 kilo base pair, of which 68% are G/C), and it carries at least 84 protein-encoding open reading frames (ORF) organized into a 126 Kb unique long segment (U_L) and 26 Kb unique short segment (U_S), tightly packed in a linear form that become circularized after the virus reach the nucleus (McGeoch et al., 1988). These regions are flanked by inverted repeat sequences, a terminal repeat termed TR_L and an internal repeat termed IR_L (Perry, 1988) containing sequences required for cleavage and packaging of the HSV-1 genome, termed "*a*" sites (Deiss, 1986). The U_S also is flanked by inverted repeats termed TR_S and IR_S (Fig. 2).

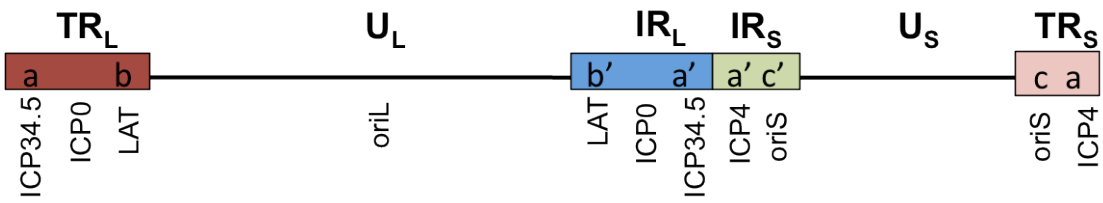


Fig. 2: *Schematic representation of HSV-1 genome*

It has been shown that the L and S units can invert relative to each other and the DNA genome produces four linear isomers of equal proportion in infected cells (Hayward et al., 1975). Moreover, the HSV-1 genome has three lytic origins of replication, two located within the U_S segment (*oriS*) and one in the U_L segment (*oriL*).

The HSV-1 replication is strictly regulated in a sequential manner: three gene classes, known as the immediate early genes (α-genes), the early genes (β-genes), and the late genes (γ-genes), are coordinately expressed following the transcriptional regulation of viral and host cell proteins (Watson et al., 1981).

The α genes don't need any viral protein synthesis for their expression but they just requires a cis-acting site (alpha TIC; with the consensus 5'-GyATGnTAATGArATTCyTTGnGGG-3') located in the promoter-regulatory domains of the alpha genes; the products of these genes are involved in the transcriptional regulation of β- and γ-genes and also can operate a positive and negative feedback regulation of α-genes (O'Hare and Hayward, 1985) though the mechanisms regulating these processes are not completely understood.

1.3.4 HSV-1 nucleocapsid

The HSV genome is packed within an icosahedral protein shell, called nucleocapsid or simply capsid, which total molecular mass is 0.2 billion daltons. The nucleocapsid displays 162 capsomers made up of four viral proteins: VP5, VP26, VP23, and VP19C (Zhou et al., 2000). Within the envelope, the nucleocapsid takes up about one third of the volume, while the tegument occupies the remaining rest two thirds of the volume. Interestingly, the encapsidation and release of viral DNA take place through a portal located within the capsid, made up by a dodecamer of the pUL6 protein (Cardone et al. 2007).

1.3.5 HSV-1 tegument

The capsid is coated with an amorphous proteinaceous filled space designated as the tegument, particularly important since is involved in transcriptional regulation of immediate early viral genes and in regulation of host-cell transcription (virion-host-shutoff protein) (Dargan et al., 1998). The tegument contains at least 20 viral proteins; some of these, such as VP16, are responsible for triggering the viral immediate early gene

expression (Campbell et al., 1984). Others contribute to creating a more suitable environment for viral replication in infected cells, such the virion-associated host shutoff protein (vhs) which degrades cellular mRNA to enhance the efficiency of viral protein translation (Kwong and Frenkel, 1989) and the protein encoded by US11, an RNA binding protein which inhibits the activation of protein kinase R inducing cellular translation arrest in host cells (McKnight et al., 1994). The tegument also includes the VP22 protein, that works like a stabilizer for important viral proteins such as gE, gD and ICP0 (Duffy et al., 2009) and is involved in viral spread during lytic infection.

1.3.6 HSV-1 envelope

Through the electron microscope, HSV-1 virions are visualized as pleiomorphic membrane-bound particles surrounded by an envelope obtained from the cytoplasmic membranes of previously infected cells. the average diameter of a spherical HSV-1 virion is 186 nm, which enlarges to 225 nm once spikes on the envelope surface are included. The lipid layer of the envelope is seen as a continuous silkily circular surface, around 5 nm thick (Grunewald et al., 2003).

The envelope consists of a trilaminar lipid membrane containing multiple viral membrane proteins with 9 different glycoproteins embedded on its surface: gB, gC, gD, gE, gG, gH, gI, gL and gM. Among these, only gB, gC, gD, gH, and gL are important for cellular attachment, fusion, and internalization of the virus (Cai, et al., 1988).

About 600-700 glycoprotein spikes have been counted on the surface of the envelope (Grunewald K, et al., 2003), non-randomly distributed since they are presented thinly at the proximal pole and compactly around the distal pole (Spear et al., 2000).

1.3.7 HSV-1 infection

HSV-1 enters host cells through a fusion event of the virion envelope with the plasma membrane. Fusion activity is triggered by receptor binding at neutral pH, inducing irreversible conformational changes that allows for viral fusion (Wittels and Spear, 1991). The virus can also spread from cell to cell by inducing the fusion of cellular membranes.

Viral entry into cells requires at least four viral glycoproteins: gD, gB and the heterodimer gH-gL. Initially, gC (WuDunn et al., 1989) and gB (Herold et al., 1994) interact independently with glycosaminoglycan (GAG) moieties of cell surface proteoglycans to promote the attachment of the virion to the host cell. Following the attachment of the

virus to the cell surface, cell entry requires the engagement of glycoprotein gD to any one of several entry receptors, to increase the stability of viral attachment and to trigger essential fusion events. Interestingly HSV gD can bind to three cellular receptors called herpesvirus entry protein A (HveA), nectin-1 (HveC) or 3-O-sulfated heparan sulfate (3-OS HS) (Montgomery et al., 1996). The binding of gD to nectin-1 induce a conformational change in this glycoprotein that enables it to interact with gB and/or the heterodimer gH-gL (Turner et al., 1998).

Following the adsorption of HSV-1 to the cell surface, the nucleocapsid is released into the cytoplasm along with the surrounding tegument proteins; these can spread into the cell in order to modify its environment for viral replication. VP1-2 localizes to the nuclear pore along with the capsid, mediating DNA translocation into the nucleus (Jovasevic et al., 2008). One of the most studied tegument proteins, VP16, also migrates into the nucleus through interaction with a cellular protein HCF-1 (Wysocka and Herr, 2003; Hancock et al., 2010). The nucleocapsid migrates towards the nucleus of the host cell (Sodeik et al., 1997); this migration rely upon the cellular microtubules and the microtubule directed motors dynein and dynactin (Dohner et al., 2002; Sodeik et al., 1997). HSV-1 genome is subsequently translocated through the capsid portal via the nuclear pore complex (Ojala et al., 2000).

1.3.8 IE gene expression

Before the viral DNA genome reaches the nucleus, it is not associated with any cellular histone proteins inside the capsid (Cohen et al., 1980). The viral genome is quickly transformed to a circular form once inside the nucleus, where it is deposited with cellular nucleosomes. It has been reported that during lytic infection, the chromatinization of HSV-1 genome occurred within 3-6h after the infection (Huang et al., 2006). The remodelling of viral chromatin by host factors and viral proteins promotes the transcription of viral genes. The first class of genes expressed during HSV-1 lytic infection are the immediate early genes, named by the initial phase of viral replication. The tegument protein VP16 is essential for viral replication since it initiates the IE gene expression and turns on viral propagation in host cells, interacting and recruiting some transcription factors and chromatin modifiers to the viral immediate early gene promoters. VP16 acts through two pathways, recruiting the RNA II polymerase to the IE promoters and promoting the active form of viral chromatin. Importantly, VP16 forms a complex with

the cellular transcription factor Oct 1 to initiate a cascade of viral gene expression (Triezenberg et al., 1988). The IE proteins are the first viral proteins expressed, requiring only the virion protein VP16 working in conjunction with the host cell transcriptional machinery for their expression. Four of the five IE proteins (ICPO, ICP4, ICP22 and ICP27) return to the nucleus after translation and play a role in regulating expression of viral and/or cellular genes throughout the course of infection. The fifth IE protein, ICP47, blocks antigen presentation and is not known to play a role in virus transcription or replication in cell culture.

1.3.9 ICP0

Infected-cell polypeptide 0 (ICP0) is a RING finger E3 ubiquitin ligase involved in inhibiting the host antiviral system, promoting the viral replication and reactivation of latency. ICP0 is a 100 kDa protein encoded by the gene α0 located in the inverted repeat sequences flanking the unique long sequence of the viral genome (Hagglund and Roizman, 2004). ICP0 is extensively processed after translation: it gets phosphorylated and nucleotidylylated by viral kinases (Blaho et al., 1993). ICP0's mechanism of action is still not well known, but several studies have shown that it works as a transcriptional activator of HSV mRNA synthesis, potentiating the function of ICP4 (Gelman et al., 1986): combinations of ICP0 and ICP4 are 20-fold more potent at driving mRNA synthesis than either ICP0 or ICP4 alone (Gelman, 1986). In addition, ICP0 is essential for viral escape to the innate interferon response (Leib et al., 1999) and, due to its nuclear localization signal (NLS) domain and its E3 ubiquitin ligase, ICP0 localizes to the nucleus early during viral replication accumulating initially at or near pro-myelocytic leukemia (PML) nuclear bodies, leading to their dispersion at early phase of infection and cotributing to the formation of adjacent, sub-nuclear replication compartments (Maul et al., 1996). Recent data have implicated ICP0 in dissociating class II histone deacetylases (HDACs) from their target genes, providing a further mechanism for ICPO-mediated transcriptional regulation (Poon et al., 2006). As the amount of protein increases, ICP0 spreads out and fills the nucleus. After viral DNA synthesis, ICP0 is found in the cytoplasm (Kawaguchi et al., 1997; Lopez et al., 2001). Late after infection, ICP0 seems to shuttle between the nucleus and cytoplasm, and it may linger in the nucleus rather than reside in the cytoplasm. Although ICP0 is not essential for viral replication in host cells, the ICP0-deletion mutant virus results in a 10-100 fold decrease in viral titer

compared to the wild type at low multiplicity of infection in the same cell line since synthesis of ICP0 causes drive HSV towards productive replication whereas absence of ICP0 produces the opposite effect. Synthesis of ICP0 is sufficient to trigger HSV reactivation in trigeminal ganglion neurons and other models of latent HSV infection (Harris et al., 1989).

1.3.10 ICP4

ICP4 is the major transcriptional transactivator of HSV-1, and is required for all post-IE gene expression (Dixon and Schaffer, 1980). ICP4 of HSV-1 is a large protein with a molecular mass of 175 kDa. ICP4 is uncoded by the two copies of the α4 gene, located in the inverted repeat region flanking the short segment (Us) of HSV-1 genome and has several functional domains, including the DNA-binding domain, at least one transcription activation domain, a dimerization domain, and a nuclear localization signal (Roizman et al, 2001). During IE infection, ICP4 is localized to the nucleus, where it exists as homodimer, and is immediately recruited to the viral genome (Everett et al., 2004). Later during viral DNA replication, ICP4 is found in the replication compartments inside the nucleus.

The structure of ICP4 consists of a DNA binding domain, a nuclear localization domain and two transactivation regions (Carrozza and DeLuca, 1996). ICP4 physically interacts with host transcriptional factors, such as TATA-binding protein, TFIIB, TFIID, and TAF250. These interactions are believed to be crucial for the ability of ICP4 to induce viral genes, inducing the transcription from promoters that minimally contain the recognizable cis-acting element of ICP4 (Shapira et al., 1987) and functioning as a regulatory protein for the activation of E and L gene expression and repressing IE gene expression. ICP4 is necessary for viral growth since it is essential for the transcription of viral early and late genes (Watson and Clements, 1980).

ICP4 increase the transcription of viral genes during viral infection and enhance the formation and the activation of transcription initiation complexes with a relatively simple set of general transcription factors (Carrozza and DeLuca, 1996). However, it is still not well known how ICP4 affects the formation of transcription initiation complexes.

1.3.11 ICP22

ICP22 is a 68 kDa protein, encoded by the α22 gene in the unique short region of the

viral genome, containing two nuclear localization signals within its structure (Stelz et al., 2002). Although the exact mechanism of repression remains unclear, ICP22 which may have multiple functions in viral proliferation but the most important is the transcriptional regulation of cellular and viral mRNAs which mediates changes on the host RNA polymerase II. This results in the inhibition of the transcription of many cellular and viral gene that precedes an increased expression of some late proteins (e.g. USll, UL38 and UL41) and the destabilization of host cell cycle proteins (e.g. cyclins A and B), leading to cell cycle deregulation and reducing the antiviral response (Orlando et al., 2006).

1.3.12 ICP27

The fourth IE regulatory protein is ICP27, expressed from the UL54 open-reading frame of the UL of HSV. ICP27 is a regulatory phosphoprotein which has a predicted molecular mass of 55kDa that becomes 63 after post-transcriptional modifications (Smith et al., 2005). Like ICP4, ICP27 is required for the productive cycle and activates E and L gene expression (Uprichard and Knipe, 1996). ICP27 has been reported to play a number of roles in the regulation of gene expression, including stabilizing mRNA, disrupting spliceosomes and inhibiting splicing, and transporting viral mRNAs to the cytoplasm (Bryant et al., 2001). It has also been shown to shuttle between the nucleus and cytoplasm and to bind to RNA lending support to the proposed role for ICP27 in mRNA export (Soliman et al., 1997). At early times post-infection, ICP27 is detected diffusely throughout the nucleus but it then moves to replication compartments (Mears and Rice 1998).

1.3.13 ICP47

ICP47 is the only IE gene that has not been shown to play a role in regulating viral and/or cellular gene expression. This 9 kDa protein instead plays a role in modulating the host immune response to infection by blocking translocation of antigenic peptides into the endoplasmic reticulum (Fruh et al., 1995). ICP47 binds specifically to transporters associated with antigen processing (TAP), blocking peptide-binding and translocation by TAP as well as subsequent loading of peptides onto MHC class I molecules in the endoplasmic reticulum. Therefore, infected cells are masked for immune recognition by cytotoxic T-lymphocytes (Ahn et al., 1996). ICP47 is not essential in tissue culture, but is essential for neuroinvasiveness in vivo (Burgos et al., 2006).

1.3.14 Virus egress

Two main models have been proposed to explain the egress newborn virion from the cell, as it is still unclear how this process occurs. In one model, the enveloped particle fuses with the outer nuclear membrane, thus releasing the capsid into the cytoplasm. The capsid then buds into the secretory pathway via the Golgi network, where it reacquires an envelope and the associated glycoproteins. Evidence for this model comes from electron microscopy studies that have shown the clear presence of non-enveloped capsids in the cytoplasm (Siminoff and Menefee, 1966). However, it is not known if these capsids eventually exit the cell, or if they are an aberration. A most recent model proposes that, upon exiting the nuclear membrane, the virion acquires a second envelope. This second envelope is lost upon fusion and release into the secretory pathway (Enquist et al., 1998). From that point on, the two models are similar, with the virion released from a secretory vesicle into the extracellular space.

1.3.15 Latency

Up today, the exact mechanisms of HSV-1 latency are not yet completely understood. During a latent infection the virus is in a relative quiescent state where the transcription is limited to a single region of the viral genome and only a group of latency-specific RNAs are detectable in the nuclei of neuronal infected cells. Only one region of the viral genome, known as the LAT locus, is actively transcribed during latency, due to the presence of a latency-associated promoter (LAP) that remains active during this phase of the infection, resulting in the synthesis of non-messenger RNA molecules of unknown function (the latency-associated transcripts, or LATs), which accumulates in the nucleus of the latently infected neurons (Farrell et al., 1991). Very recently, the LAT locus has been shown to express miRNA molecules that can downregulate expression of lytic viral genes (Umbach, et al., 2008). The mechanism of reactivation is still uncertain, although several models of reactivation are proposed: the causes of viral reactivation usually result from some stimulus that can be linked to stress (physical of psychological) trauma, pneumococcal or meningococcal infection, UV irradiation, fever, hormonal changes or other unknown causes.

Upon reactivation, virus travels via anterograde transport from the infected neurons back to the epithelium that is innervated by the nerve endings. This is usually the site of

primary infection such as the lip or can be to other innervated areas such as the eye. This reactivation could lead to the formation of an ulcer caused by the cell to cell spread and replication of the virus. This, while relatively benign on most areas of the face, can have serious consequences in the eye. In rare cases virus can move from the peripheral nervous system to the central nervous system resulting in encephalitis, meningitis and bells palsy all of which, if left untreated, can result in death of the patient. Fortunately this rarely occurs and the factors that predispose a person to encephalitis are not known. Thus, two of the most important cellular targets in HSV disease are epithelial cells of the mucosa (replication and spread), and neurons (for latent infections).

1.4 GENE THERAPY

1.4.1 Overview

Loosely defined, gene therapy is the transfer of genetic material either from a virus, naked DNA or RNA, or liposomes to specific cells in order to intercede in cell fate control (i.e. restoring protein production that is absent or deficient due to a genetic disorder) and to treat a certain disease state.

With the advent of the human genome project and recent advances in stem cell technology, this fairly new field is destined to become the best bet for new treatments of life threatening diseases, after the residual technical problems will be overcome. For a successful gene therapy is necessary to have a suitable disease model, specificity of gene delivery, efficient gene transduction, and limited or null toxicity. The biggest limiting step for these criteria is the effective targeting of specific affected cells within a myriad of cellular receptors while avoiding or minimizing toxic effects to normal tissue. Recent efforts have shown that retargeting of viral vectors is possible through genetic modification of the nucleocapsid and glycoprotein coat of different viruses mainly for cancer therapy (Kritz et al., 2007; Morizono et al., 2005; Uchida et al., 2012). Removal of toxicity is commonly achieved by the deletion or the modification of essential viral gene functions (Bozac et al., 2006, Hong et al., 2006).

Once these processes have been optimized for efficient large-scale manufacture the full potential of gene therapy can be realized.

1.4.2 Vectors for gene therapy

Gene transduction is the process of expressing an ectopic gene in specific cells. The vector has the role in delivery of the genetic payload to the target tissue and, also, to protect it from degradation mediated by host immune surveillance. Vector mediated gene transfer can be carried out by one of two ways either viral or non-viral.

Most non-viral methods are composed of synthetic carriers such as liposomes, polyplexes, nanoparticles, or alternatively naked DNA injection.

Viruses are naturally derived vehicles that offer the advantage of tissue tropism as well as large genetic payload.

Interesting pre-clinical and clinical studies has shown the potential of genetic therapies (Kohn et al., 2003, Cavazzana-Calvo and Fischer, 2007): encouraging results came from

clinical trials for the treatment of monogenetic diseases (Bainbridge et al., 2008; Aiuti et al., 2009). However, recent studies have shown that other avenues can be explored such as chronic pain, bone regeneration, oxidative injury and cancer therapy (Atencio et al., 2004, Heavner et al., 2007).

Gene transduction is the process of expressing an ectopic gene in specific cells. The vector has to deliver the genetic payload to the target tissue and, also, to protect it from degradation mediated by host immune surveillance. Although the basic principle of gene therapy is quite simple, successes rely considerably on the development of the gene transfer vectors. Over the years, a number of gene transfer vehicles have been developed that can roughly be divided into two categories: non-viral and virus-based gene delivery systems. Most non-viral gene delivery systems depend on direct delivery of genetic information into a target cell and include direct injection of naked DNA or encapsulation of DNA with liposomes, polyplexes or nanoparticles. Although these delivery systems exhibit low toxicity, gene transfer in general is inefficient and often transient (Huebner et al., 1999). Non-viral vectors are promising, but still big challenges regarding gene-transfer efficiency need to be engaged (Boulaiz, H., 2005; Rolland, 2006). For this reason, development of gene therapy elicited greater progresses during last years especially using viral vectors, which have been the first choice for clinical trials (Edelstein et al. 2004).

Viral vectors make it possible to dramatically improve the transfer efficiency and to control the number of recombinant DNA molecules introduced in the genome of a transgenic cell.

This explains why viruses have had to consistently develop into efficient vehicles for gene transfer. Gene therapy based on viruses shows great promises for both basic research and therapeutic applications and has been the focus of many institutions and each vector system has their own intrinsic limitations and advantages. Several groups all over the world are making huge efforts to improve at the best the features of candidate virus to get the perfect vector.

It is important to choose the best vector for each specific application. Several factors must be considered when making this decision such as the route of delivery, the duration of gene expression, the viral tropism and its toxicity.

1.4.3 Retroviruses & Lentiviruses

Retroviruses are one of the main tool of current gene therapy approaches. Following entry into the cell, the viral RNA is reverse transcribed into linear double-stranded DNA and transported to the nucleus, where it becomes integrated into the host genome. In retroviral/lentiviral vectors, essential viral genes are replaced by the gene of interest, which is flanked by the virus long terminal repeats (LTR) and packaging signal. Vector production utilises packaging cell lines that express the deleted viral genes from separate plasmids to minimise the possibility of generating replication-competent virus through recombination. Retroviral vectors derived from Moloney murine leukemia virus (MoMLV) have limited applications as delivery vectors to the nervous system, due to their inability to deliver genes to non-dividing cells and thus, post-mitotic neurons. They have, however, been extensively used for *ex vivo* transplantation strategies and delivery to neural precursors.

The majority of retroviral vectors are based on the betaretrovirus Moloney murine leukemia virus (MoMLV). The MoMLV genome encodes for three essential genes: *gag*, *pol*, and *env*. The *env* gene is required to produce transmembrane subunits for the binding to cell surface receptors of the target cell. Once inside the cell, the viral RNA is reverse transcribed into linear double-stranded DNA by the *pol* gene and subsequently transported to the nucleus for integration into the host genome. All that is required for viral replication are the 3' end and 5' LTRs, which contain the sites of DNA transcription initiation and polyadenylation. These sites are essential for production of progeny viral genomes and viral mRNA. A packaging signal termed psi is also located near the 5' repeat sequence which directs incorporation of viral DNA into the capsid. As the viral DNA is packaged, the *gag* gene directly binds to the viral RNA to promote packaging into the virion.

Retroviral vectors can either be replication-competent or replication-defective. In retroviral replication-defective vectors, the three essential genes just described above are deleted and replaced by the gene of interest, which is flanked by LTRs and packaging signal. These viruses are capable of infecting their target cells and delivering their viral payload, but then fail to continue the typical lytic pathway that leads to cell lysis and death. In this case, for retroviral-vector production are needed packaging cell lines that express the deleted viral genes from separate plasmids to minimize the possibility of generating replication-competent virus through recombination: typically the propagation

take place in murine based cell lines that provide the missing essential genes *in trans*. These cell lines have been developed to complement for env, gag, and pol genes on separate plasmids to reduce the possibility of generating replication competent retroviral vectors (Markowitz et al., 1988).

The primary limitation to the development of this system for gene therapy involves the requirement of actively dividing cells for transduction: systems based on simple retroviruses cannot deliver genes to quiescent cells because a complex of a retrovirus provirus with internal viral proteins is incapable of entering the cell nucleus with the intact cell membrane. Hence, infection of quiescent cells with simple retroviruses is aberrant and does not lead to provirus integration in the genome of an infected cell. As a result, non-dividing cells such as neurons are very resistant to infection and transduction by retroviruses. The main drawback to this system of gene transfer is the issue of insertional mutagenesis due to integration into the host genome might lead to cancer or leukemia (Herzog and Arruda, 2003). Despites these limitations, retrovirus-based systems are now most widely used to deliver various genes to higher animal and human cells in vitro and in vivo, since their stable integration in the infected cell genome can achieve high efficiency of transduction and long-term expression. In addition, they have been used in a number of FDA-approved clinical trials (Cavazzana-Calvo, et al., 2000).

Compared to retroviruses, lentiviral-based vectors have the big advantage to produce long-term transgene expression by integration into the genome of both dividing and non-dividing cells, including neurons and induce a minimal inflammatory response when introduced into the brain (Brooks et al., 2002). However, the use of these vectors don't reduce the risk of insertional mutagenesis by activation of cellular proto-oncogenes, even though this risk is lower for neurons then glial cells, which retain a mitotic competence. The viral safety could be increased by directing integration to specific target sites through the use of viral integrase fused with a sequence-specific DNA-binding protein (Tan et al., 2004) or through the use of integration-defective LV vectors (Saenz *et al.*, 2004). The complex genome and replication cycle of lentiviruses have made the development of vectors and stable packaging cell lines difficult. Moreover, the restricted host range, low titres and pathogenic characteristics of HIV-1 have limited its use.

1.4.4 Adenovirus

Adenoviruses are the largest non-enveloped icosohedral viruses containing double-

stranded DNA that causes endemic and epidemic respiratory and intestinal infections in humans. The virions bind to the coxsackie-adenovirus receptor and integrins on the plasma membrane and enter the cell by receptor-mediated endocytosis (Harrison, 2010). The acidification of the endosome allow the nucleocapsid release into the cytoplasm and the linear DNA enters the nucleus through the nuclear pore, where it remains in an episomal form. The adenoviral genome is a linear, double-stranded DNA (dsDNA), varying from 26 to 40 kb in length, depending on the subtype. (Campos and Barry, 2007). This linear form is organized into a compact, nucleosome-like structure within the viral capsid and is known to have inverted terminal repeat (ITR) sequences (103 base pairs in length) on each end of the strand (Douglas, 2007). The viral genome has two major transcription regions, termed the early region and the late region, and two inverted terminal repeats at both end of the strand. Gene expression proceeds in a cascade fashion, with activation of early genes followed by the onset of viral DNA replication and subsequently expression of late genes, leading to the production of infectious viral particles.

Adenoviruses, in particular Human adenovirus serotypes 5 (Ad5), have been attractive candidate for use as a vector in basic studies and gene therapy (Morgan and Anderson, 1993; Crystal, 1995), since their genome is relatively easy to manipulate by recombinant DNA techniques, the viral particle is fairly stable and they can be grown to high titer (Kanegae et al., 1994). Moreover, they have a broad host range and a variety of different cell-type specificity, independently from cell division. As opposed to lentiviruses, adenoviral DNA does not integrate into the genome and is not replicated during cell division. This is a double-edged sword, since it limits their use just for transient expression assays, although adenoviral vectors are occasionally used in *in vitro* experiments. Adenoviruses are able to host moderate DNA inserts (up to 8 kb) but some viral proteins are expressed after administration into the host and, being one of the most common human pathogen, *in vivo* delivery may be hampered by prior host immune response.

1.4.5 HSV-based vectors

Due to their permissive characteristics, HSV-1, HCMV and EBV are the mostly chosen herpes virus subtypes for the production gene delivery vectors. However, the ability to infect a broad host range, the natural neuronal tropism and especially the wide

knowledge of its biology have made HSV-1 the first choice for the development of new HSV-based viral vectors.

As mentioned before, we can distinguish two serotypes of HSV, HSV-1 and HSV-2. Lesions caused by HSV-1 strains cannot be distinguished from those caused by HSV-2 but there are distinct genetic and biological differences between members of the two serotypes. For example, although both HSV-1 and HSV-2 can infect either oral or genital sites, HSV-1 is more likely to reactivate frequently from oral sites and HSV-2 is more likely to reactivate from genital sites (Lafferty et al., 1987). The most common manifestations of HSV infection are mucocutaneous lesions, commonly called cold sores or fever blisters if they occur on or near the lips.

One advantage of using HSV-1 as viral vector is the availability of several animal models that support HSV-1 infection and latency include the mouse, rat, hamster, guinea pig, rabbit, dog, and cat.

A variety of HSV-1 vectors have been developed for use as gene transfer vehicles. The main differences are in the number of genes mutated or deleted in the host strain.

1.4.6 Wild-type HSV-1 vectors

Wild-type HSV-1 induces encephalitis at very low titers when injected intracranially. Thus, these vectors are unlikely to be useful for direct intracranial injection. One potential use of vectors based upon wild-type strains is that they are transported to the central nervous tissues when injected peripherally (Deshmane et al., 1995), similar to a natural infection.

However, since attenuated HSV-1 strains can also reach the nervous system after peripheral injection (Coffin et al., 1996; Marshall et al., 2000; Perez et al., 2004), it is not likely that vectors based upon wildtype strains will be suitable for *in vivo* applications. Some non-neurotropic viral vectors can be transported to the CNS after peripheral inoculation, but the efficiency compared to HSV-1 has not been examined (Azzouz et al., 2004; Wong et al., 2004).

1.4.7 Replication defective vectors

Expression of viral genes is generally toxic to cells, as the central purpose of viral gene expression is to redirect cellular activities towards replication of the virus. This applies to HSV-1 (Lilley et al., 2001). For example, the function of the HSV-1 *vhs* gene is to prevent expression of host genes (Smiley, 2004), which has an obvious negative effect on an

infected cell. This has obvious effects on the health of an infected cell.
However, an ideal gene transfer vector will not be cytotoxic. Thus, studies have been carried out to determine the most effective way to prevent viral gene expression in order to limit toxicity. The herpesviruses share a common mode of control of gene expression where genes are expression in a temporal order.
Immediate-early genes are the first to be expressed and one of their primary functions is to transactivate expression of other genes. Thus, one strategy to block expression of most viral genes is to mutate these genes, thus preventing the transactivation of all other genes (Glorioso and Fink, 2004).
Each of the five immediate-early genes (ICP 0, ICP 4, ICP 22, ICP 27, ICP 47) has been mutated in the various HSV -1 vector strains created to date (Chiocca et al., 1990; Goins et al., 2001; Howard et al., 1998; Huard et al., 1997; Krisky et al., 1998; Lilley et al., 2001). One or more immediate early genes are usually deleted. The immediate-early genes are either essential for replication *in vitro,* or their deletion can result in lower titers (the exception being ICP47, which is only required for *in vivo* replication). In order to be able to produce these vectors, complementing cell lines have been created in order to provide the necessary viral genes in *trans.*

1.4.8 Replication defective vectors

Viral gene expression is generally toxic to cells, since the virus aims at redirect cellular activities towards its own replication preventing the expression of essential genes of the infected cells (Smiley, 2004).
However, an ideal gene transfer vector must not be cytotoxic. Thus, studies have been carried out to determine the most effective way to prevent viral gene expression in order to limit toxicity.
Gene expression in HSV is activated in a specific temporal order (Honess and Roizman, 1974). Since immediate-early genes are the first to be expressed and one of their primary functions is to transactivate expression of other genes, their inhibition could be the best way to prevent the transactivation of all other genes (Glorioso and Fink, 2004).
Each of the five immediate-early genes has been deleted or mutated in the various HSV -1 vector strains created to date (Chiocca et al., 1990; Dobson et al., 1990; Goins et al., 2001; Howard et al., 1998; Huard et al., 1997; Krisky et al., 1998; Lilley et al., 2001; Puskovic et al., 2004). The deletion of these genes make the virus unable to replicate *in*

vitro in “regular” cell lines. To overcome this problem, complementing cell lines have been created in order to provide the necessary viral genes in *trans* making possible to get viral titers comparable to wild-type or replication attenuated vectors (DeLuca et al., 1985; Samaniego et al., 1995; Marconi et al., 1996). The deletion of viral genes make possible the insertion of larger transgene into the viral genome of this replication-defective vectors. The drawback of this system is the possibility for the virus of reacquiring the complementing viral genes inserted *in trans* in the complementing cell lines by recombination with the homology sequences cloned in the cell genome reverting to a replication competent virus after several passages *in vitro*; these virus stocks need to be periodically screened to check for wild-type revertants. However, as many of these vectors have been deleted for different essential genes, there are only very few probability of complete reversion to wild-type.

1.4.9 Replication attenuated vectors

The HSV genes can be categorized in essential or non essential for viral replication. Essential genes are required to produce new infectious viral particles in permissive cell culture infections. Non essential genes are only required for optimum lytic replication or affect the natural life cycle of the virus in vivo, contributing to host range, pathogenesis, or latency. HSV-1 needs about half of its genes for replication *in vitro*. However, many more genes are required for replication *in vivo* (i.e. thymidine kinase, ICP6, ICP 34.5); these genes have been identified to attenuate the ability of the virus to replicate in animals, while preserving the ability to replicate to high titers in cultured cells (Lilley et al., 2001). Since these viruses don’t need to be grown on complementing cell lines, the probability of reversion to wild-type is low.

1.4.10 Amplicons & BAC

For several years, HSV vectors have been routinely generated through extremely tedious and long processes depending on rare recombination events in susceptible eukaryotic cells and the co-transfection of these cells with HSV DNA and a plasmid containing exogenous sequences flanked by viral sequences homologous to the insertion site. This process allows the deletion or mutation of the gene of interest in the virus genome, but unfortunately the deletion of essential viral genes is not always possible because of the strong selection for progeny viruses that efficiently replicate *in vitro* and can results in

compensatory mutations on the viral genome after several virus passages. These problems have been partially solved with the creation of transcomplementing cell lines that compensate for the absence of the deleted viral genes; this solution works well but sometimes is not feasible because of the high toxicity of HSV proteins for the transduced cells.

The solution to overcome all these obstacles was to mantain and modify the HSV genomes in bacteria, taking advantage of the accuracy of the bacterial polymerase for clonal maintenance of viral sequences and of the absence of selective pressure since the viral promoters cannot be activated in bacteria: the entire viral genome can actually be carried as an episome in prokaryotic cells, where it can be easily manipulated by prokaryotic genetic techniques and then transfected into eukaryotic cells to produce virus particles.

Amplicon vectors are HSV-1 virions carrying a concatemeric DNA plasmid instead of the viral genome but which don't differ from wild type HSV-1 regarding the structural, immunological and host range points of view. The HSV amplicon plasmid is a standard Escherichia coli plasmid containing the bare minimum of viral sequence, the HSV-1 packaging sequence, or *pac* sequence (Hershey & Chase, 1952), and an HSV-1 origin of replication, the oriS sequence (McGeoch et al., 1986). This allows for up to 150kb DNA of foreign DNA to be incorporated. It typically also house a bacterial origin of replication for propagation in E. coli, a marker gene, and transgenes of interest.

Thanks to the ability to package very large transgenic sequences, amplicons can be used to deliver complete DNA genomes, or DNA sequences that regulate chromatin structure and function or subnuclear localization. This may be useful to design improved gene therapy vectors having advantages such as prolonged, physiological and tissue-specific expression, generation of multiple splice variants from primary genomic transcripts, or synthesis of the full set of proteins required to assemble complex structures as well as metabolic or signalling pathways.

The use of amplicons as vector gene transfer take the advantage from the fact that they carry no virus genes and consequently do not induce synthesis of virus proteins and strongly reduces the risk of reactivation, complementation or recombination with latent HSV-1 genomes. Therefore, these vectors are fully nontoxic for the infected cells and nonpathogenic for the inoculated organisms. Moreover, they have the ability to infect a wide variety of cell type and they are not particular easy to manipulate for vector construction. Classically, amplicon vectors were prepared in cells transfected with the

amplicon plasmid and superinfected with helper HSV-1, which supplied the full set of proteins required to amplify and package the amplicon DNA into HSV-1 particles. As the helper virus used was generally a replication-defective mutant of HSV-1, the amplicon stocks were produced in transcomplementing cell lines (Geller et al., 1990). However, the use of defective HSV-1 as helper resulted in the production of helper-contaminated vector stocks, and the contaminant helper particles, even if defective, induced significant cytotoxicity and inflammatory responses (Johnson et al., 1992) therefore preventing their use in gene therapy of vaccination protocols. To overcome these obstacles, different helper systems that produce essentially helperfree vector stocks have been recently developed. The first of these systems was based on the cotransfection of amplicon plasmids with a set of five overlapping cosmids that supplied the full-set of transacting HSV-1 functions required to build amplicon vectors. These cosmids however lacked the pac sequences, thus avoiding packaging of the helper virus genomes that could emerge following homologous recombination between the cosmids. As a consequence, these cosmids allowed producing amplicon vectors with only very low levels of contamination with helper particles.

However, the amplicon could be packaged into virus using a helper-virus free method. A limitation of herpes amplicons packaged by either method is the inability to transcribe a transgene for an extended duration and the difficulty to prepare in high amounts than recombinant-defective vectors. *In vivo* studies show that HSV amplicons delivered to the central nervous system (CNS) can express their transgene from weeks to months depending on the promoter and expression is typically limited to a small number of cells (Jln et al., 1996; Kaplltt et al., 1994; Brooks et al., 2000).

Sometimes may happen that viral sequences inserted in high copy plasmids are toxic even for bacteria; this problem has been circumvented by use of low copy plasmids, like bacterial artificial chromosomes (BACs).

These DNA constructs have a huge capacity (up to 300 kbp) for the cloning of exogenous genetic sequences and they can be faithfully replicated in *E. coli* facilitated by components encoded in the minimal fertility factor replicon (mini-F) included in the backbone of the BAC. A typical BAC vector is about 10 kilobase pairs long and its replication initiates at the *origin of replication S (oriS*) and it is regulated by the *repE* and *repF* gene products encoded in the mini-F, necessary for BAC replication, and by genes to control the rate of replication in order to limit the copy number to one or two BACs per bacterial cell, such as parA and parB. In addition, two loxP sites are often included at

both ends of the BAC sequence so that the BAC vector can be excised out when recombinant viruses are generated (see discussion below). Moreover, many BAC vectors harbor a variety of expression cassettes that allow visualization of BAC-containing sequences in transfected cells selection in mammalian cells (such as GFP, beta-galactosidase, antibiotic resistance genes, or metabolic genes). Recent advances in bacterial artificial chromosome (BAC) technology have enabled cloning of the whole HSV genome as a BAC plasmid and subsequent manipulation in *E. coli* (Stavropoulos et al., 1998; Tanaka et al., 2003).

Due to the big size of HSV genome, the manipulation of BAC-cloned HSV DNA with conventional methods (restriction enzyme digestion or ligation) is almost impossible. To overcome this limitation, severeal techniques have been adapted to create new possibilities for the targeted mutagenesis of the viral genome in *E. coli*, such site-specific or homologous recombination and transposon integration (Horsburgh et al., 1999; White et al., 2003). This technology arouses new possibilities for the targeted mutagenesis of the viral genome (Brune *et al*., 2000; Wagner *et al*., 2002). To reduce the possibility of harmful effects for bacterial sequences on eukaryotic cells and potential transmission between bacteria and man, some herpesvirus BAC clones have been constructed cloning the BAC sequences between 2 loxP sites, allowing its removal by Cre recombinase so that the BAC vector can be excised out when recombinant viruses are generated (Tanaka et al., 2003).

1.5 LIM-HOMEODOMAIN PROTEINS

1.5.1 Lhx-protein overview

The LIM homeobox genes are widely expressed in various developmental and physiological processes including embryogenesis, development of central nerve system (CNS) and endocrine regulation. LIM-homeodomain (LIM-HD) proteins are important in CNS development (Hunter and Rhodes, 2005) and are one of the subfamilies of homeobox-containing genes found to be essential in eukaryotes (Kadrmas and Beckerle, 2004) for many developmental processes and organogenesis (Hunter and Rhodes, 2005). LIM-HD proteins are composed of two LIM domains located at N-terminal, and a DNA-binding homeodomain at C-terminal (Hobert and Ruvkun, 1998; Hobert and Westphal, 2000; Kadrmas and Beckerle, 2004). LIM domain is a zinc-binding domain (Michelsen et al., 1993; Archer et al., 1994) consists of approximately 55 amino acids with 8 highly conserved, cysteine and histidine-rich residues. Each LIM domain coordinates two zinc ions, the first four highly conserved residues bind to the first zinc ion while the other four binds to the second zinc ion to form zinc-finger structure (Kadrmas and Beckerle, 2004). This tandem zinc-finger topology of LIM domain can be linked to other protein targets for various functions (Agulnick *et* al., 1996; Breen et al., 1998; Dawid et al., 1998; Matthews and Visvader, 2003; Matthews et al., 2008).

1.5.2 LIM-homeodomain protein 2 (Lhx2)

Lhx2, a subfamily member of LIM homeobox genes, was originally identified in pre-B cell lines (Xu et al., 1993).

Lhx2 plays a pivotal role in embryogenesis, as Lhx2 -/- mouse embryos develop liver hypoplasia and die at embryo day E15–E17 (Wandzioch et al., 2004). Lhx2 is also required for the development of eye, forebrain, and erythrocyte (Porter et al., 1997) and interacts with the thyroid stimulating hormone b-subunit (TSHb) promoter, enhancing its expression (Kim et al., 2007).

One of the most important roles played by Lhx2 at the very early step of embryogenesis is to ensure the proper patterning of telencephalon by regulating a crucial boundary in the early cortical neuroepithelium. In Lhx2 -/- mouse, the cortical primordium that should give rise to hippocampus and cortex in the embryo is replaced by alternative cortical hem and anti-hem (Wnt- and Bmp-rich signaling centers that are normally restricted to the medial

extreme of the dorsal telencephalon), resulting in an expanded and mislocated cortical hem (Bulchand et al., 2001). During these early developmental stages, coordinated signalling at key boundaries between compartments finely regulates brain development. We can distinguish three distinct structures of the telencephalon involved in forebrain arrangement: the thalamic eminence, the cortical hem and the septum. Lhx2 plays a fundamental role in regulating both size and location of this dorsal signaling center structure and, moreover, to maintain neuroepithelial stem cells in a proliferative state (Roy et al., 2013).

In mouse forebrain hem system, Lhx2 expression is significantly downregulated after E10.5 to allow the structures the proper acquirement of their identity (Mangale et al., 2008). However, it has been demonstrated that Lhx2 expression is not just limited to the early embryonic neuroepithelium but continues in the telencephalic ventricular zone even after E10.5, when remains strongly expressed in the hippocampal ventricular zone with the ongoing hippocampal neurogenesis (Bulchand, 2003). During these midlate gestation phases, the progenitor cells of the CNS give rise to neurons and, further on in time, glial cells (Miller and Gauthier, 2007). Although we still don't know which are exactly the molecular pathways beyond this cell fate switch, experimental analysis identified in Notch signaling one of the most important actors involved in this process, since it is essential at early stages to keep the undifferentiated state of telencephalic progenitors (Mizutani and Saito 2005) and, later, to direct astrogliogenesis through activation the transcription factor Nfia, which is necessary and sufficient for astrocytic cell fate (Ge et al., 2002; Piper et al., 2010). Even though Notch signaling is active from early stages in the telencephalic ventricular zone, astrocytes are not generated during the neurogenic phase. In a recent study has been identified in Lhx2 a major role in repressing the astrogliogenesis selectively in the hippocampus by repression of the GFAP promoter, target of Nfia, by breaking the Notch-Nfia pathway to prevent premature gliogenesis until neurogenesis is complete (Subramanian et al., 2011).

2. MATERIALS AND METHODS

2.1 Cell lines

U2OS Human Osteosarcoma (HTB-96) and HEK 293T/17 Human Epithelial Kidney (CRL-11268) cell lines were purchased from ATCC (Manassas, VA) whereas primary Mouse Embryo Fibroblasts were purchased from Millipore (PMEF-CFL). U2OS-ICP4 complementing cell line has been generated (and kindly donated) by Yoshitaka Myagawa through lentiviral transduction of ICP4 HSV-1 gene into U2OS cells, allowing the stable expression of the transgene. All these cells were maintained at 37°C with 5% CO_2. All these cells were grown in Dulbecco's Modified Minimum Essential Medium (DMEM, HyClone) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 2 mM L-glutamine (Cellgro), 100 U/mL Penicillin (Cellgro), and 100 µg/ml Streptomycin (Cellgro). Undifferentiated mouse ES-D3 cells, generously donated by Thomas E. Smithgall, were maintained on a feeder cell layer of mitomycin C-treated mouse embryonic fibroblast (PMEF-CFL, Millipore) in Dulbecco's Modified Minimum Essential Medium (DMEM, Gibco), supplemented with 15% knockout serum replacement (KSR, Gibco), 1 mmol/L glutamine (Gibco), 0.1 mmol/L β-mercaptoethanol (Gibco), 100 mM/L nonessential amino acids (Sigma), 100 mg/ml penicillin and 100 mg/ml streptomycin (Sigma). To maintain the pluripotent status, 1000 U/ml recombinant mouse leukemia inhibitory factor (mLIF, Chemicon) has been added to the culture medium. Medium was replaced daily until cells reach confluence and they were dissociated using TrypLE Express (Gibco). To make the feeder layer, mouse embryonic fibroblasts (MEF) were cultured at 37°C and 5% CO_2 in DMEM + 10% FBS; at approximately 80% confluence, the cells were incubated with 10 µg/ml mitomycin C (Sigma) for 2.5 h and washed three times with PBS. After this treatment, about $1x10^6$ inactivated MEF were plated in 6cmø petri dishes coated with 0.1% gelatin (Sigma).

2.2 DNA restriction digest and purification from agarose gel

Plasmids, BAC DNAs and PCR products were digested with restriction enzymes (NEB), according to rre's instructions. The DNA fragments were run on a 1% agarose gel

supplemented with 0.001% Ethidium Bromide. Gel purification have been done using the MiniElute Gel extraction kit (QIAGEN), according to the manufacturer's instructions.

2.3 Ligation of DNA fragments and transformation of competent bacteria

Linearized DNA fragments were ligated together (generally at a 1:10 backbone/insert ratio) through overnight incubation with T4 DNA ligase (NEB), at 16° C.

2.4 PCR analysis

Routine PCR for detection of an amplification product or estimation of a product size was performed using Go-Taq (Promega) according to the manufacturer's instructions.
DNA fragments for subcloning were amplified by Polymerase Chain Reaction (PCR) using either High Fidelity Accuprime PFX DNA Polymerase (Invitrogen).

2.5 Chemical transformation of competent bacteria cells

DNA ligation products (about 10 ng) were chemically transformed into DH10β competent bacteria (NEB) through the following steps: a single aliquot of bacteria (50 **µ**l) was placed on ice for 30 minutes with the ligated DNA before receive heat shock (30 seconds in a water bath at 42° C); the tubes were immediately placed on ice for 5 minutes and incubated for 1 hour at 37°C with shaking at ~225 rpm upon be added with 900ul of SOC or Luria-Bertani (LB) broth (1% NaCl, 1% Bacto-Trypton, 0.5% Yeast-Extract, 0.02% NaOH). About 100 ul of the transformation mix were plated on LB-agar plates (1% NaCl, 1% Bacto-Trypton, 0.5% Yeast-Extract, 0.02% NaOH, 1.5% agar) containing the appropriate selection drug and incubated overnight at 37°C.

2.6 DNA extraction for screening of transformed bacteria colonies

Transformed bacteria colonies grown on agar plates were picked, expanded overnight in LB broth plus the appropriate selection drug and processed to extract plasmid DNA using the QIAprep Spin Miniprep Kit (QIAGEN), according the manufacturer's instructions. Quantification of DNA was performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.7 Gateway® recombination

The Invitrogen Gateway® technology employs *in vitro* site-directed recombination to clone a DNA fragment from an entry vector to a donor vector, while maintaining both its orientation and the open reading frame. This reaction exploit the LR recombination between an attL containing entry clone and an attR containing estimation vector, exchanging the region between L sites in the entry vector with the region between R sites in the destination vector, giving rise to an attB-containing Expression cloneLR reactions were performed in 10 µl, adding 100ng of entry plasmid (containing the transgene flanked by attL sites), 1ug of destination DNA vector (with attR sequences) and 1 µl of LR Clonase II enzyme mix (Invitrogen), enough to achieve an efficient recombination. Recombination reactions were incubated for 1h at room temperature and followed by Proteinase K treatment (15 min at 37°C) to inactivate the enzyme before transformation. Transformation of expression vectors was performed using 1–2 µl of recombination reaction and 40 µl of electrocompetent bacteria (ElectroMAX™ DH10B™ T1 Phage-Resistant Competent Cells, NEB).

2.8 Electroporation

The transformation of the BAC requires electroporation. Electrocompetent bacteria (ElectroMAX™ DH10B™ T1 Phage-Resistant Competent Cells, NEB) were thawed on ice for 5–10 min. For a single transformation, 1 aliquot (50 µl) of bacterial suspension was mixed in a cold transformation cuvette with 3–5 µl of the ligation product (approximately 10 ng of BAC DNA) using a Biorad GenePulserXcell electroporator (Voltage 1650 V, Capacitance 25 uF, Resistance 150 Ω, Cuvette 1 mm). Immediately after the electroporation, 1ml of SOC-medium was added and this suspension was transferred to an Eppendorf tube. The cells were incubated on a rotor at 37°C for 1 h; 100 µl of this solution were plated on an agar plate containing the chloramphenicol selection and grown at 37°C over night.

2.9 BAC minipreps and midipreps

Following electroporation and selection on agar plates, single BAC-transformed bacteria colonies were picked and BAC–DNA extracted following the “Miniprep-Alkaline Lysis for

BACs" protocol adapted from Untergasser's Lab (http://untergasser.de/lab/protocols/miniprep_alkaline_lysis_for_bacs_v1_0.htm).
The BAC-DNA was then checked for correct manipulation using both diagnostic PCR and FIGE (Field Inversion Gel Electrophoresis) analysis. Positive clones were furthermore propagated using Midipreps kit (Promega), according to manufacturer's instructions.

2.10 Transfection of BAC-DNA into U2OS-ICP4 to generate JΔβββ4 and JΔNI4 viruses

BAC HSV-1 DNAs were transfected into U2OS-ICP4 cells to ensure the ability of the BAC-DNA to produce virus. On the day before transfection, cells were plated in a 6 multiwell plate at a density of $1.2x10^6$ cells/well. 24 h later 80% confluent cells were transfected with 30 µl of BAC HSV-1 DNA using Lipofectamine LTX (Invitrogen) following the manufacturer's instructions. Cells were incubated at 37°C in 5% CO_2 in DMEM+10%FBS until viral plaques formation and spreading, observed by fluorescence microscopy for mCherry expression. Fluorescent pictures of transfected cells were taken using a Nikon Diaphot fluorescent microscope.

2.11 Viral titration in plaques forming units (p.f.u/ml)

Titration of viral supernatant was performed through infection of a 48 well of either U2OS-ICP4 with serial 10-fold dilutions of viral supernatant. The day before infection, cells were plated as a monolayer on a 48 well dish at a density of $1.8x10^5$cells/well and incubate at 37°C overnight or until monolayer is confluent (triplicates were used for statistical analysis). At this point cells were infected with serial 10-fold dilutions of viral supernatant in 120 µl of serum-free media and incubated at 37°C in 5% CO_2, shaking multiwell plates 20-30' to ensure that the virus is spread evenly over the entire monolayer. 3h after the infection each 120 µl of growth media plus 5% methylcellulose (MeOH) were added to each well. The infected cells were maintained at 33°C in 5% CO_2 until plaques formation. Since HSV-1 infected cells strongly express mCherry, 3-4 days after infection was possible to detect red expanding plaques made by dying cells. Plaques counting has been done by fluorescence microscope; the titer results were then calculated in p.f.u /ml (plaque forming units per ml of viral preparation).

2.12 Infection to test viral growth

The day before infection, U2OS-ICP4 cells were plated as a 60% confluent monolayer. 24 h later 100% confluent wells were infected at an established multiplicity of infection (MOI) in serum free media and incubated at 37°C in 5% CO_2. 2 h after the infection each well was overlaid with DMEM plus 10% FBS and maintained at 33°C in 5% CO_2 a condition that does not interfere with viral growth but stops cells from becoming over-confluent in the plate. At different time-points after initial infection, viral supernatant was collected and titered to quantify viral yield.

2.13 High scale viral production

Viruses were propagated on U2OS-ICP4 cells plated in T150 tissue culture flasks. In order to get high titer stocks, about 20 T150 flasks per virus have been used. 24 h before infection, U2OS-ICP4 were plated as a 50% confluent monolayer in order to have about 90-100% confluent cells the day after. The amount of virus for infection were established by calibrating the multiplicity of infection (MOI, between 0.01 and 0.05), in serum free media; the infected cells were incubated at 33°C in 5% CO_2.

4-5 days after infection, supernatant was collected and separated from cellular debris by centrifugation at 3000 revolutions per minute (rpm) for 10 minutes, followed by filtration through a 0.8 μm Versapor filtering membrane (PALL Corporation). The virus was then concentrated by 19500 rpm centrifugation for 45' and the viral pellet resuspended in about 250μl Phosphate-Buffered-Saline (PBS) 1X supplemented with 10% glycerol by slow overnight rotation at 4°C. The resulting concentrated virus was divided in 10/20 μl aliquots and stored at -80°C. The day after, one aliquot was used for titration.

2.14 Immunofluorescence of adherent cells

Cells for immunofluorescence labelling were cultured at sub-confluence in 6- or 12-wells. Cells were washed from supernatant and fixed for 30' at RT. Upon permeabilization of membranes (0.1% Triton X-100, 10', RT), cells were incubated with blocking solution (10% horse serum in PBS) for 1h and consequently incubated O/N at 4°C with Lhx2 primary antibody (goat polyclonal, santa cruz, 1:100). The day after, upon 3x washing in PBS, cells were incubated with secondary antibody (488 donkey anti-goat, Invitrogen, 1:1000 or 594 rabbit anti-goat, Sigma, 1:2000).

2.15 Western blot

To extract proteins, adherent cells were washed from their supernatant and incubated on ice with pre-chilled RIPA buffer for 10'. Cells were then scraped from the bottom of the well, moved to pre-chilled tube and sonicated 3 x 15''. Debris were removed upon 15' of centrifugation at 4°C; supernatant was used to calculate the amount of proteins through Bio Rad® protein assay, according to manufacturer's instructions. Upon polymerization of polyacrylamide separating and stacking gel, 6X protein loading buffer to protein samples was added and then boiled at 100°C for 5'. 20µg of protein samples were loaded on each lane of stacking gel and run on Tris running buffer (110 V for ~1-2 hr or until the loading buffer reached the bottom edge of separating gel). Proteins were transferred onto a PVDF membrane for 90' at 200 mA using SD Semi-dry Transblot Apparatus (Bio-Rad). The blotted membrane was incubated in blocking buffer (5% dry milk in PBS) for 1h at RT to reduce unspecific binding and finally incubated O/N at 4°C with anti-Lhx2 primary antibody (goat polyclonal, 1:200, diluted in blocking buffer). A horseradish peroxidase-conjugated antibody (donkey anti-goat, 1:6000) was then added, and secondary antibodies were detected through autoradiography using enhanced chemioluminescence (ECL Plus, General Electric Healthcare, Milwaukee, WI).

2.16 Viruses

Both the JΔβββ4 and JΔNI4- HSV vectors have been generated by engineering of the bacterial artificial chromosome (BAC) containing the HSV-1 genome from the strain KOS (KB), donated to our lab by Dr. D.A. Leib (Gierasch et al., 2006). As discussed before in section 1.4.10, the advent of recombinant approaches to manipulate BAC DNAs have dramatically increased the possibilities to generate novel HSV-based vectors, taking advantage from the wide versatility of biological features of bacteria, in which the BACs autonomously can propagate.

The Leib's BAC-DNA construct contains the LacZ reporter gene under the control of the SV40 promoter and the sequence encoding the chloramphenicol resistance protein, which allows the selection of bacteria transformed with this BAC.

In this construct the BAC sequences are located between the UL37 and UL38 intergenic regions of HSV-1 genome, flanked by *loxP* recombination sites that allow their excision by Cre recombination (Sternberg and Hamilton, 1981), in order to remove any residual bacterial sequence and, therefore, to avoid potential negative effects of on viral

replication and on cellular health.
Important modifications of this backbone have been made by Dr Yoshitaka Miyagawa (see section).

2.17 Primary cell cultures

To establish primary neuronal cultures, sterilized glass coverslip were specifically treated for cell attachment. All steps were done in sterile hood.
The day before the experiment, upon immersion for 15' in absolute ethanol, 12 mm ⌀ glass coverslips were placed under UV light for further 15' and, eventually, quickly passed through the flame before been individually put into the central wells of a 24 multiwell plate.
Hence, the sterilized coverslips were double coated: firstly, 200ul of 1mg/ml Poly-L-Ornithine solution (Sigma) was added in each well containing the coverslip and let it incubate for 1h at R/T; after washing (2X with sterile ddH2O) and drying, 200ul of 10ug/ml Laminin solution (Sigma) was further added in the same wells. The multiwell was then moved in the incubator at 37C for an O/N coating.
C57BL newborn mice pups were sacrificed at postnatal day 1 (P1). Hippocampi were carefully separated from the rest of brain tissue and washed 3X with Hanks Balanced Salt Solution (HBSS, Sigma). To achieve their disassociation, brain tissues were incubated for 30' at 37C in a solution of HBSS with 2mg/ml of the proteinase Papain (Sigma). The enzymatic reaction was stopped by washing cell pellet with Neurobasal (Gibco) + 2% FBS (Gibco), 10ul/ml Hepes (Sigma), 10ul/ml Na^+-Pyruvate (Sigma), 2ul/ml Penicillin-Streptomycin (Gibco) and 2% FBS (Gibco). The disassociation was mechanically completed by manual pipetting with a glass pasteur pipette and pelleted down for 10' at 500rpm. The pellet was resuspended with Neurobasal (Gibco) supplemented with 2% FBS (Gibco), 10ul/ml Hepes (Sigma), 10ul/ml Na^+-Pyruvate (Sigma), 2ul/ml Penicillin-Streptomycin (Gibco) and 2,5 ul/ml L-Glutamine (Gibco). To get single cells, the resuspended solution was passing through a 25G needle and a 70um cell strainer and, eventually, seeded on previously coated glass coverslips ($\sim 5x10^5$ cells/well).
The day after, the medium was replaced with Neurobasal (Gibco) supplemented with 20ul/ml B27 (Gibco), 10ul/ml Hepes (Sigma), 10ul/ml Na^+-Pyruvate (Sigma), 2ul/ml

Penicillin-Streptomycin (Gibco) and 2,5 ul/ml L-Glutamine (Gibco).

2.18 Animals

Newborn C57BL (P1) were used for in vitro experiments. Male Sprague-Dawley rats (240–280 g; Harlan Italy) were used for in vivo infusion of the vectors. All procedures were carried out in accordance with guidelines by the European Community and national laws and policies.

2.19 Pilocarpine model and HSV infusion

Male Sprague-Dawley rats (280 g, Harlan Laboratories) were injected with methyl-scopolamine (1 mg/kg) 30 minutes before the pilocarpine administration (370 mg/kg). All the animals that experienced 2 hours of status epilepticus (SE) were infused 3 days after pilocarpine with HSV vectors. Under ketamine and xylazine anesthesia (87 mg/kg ketamine, 13 mg/kg xylazine), a borosilicate glass needle connected to a perfusion pump was stereotactically implanted in the CA1 sector of dorsal hippocampus, using the following coordinates (calculated from bregma): AP (anterior-posterior) -1.7; ML (Medial-Lateral) -1.7; DV (Dorsal-Ventral) +3.2. A total of $1x10^6$ pfu of vector were injected in a volume of 2 ul at a flow rate of 0.1 ul/min. The animals were sacrificed 12 days after SE (to verify the virus toxicity) and 28 days after SE (that is 25 days after infusion of the vectors). Control animals were vehicle-injected or naïve rats.

2.20 Fixation Procedure

At different time points, anesthetized animals were sacrificed by decapitation and brain tissue was immediately collected and frozen. By criostatic microtome (Leica), 20 μm coronal sections were cut and subsequently fixed in paraformaldehyde solution, that stabilizes cell structures by introducing covalent crosslinks between proteins. The protocol used was the following:

- 30’ in paraformaldehyde 4% solution (prepared in PBS)
- 10’ in PBS 3X
- 10’ in PBS 1X

When the slices were not immediately processed, they were dehydrated through a series of graded alcohol baths of increasing concentration (5’ in EtOH 80%, 5’ in EtOH 95%, 5’ in EtOH 100%) and finally stored at -80^{o}C.

2.21 Hematoxilin and Eosin staining

Frozen coronal sections (20 μm thick) were cut at the level of the dorsal hippocampus (plate 45, Paxinos and Franklin, 2001) and mounted onto polarized slides (Superfrost slides,Diapath).

The frozen brains were subjected to standard histological procedures and stained with hematoxilin and eosin to accurately identify the regions of virus injection.

2.22 Fluoro-Jade C and NeuroTrace staining

Damaged neurons were then detected using the Fluoro-Jade C (FJ) (Schmued and Hopkins. 2000), on coronal sections cut at the level of the dorsal hippocampus (Paxinos and Franklin 2004). NeuroTrace 594 red fluorescent (NT; Molecular Probes, Inc., Eugene, OR, USA) staining has been performed after FJ staining, to identify degenerating neurons. After rehydration in 0.1 M PBS, (pH 7.4), sections were treated with PBS plus 0.1% Triton X-100 10 min, washed twice for 5 min in PBS then stained by NT (1:100), for 20 min at room temperature. Sections were washed in PBS plus 0.1% Triton X-100, twice with PBS, then let stand for 2 h at room temperature in PBS.

2.23 GFAP immunofluorescence and Neurotrace staining

After a PBS wash, frozen coronal sections were preincubated with PBS containing 0.3% Triton X-100 for 10 min. After two rinses in PBS, they were blocked for 30 min in a solution of PBS containing 5% bovine serum albumin (BSA) and 5% normal goat serum. Finally, sections were incubated overnight at 4°C with the primary antibodies, anti-GFAP (rabbit polyclonal; 1:100; Sigma). The next day, sections were rinsed twice with PBS and preincubated with PBS containing 0.3% Triton X-100 for 30 min. Then, they were rinsed with PBS again and then incubated for 3 h at room temperature with a secondary antibody mixture containing goat anti-rabbit, Alexa 594-conjugate (1:500; Life Technologies-Invitrogen). Finally, samples were washed three times with PBS, then they were stained by NT (1:100), for 20 min at room temperature. Sections were washed in PBS plus 0.1% Triton X-100, twice with PBS, then let stand for 2 h at room temperature in PBS. Coverslips were mounted using anti-fading Gel/Mount water based media (Biomeda). The specificity of immunolabeling was verified in all experiments by controls in which the primary antibody was omitted.

3.RESULTS

3.1 Lhx2 cDNA cloning into pENTR1A® plasmid

Lhx2 human cDNA (NCBI Reference mRNA Sequence: NM_004789.3), kindly donated from Elena Cattaneo's lab, was cloned in PCR4-TOPO® plasmid. The cDNA was firstly cut out from the original plasmid with EcoRI restriction enzyme and then ligated to the linearized pENTR1A® plasmid, cut as well with EcoRI. Before ligation, 5´ phosphate groups were removed from linearized pENTR1A by treatment with Alkaline Phosphatase (CIP), to prevent simply recircularising during the ligation step.

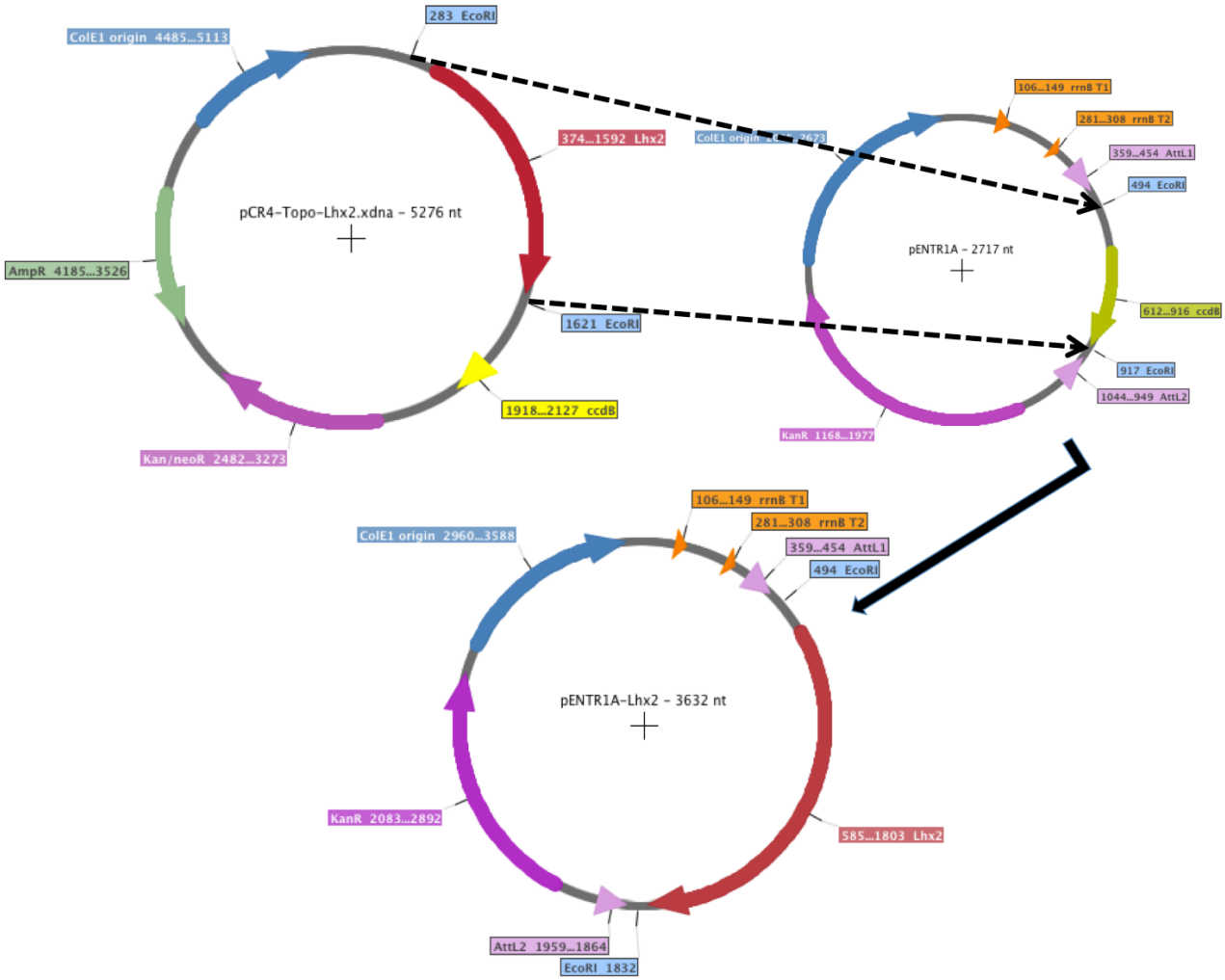


Fig. 4: *Schematic representation of pENTR-Lhx2 generation.*

The importance of cloning Lhx2 gene into pENTR1A plasmid conferred the possibility to

easily move the transgene from this entry vector to any destination vector endowed with the Gateway Cassette (see section…).

The resultant pENTR-Lhx2 plasmid was transformed into competent E.Coli; several bacterial colonies grew after plating these transformed bacteria on agar dishes with kanamycin selection, predicting the incorporation of kanamycin-resistance gene and the removal of ccdB gene (encoding for a lethal bacterial toxin, essential to avoid background) from original pENTR1A.

To check if the cloning was actually successful, it has been done a diagnostic digestion using SalI restriction enzyme that should cut once the "empty" pENTR1A and twice the pENTR-Lhx2. Every screened clone showed the expected digestion pattern after gel running of DNA fragments (Fig. 5), confirming that the gene inserted into the entry plasmid and, importantly, with the correct orientation.

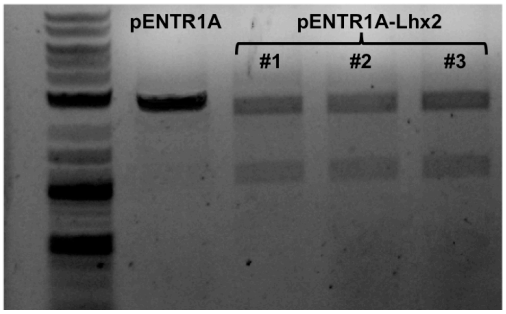


Fig 5. *Diagnostic restriction digestion of pENTR-Lhx2 with SalI, followed by DNA sequencing, verified that the Lhx2 gene was cloned into pENTR1A plasmid correctly.*

3.2 Construction of the fusion gene Lhx2-T2A-eGFP

To check Lhx2 transgene expression in living cells, a fusion gene has been created by insertion of the enhanced green fluorescent protein (eGFP) gene in-frame with Lhx2 gene, separated by the viral 2A oligopeptide (T2A) that mediates ribosomal skipping (Ryan et al., 1981, Donnelly et al., 2001). As a result, the Lhx2 and eGFP genes might be efficiently co-expressed from the same promoter but translated separated, making eGFP expression a useful marker for Lhx2 for both *in vitro* and *in vivo* studies. Three consecutive PCR reactions were used to generate the Lhx2-T2A-eGFP cassette. Lucia Mazzacurati kindly donated the BAC-DNA in which she inserted the T2A-eGFP cassette, which served as DNA template for the first PCR reaction. This reaction was run using an upper 5'-tailed primer (*Lhx2endΔSTOP-T2A*-F), complementary for the first nucleotides of

T2A but carrying a non-complementary sequences at its 5' ends, corresponding to the final part of Lhx2 gene without its STOP codon; the lower primer (*eGFPend_XbaI-EcoRV*-R) annealed to the end of eGFP gene, and it has as well a non-complementary sequence corresponding to XbaI and EcoRV restriction sites, introduced to facilitate the following cloning. This reaction produced a 0.8Kb amplicon. For the parallel PCR reaction, I used a forward primer (*Lhx2_preSalI*-F) that anneal just before the unique SalI site of Lhx2 gene (nt 1004) and a reverse primer (*Lhx2endΔSTOP*-R) complementary to the end of Lhx2 gene, but not to the STOP codon that was so deleted (the deletion of STOP codon is essential to keep the RNA polymerase transcription until the end of eGFP gene). The size of the amplicon generated with the latter reaction was 0.25Kb.

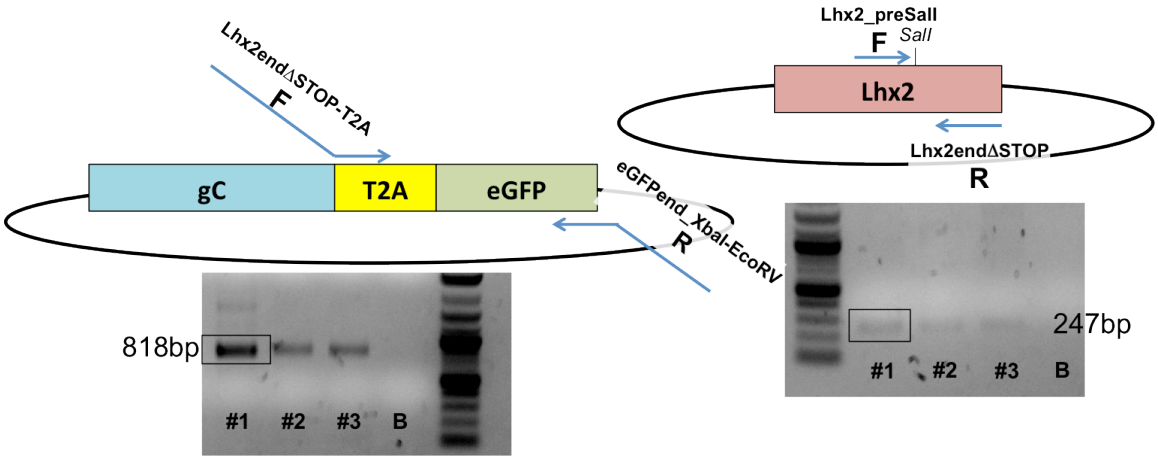


Fig. 6: *Schematic representation of Lhx2-T2A-eGFP generation. Two partially overlapping amplicons were generated by independent PCRs. These two fragment were then linked together through an overlap PCR.*

The two resultant amplicons were purified from gels and finally linked together through an overlap PCR, by using two of the four primers involved in the previous PCR (*Lhx2_preSalI*-F and *Lhx2endΔSTOP*-R). The DNA fragment SalI_Lhx2end-T2A-eGFP_XbaI-EcoRV has been finally got (1 Kb) and it was then cloned in the pENTR-Lhx2 plasmid (conveniently deleted of SalI restriction site present in polylinker site) through digestion with SalI and XbaI and following ligation.

Upon bacteria transformation and DNA purification, SalI-XbaI diagnostic digestion confirmed the successful cloning for 5 of 6 Kan-resistance screened growing bacteria, (Fig. 7). DNA sequencing further confirmed the accuracy of this cloning.

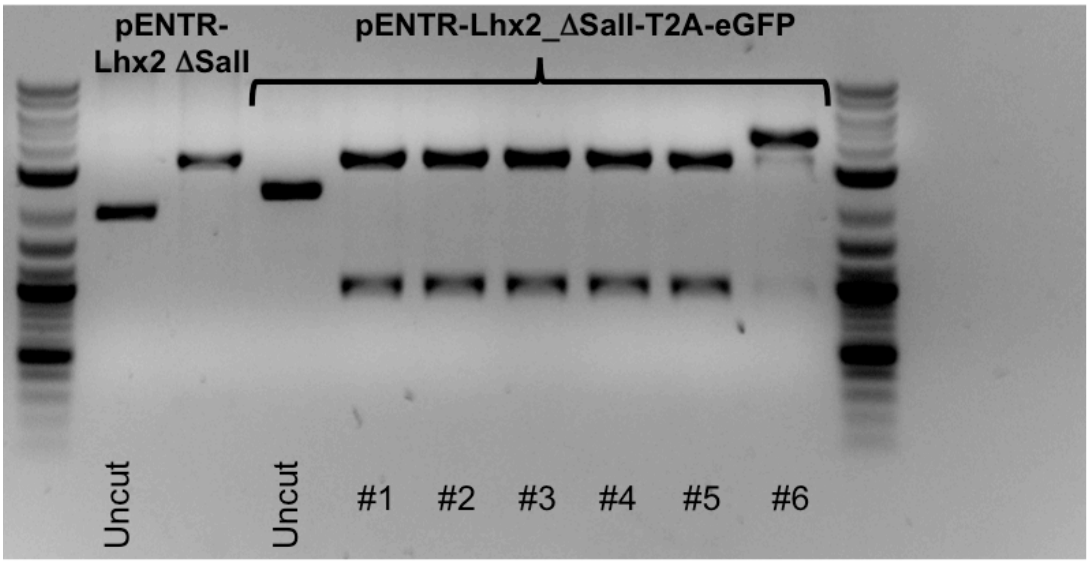


Fig. 7: *Restriction pattern of pENTR-Lhx2_ΔSalI-T2A-eGFP after double restriction digestion with SalI and XbaI confirmed the successful cloning of the fusion gene Lhx2_ΔSalI-T2A-eGFP into pENTR1A plasmid*

3.3 Construction of the expression plasmid pcDNA-DEST_Lhx2 and pcDNA-DEST_Lhx2-T2A-eGFP

To confirm the ability to express the eGFP protein, the Lhx2-T2A-eGFP construct was cloned into the expression plasmid pcDNA-DEST, conveniently engineered by Yoshitaka Miyagawa with the introduction of the Gateway cassette, which allows the insertion of transgenes deriving from an entry vector in just one step, through LR recombination. (Fig. 8).

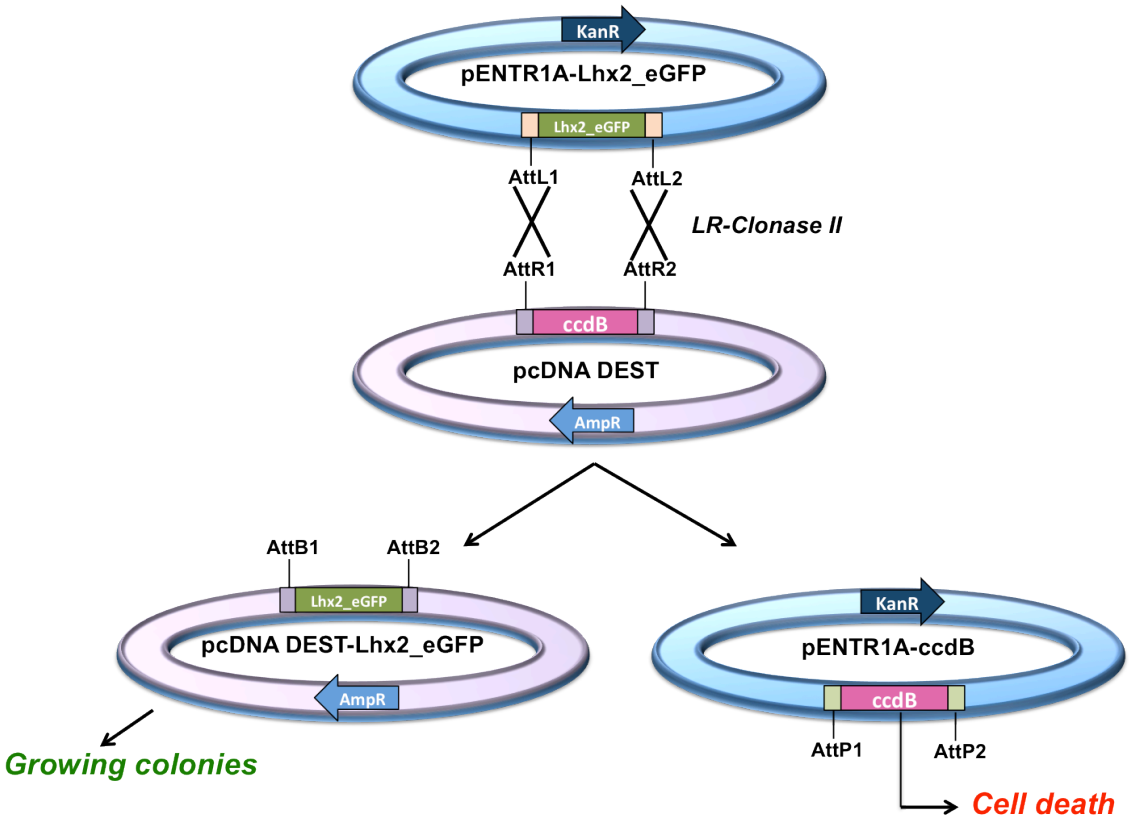


Fig. 8: *Schematic representation of Lhx2 cloning by LR reaction into the expression plasmid pcDNA-DEST.*

This reaction replaced the ccdB gene of original pcDNA-DEST with the Lhx2-T2A-eGFP, guaranteeing the growth of only recombinant bacterial colonies upon transformation. In parallel, another LR recombination was run to insert the only Lhx2 gene into the same pcDNA-DEST. Ampicillin-resistant colonies were screened for correct insertion of transgenes by diagnostic digestion, again with SalI and XbaI (Fig. 9)

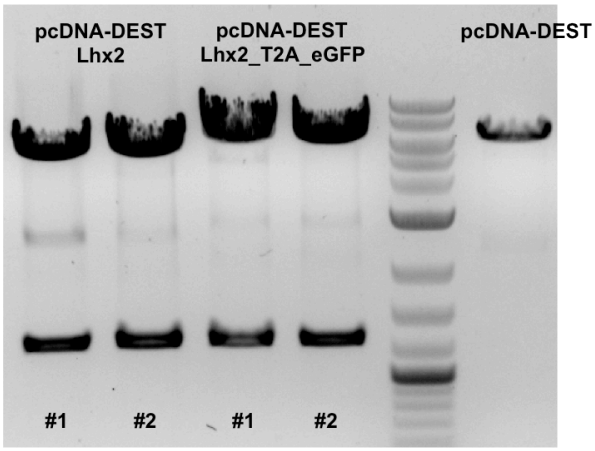


Fig. 9: *Restriction pattern of pcDNA-DEST_LHX2_T2A_eGFP and pcDNA-DEST_LHX2 upon double digestion with SalI and XbaI*

Both the pcDNA-DEST-Lhx2 and pcDNA-DEST-Lhx2_T2A_eGFP were afterwards transfected in HEK293T cells, to demonstrate the in vitro capability for Lhx2 and eGFP expression. pcDNA-DEST alone was transfected as a control. 24h after transfection, cells were fixed and labeled through indirect immunofluorescence (Fig. 10)

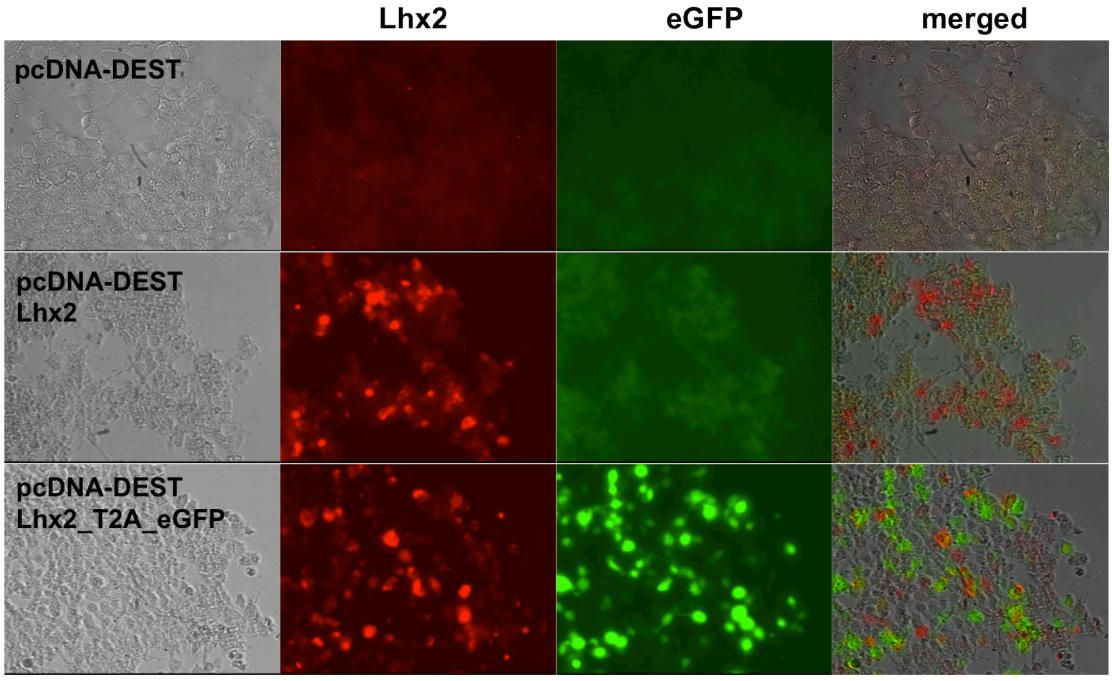


Fig. 10: *Immunofluorescence on HEK293T 24h after transfection with pcDNA-DEST, pcDNA-DEST_Lhx2, pcDNA-DEST_Lhx2_T2A_eGFP. Cells transfected with both the recombinant expression plasmids pcDNA-DEST_Lhx2 and pcDNA-DEST_Lhx2_T2A_eGFP are positive for Lhx2 upon immunofluorescence. Moreover, the newly generated fusion protein Lhx2_T2A_eGFP cloned in this expression plasmid could efficiently express both Lhx2 and eGFP protein.*

Immunofluorescence analysis confirmed the expression of Lhx2 protein from both backbones and, moreover, the co-expression of Lhx2 and eGFP in HEK293T cells transfected with pcDNA-DEST-Lhx2_T2A_eGFP. In the latter case, eGFP fluorescence looks higher then Lhx2 labeling; this could be due to a differential fluorescence emission.
The lysates of HEK293T transfected cells was collected to further check Lhx2 expression, this time by Western Blot analysis. As shown in Fig 11, from both plasmids Lhx2 is strongly expressed.

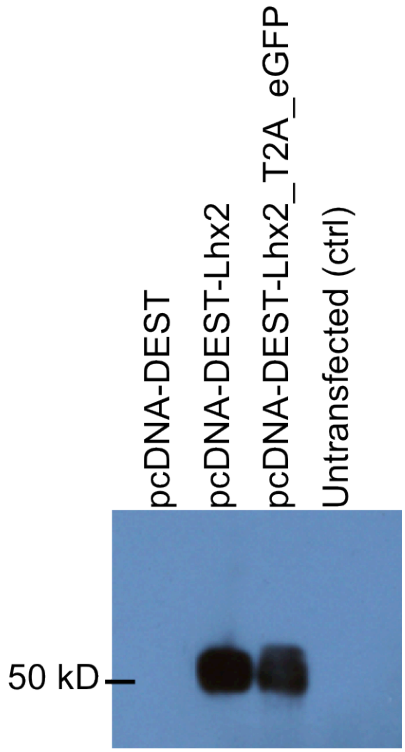


Fig. 11: *Western blot analysis of HEK293T lysates 24h upon transfection with pcDNA-DEST, pcDNA-DEST_Lhx2, pcDNA-DEST_Lhx2_T2A_eGFP. 20mg of protein were load in each lane. Last lane is loaded with the lysate of untransfected HEK293T cells. Both the pcDNA-DEST_Lhx2 and pcDNA-DEST_Lhx2_T2A_eGFP strongly express Lhx2 protein. This latter showed an additional higher band, probably due to a residual uncut Lhx2-eGFP protein.*

3.4 Generation and characterization of JΔβββ4-Lhx2 and JΔNI-Lhx2 viruses

Once verified the ability of hLhx2 cDNA to successfully encode for Lhx2 protein, Lhx2 cDNA could finally be cloned into the HSV-1 BAC-DNA. Important modification of HSV-1 genome have been made by Yoshitaka Miyagawa, to make the virus unable to replicate in non-complementing cells and to remove or, at least, dramatically drop its toxicity anyhow keeping a stable transgene expression (Fig. 12). The oldest version, named JΔβββ4, has been deleted of the immediate early (IE) genes encoding for ICP4 and ICP47 proteins; in addition, ICP0, ICP22 and ICP27 IE genes have been converted to early genes by replacement of their original promoters with a promoter controlling the expression of early genes, in order to allow their expression only in case of ICP4 expression in complementing cell lines. Three additional and undoubtedly not less important variation of this backbone have been made: 1) cloning the gene for the fluorescent protein mCherry, driven by the ubiquitin promoter, to constitutively

express this marker protein for the detection of HSV-1 occurred infection in target cells; 2) introduction of the Gateway cassette, that have been strategically inserted for a rapid and efficient cloning of any gene into this BAC DNA (placed downstream the strong and constitutively express promoter EF1α); 3) the double mutation on gB protein (D285N/A594T), which has been demonstrated to enhance the viral infectiveness (Uchida et al., 2010).

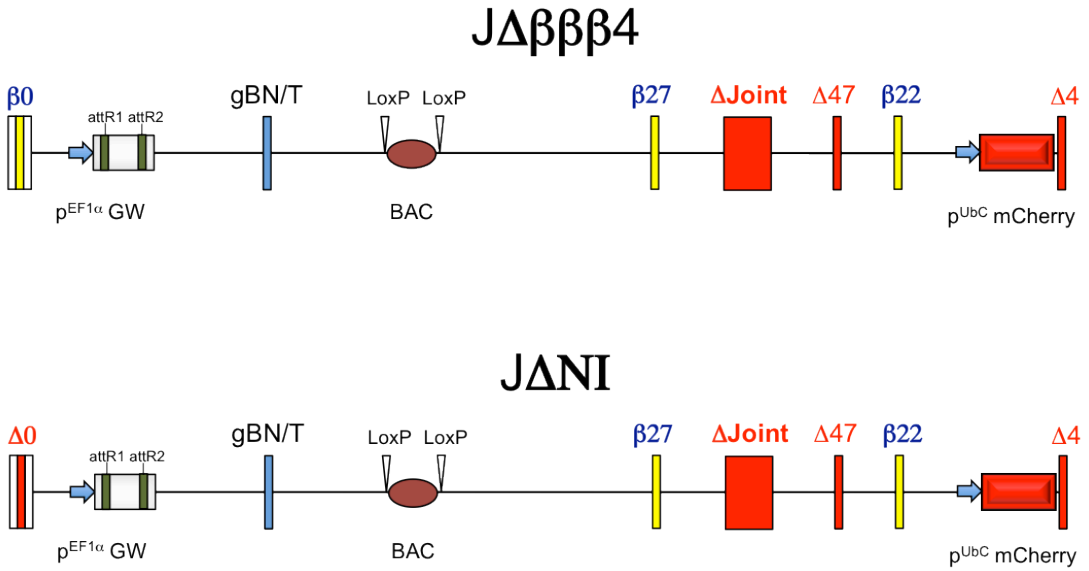
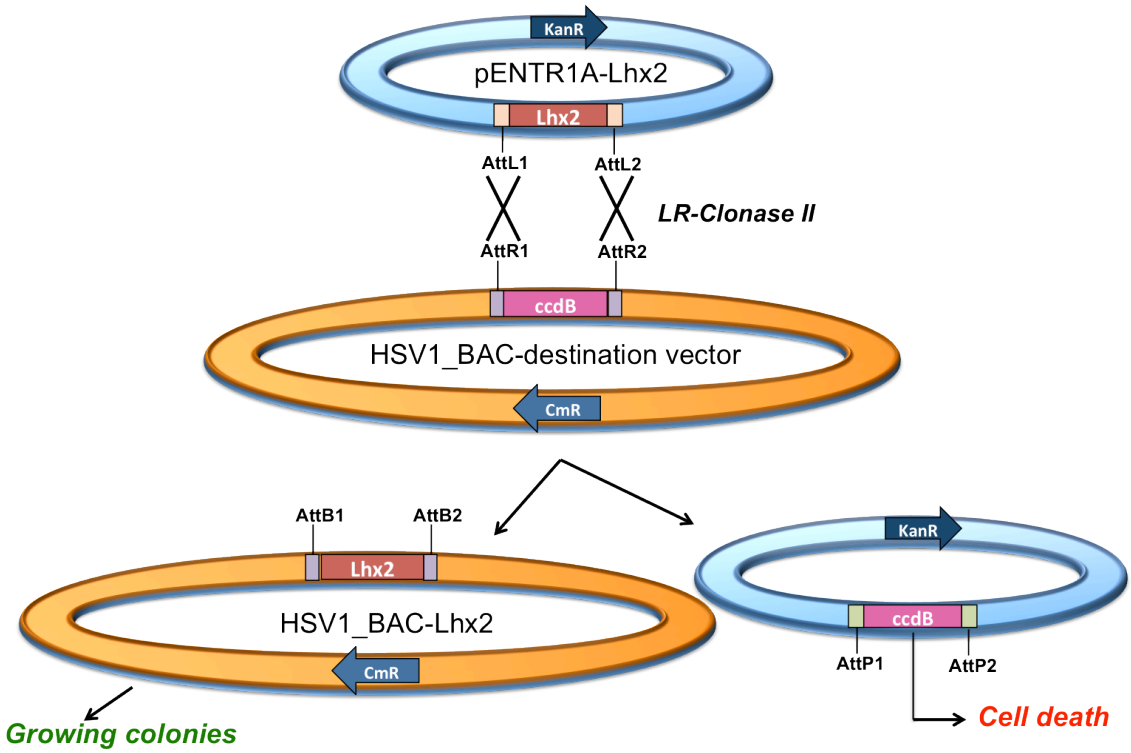


Fig. 12: *Schematic representation of JΔβββ4 and JΔNI BAC-DNAs. The only characteristic differentiating the JΔNI from the JΔβββ4 (from which the first one is derived) is the deletion of the ICP0 immediate early gene.*

The JΔβββ4 vector, however, may exert some toxic effect in vitro or in vivo, making it useless for particular assays. To reduce residual viral toxicity, a newer HSV-1 vector has been generated: this novel recombinant virus, called JΔNI, has been engineered to have only one but essential difference to its predecessor JΔβββ4 (from which has been derived), that is the deletion of ICP0 gene (unpublished data).

Both of them were engineered to carry the gateway cassette between UL3 and UL4 region of HSV-1 genome, allowing a fast and efficient recombination with any DNA fragment previously cloned into an Entry Vector.



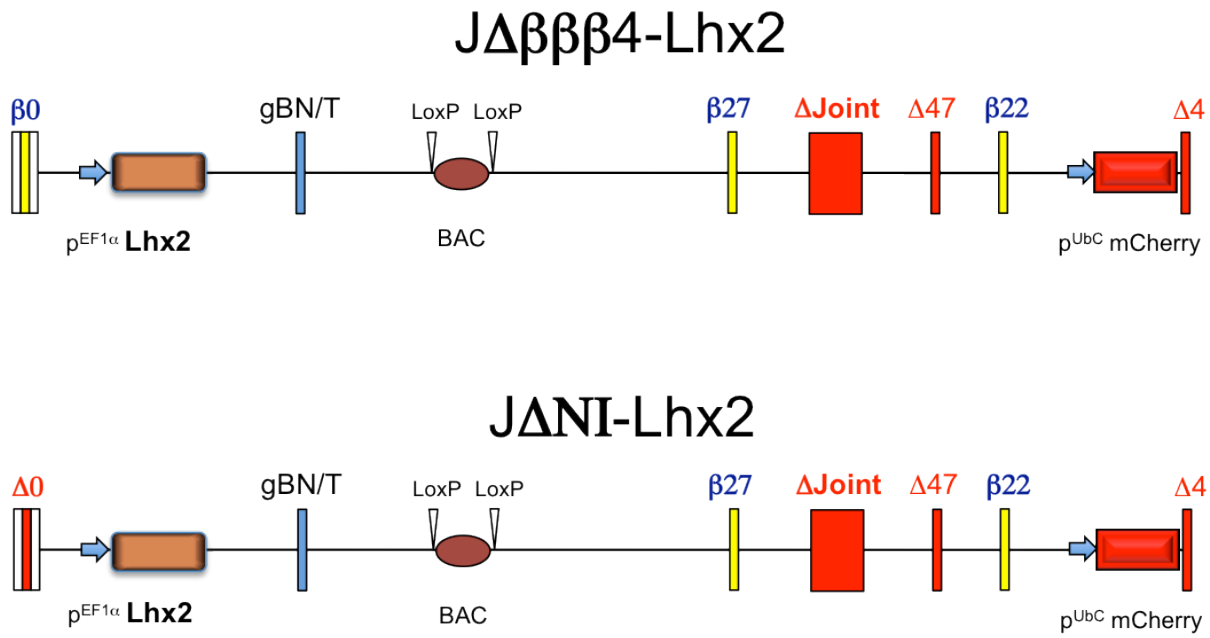


Fig.13: *Schematic representation of Lhx2 cloning by LR reaction into the HSV-1 BAC DNA and resultant recombinant vectors*

Following LR reaction, BAC DNAs were transformed in electrocompetent bacteria to achieve their propagation; only the clones that successfully incorporated the recombinant BAC DNA might form colonies.

Bacteria transformed with both JΔβββ4-Lhx2 and JΔNI-Lhx2 BAC DNAs gave rise to several colonies after growing O/N on LB-agar plates supplemented with the selector drug chloramphenicol (CM). Single colonies were picked with a sterile tip and transferred into tubes containing LB broth + CM for propagation. Additionally, single bacterial colonies previously transformed with the parental JΔβββ4 and JΔNI were picked and propagated, to serve as negative controls for following assays. BAC-DNAs were purified by BAC minipreps and overnight digested with BglII. The presence of a BglII restriction site in the Lhx2 cDNA (at nt 175) produced an extra DNA fragment for both JΔβββ4-Lhx2 and JΔNI-Lhx2 backbones if compared to their parental JΔβββ4 and JΔNI. This was confirmed upon FIGE gel analysis of BAC-DNA digestions (Fig. 14).

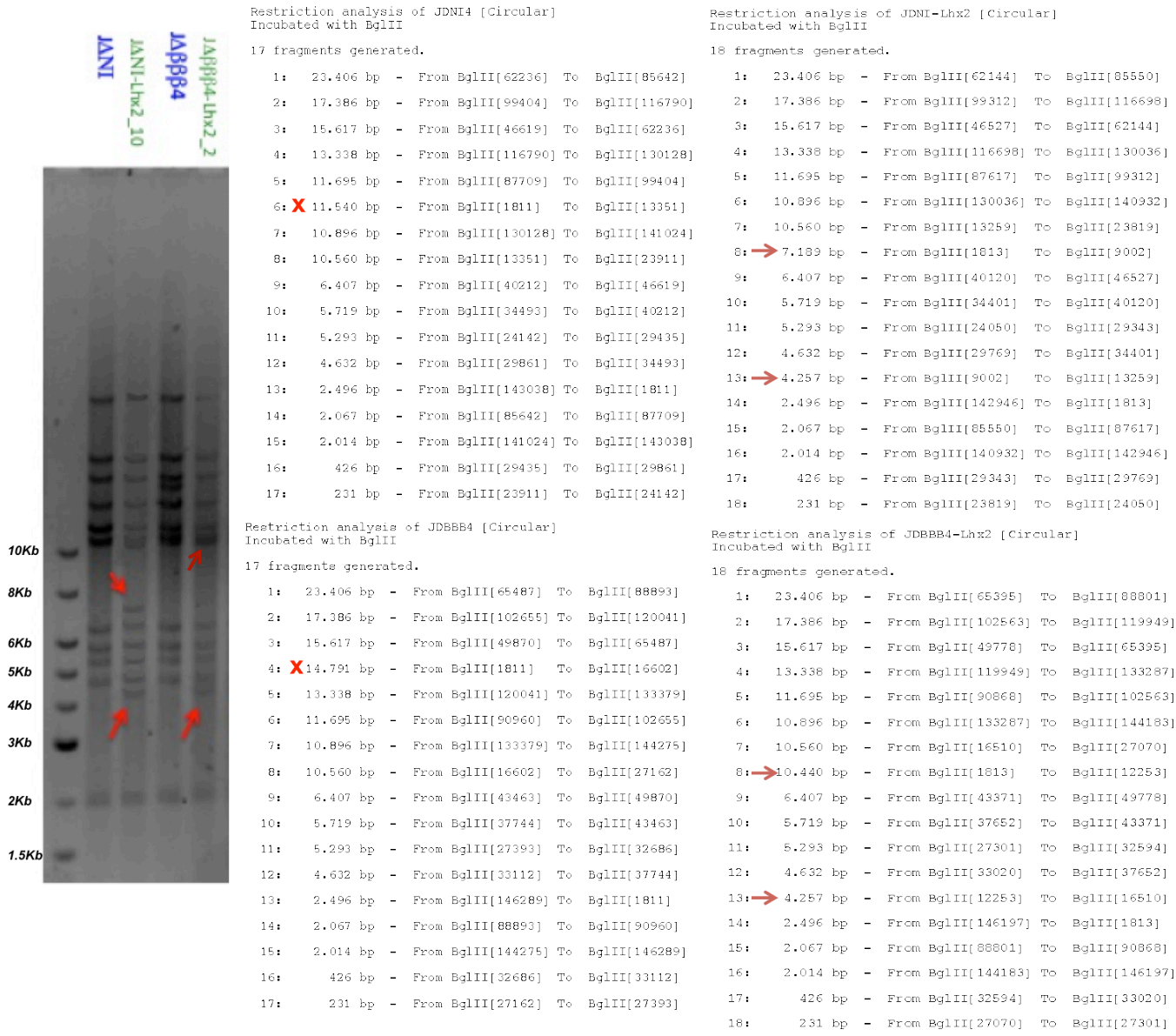


Fig. 14: *Diagnostic FIGE digest (left pictures) of JΔNI, JΔNI-Lhx2, JΔβββ4, JΔβββ4-Lhx2 BAC-DNAs with BglII and predicted digestion patterns resulting from Serial Cloner® software (right pictures) with same restriction enzyme. BglII BAC digestion generated one extra band of 4,2-Kb in both JΔNI-Lhx2 and JΔβββ4-Lhx2 backbones compared to their parentals, predicting Lhx2 insertion; moreover, both digests showed the expected shift of the DNA fragment containing the site of insertion from 11.5-Kb to 7.1-Kb in JΔNI-Lhx2 digest and from 14.7-Kb to 10.4-Kb in the JΔβββ4-Lhx2 digest, without any changes in other bands.*

To confirm the positive result coming from diagnostic digestion and the correct insertion of the Lhx2 gene, a diagnostic PCR was run. The DNA templates were the same BAC DNAs used for FIGE analysis. A couple of primers has been selected that should generate a PCR amplicon only in case Lhx2 was cloned into the destination vector with the right orientation: the forward primer annealed at nt 671 of Lhx2 gene and the reverse primer annealed on UL4 region, giving rise to a 1.2kb PCR product (Fig. 15).

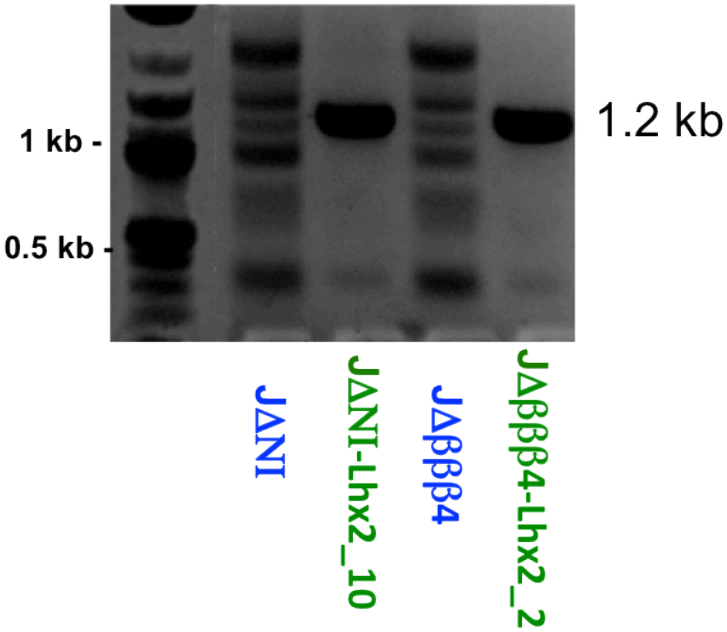


Fig. 15: *Diagnostic PCR on recombinant BAC DNAs. Clear differences between the parental and the recombinant backbones confirmed the successful cloning of Lhx2 cDNA in both the JΔNI and JΔβββ4 BAC DNAs. In particular, the PCR on JΔNI-Lhx2 and JΔβββ4-Lhx2 produced a strong band of expected size (1.2kb) after gel running, while a series of not specific bands appeared on the lane loaded with the PCR products generated from parental backbones JΔNI and JΔβββ4, indicating the absence of Lhx2 gene inside.*

To test if the virus could efficiently generate replicating viral particles in complementing cell line, JΔNI-Lhx2 and JΔβββ4-Lhx2 BAC DNAs were transfected in U2OS-ICP4 cells, seeded in 6 multiwell plate. Recombinant virus can be actually reconstituted by transfection of purified virus BAC DNA into susceptible eukaryotic cells. Upon uptake of the virus genome into transfected cells HSV-1 proteins start to be expressed, initiating the replication. Since ICP4 IE gene (essential for viral replication) have been deleted from both viral vectors, the expression in trans of this protein mediated by U2OS-ICP4 can compensate its missing. However, JΔNI-Lhx2 backbone is additionally missing of ICP0 IE gene; although not essential for viral replication, ICP0 confers a significant advantage for the growth of the virus. Experimental analysis showed that U2OS cells could functionally substitute for ICP0. This makes U2OS-ICP4 cells an ideal tool even for JΔNI-based vectors propagation. Indeed, U2OS-ICP4 cells transfected with whether JΔβββ4-Lhx2 or JΔNI-Lhx2 can both form plaques clearly visible 3 days post BAC-transfection with fluorescence microscope, as shown in Fig. 16.

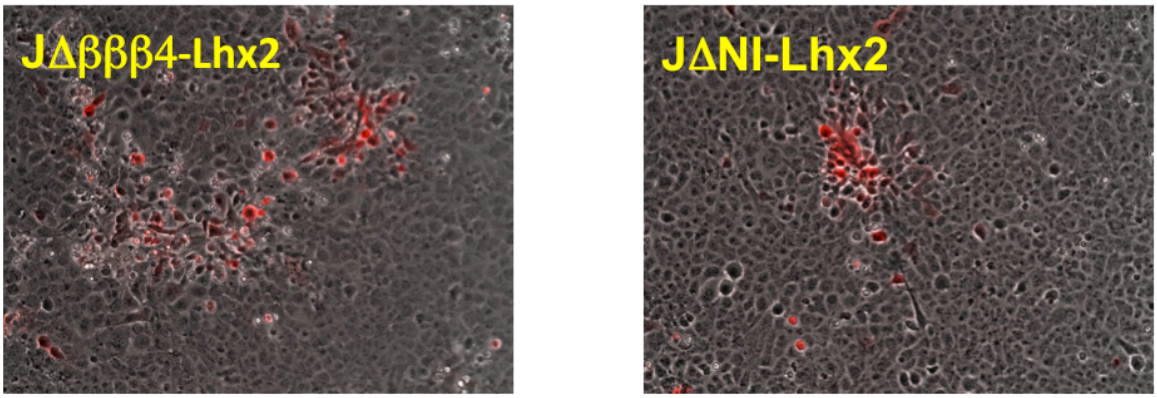


Fig. 16: *U2OS-ICP4 cells 3 days post transfection with JΔβββ4-Lhx2 and JΔNI-Lhx2 BAC DNAs. Both viruses could form plaques even though, as expected, viral growth is faster for JΔβββ4-based vector, due to the presence of ICP0 gene into the viral backbone.*

Generally, after BAC-transfection viral growth is rather slower than usual, since a percentage of viral particles generated are not able to spread and infect other cells. Indeed, after transfection were necessary 7 days to collect the virus: at this point, every cell was infected and most of them underwent cytolysis, releasing virions in the growing medium. However, several cells were still attached on the well bottom; to collect as much as possible virus, these cells were scraped out from their adhesion surface and sonicated in their own supernatant to free the virus still trapped inside cells. This procedure must be done only when the purpose is to collect as much virus as possible for its propagation, since the massive cytolysis mediated by sonication also free a big amount of cellular debris in the medium, making the virus preparation too “dirty”. To reduce the cellular contaminants, the sonicated supernatant was spun down at 3000 rpm for 10 minutes and passed through a 0.8um filter. This viral preparation was used to propagate the amount of virus by infection of U2OS-ICP4 cells, seeded in 10cm ø petri dishes at M.O.I. of 0.1. after 3 days from infection with both JΔβββ4-Lhx2 and JΔNI-Lhx2, a great percentage of U2OS-ICP4 cell was mCherry positive (Fig. 17), meaning that both these viruses can efficiently infect and spread in U2OS-ICP4 cell monolayer.

JΔNI-Lhx2 **JΔββββ4-Lhx2**

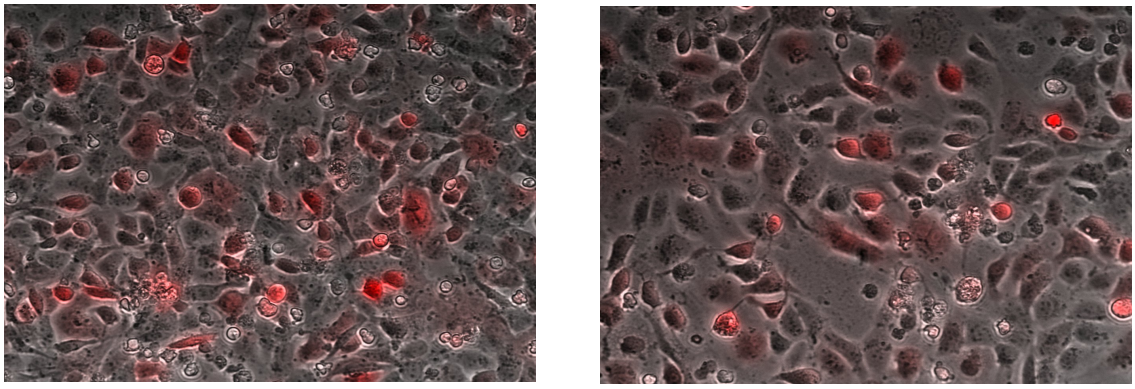


Fig. 17: *U2OS-ICP4 cells 3 days post infection with JΔNI-Lhx2 and JΔββββ4-Lhx2*. Both the viruses rapidly form spreading plaques. *The kinetic of propagation of JΔββββ4-Lhx2 virus is visible faster then the JΔNI-Lhx2 one, due to additional presence of ICP0 gene into its genome.*

Upon virus collection from 10cm ø petri dishes, viral propagation was scaled up by infection of U2OS-ICP4 cells seeded and brought to confluency in T150 flasks.

JΔββββ4-Lhx2 virus was propagated in 15 T150 flasks confluent with U2OS-ICP4 cells. On the other hand, JΔNI-Lhx2 virus (that, missing ICP0, grow slower) was propagated into Corning® CellBIND® Surface CellSTACK® cell culture 10 chambers, a very useful tool to greatly scale-up cell cultures and, in this case, viral production. Indeed, after titration of purified virus, the amount of JΔββββ4-Lhx2 virus got from 15 T150 flasks was about $1x10^8$ pfu (resuspended in 250ul of PBS + 10% glycerol, for a concentration equal to $4x10^8$ pfu/ml) 10 times less of JΔNI-Lhx2 virus purified from 10 chambers ($1x10^9$ pfu in 1ml of PBS + 10% glycerol).

To test whether JΔββββ4-Lhx2 and JΔNI-Lhx2 viruses were able to express Lhx2 protein, confluent U2OS-ICP4 cells were infected at M.O.I. 0.1 to perform an immunofluorescence against Lhx2 protein 24h after infection. As shown in Fig. 18, U2OS-ICP4 cells infected with both viruses were positive for Lhx2 after immunofluorescence, 24h after infection.

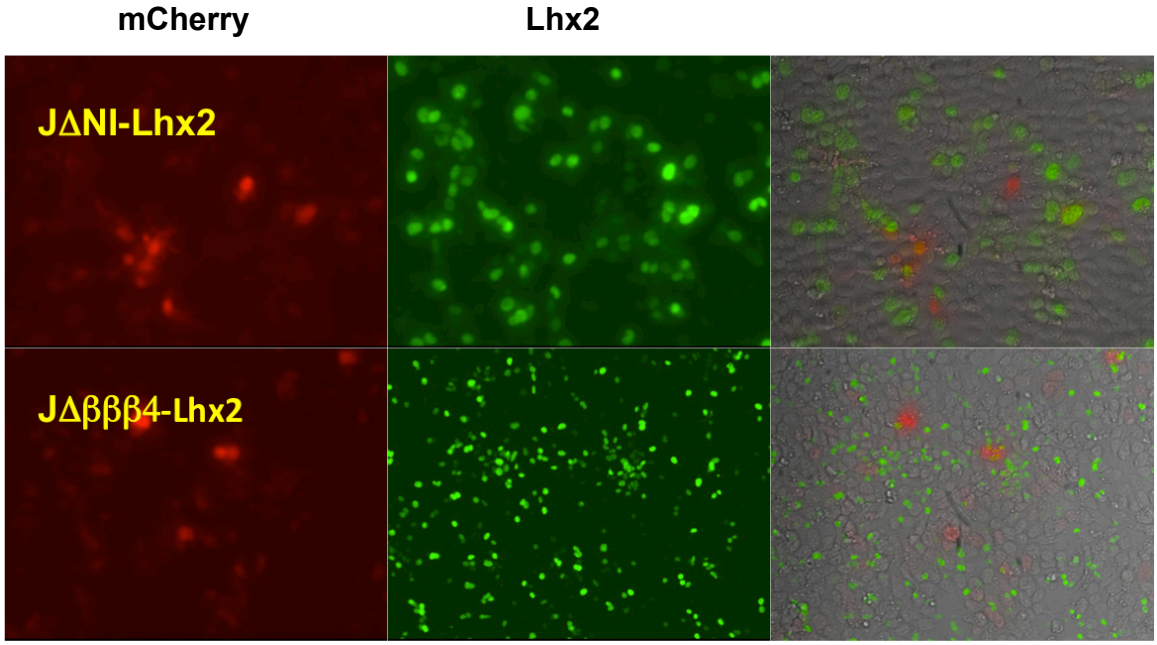


Fig. 18: *Immunofluorescence on U2OS-ICP4 infected cells. Infection was performed at MOI 0.1 and cells were processed 24h after infection. Both JΔNI-Lhx2 and JΔβββ4-Lhx2 infected cells are positive after Lhx2 staining, confirming that the two viruses could express Lhx2 protein after infection of this complementing cell line.*

These data were further confirmed by western blot analysis: lysates from U2OS-ICP4 infected cells were collected 24h after infection at M.O.I. 0.1 (same conditions as above); the samples were loaded onto an SDS-polyacrylamide gel and run under standard conditions. Interestingly, the band corresponding to Lhx2 protein in JΔNI-Lhx2 infected cell lysate appeared stronger then the one revealed in JΔβββ4-Lhx2 lysate. This might be due to the greater toxicity evoked by JΔβββ4-based vector (extensively demonstrated in Glorioso's lab in previous studies, unpublished data) on U2OS-ICP4 cells: cell death lead to protein degradation and, as a consequence, Lhx2 protein levels might decrease (Fig. 19)

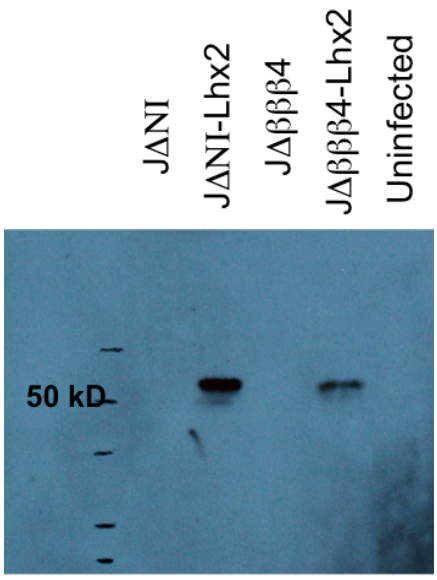


Fig. 19: *Western Blot analysis on U2OS-ICP4 lysates 24h upon infection (MOI 0.1). 20 μg of protein were loaded on each lane. The band corresponding to Lhx2 appeared in both lanes corresponding to cells infected with JΔNI-Lhx2 and JΔβββ4-Lhx2 infected cells. The differential intensity of the two bands may indicate the higher toxicity evoked by the JΔβββ4-Lhx2 viral backbone.*

However, we didn't know if the complementation of these cells for ICP4 could affect the transgene expression. Hence an immunofluorescence against Lhx2 were performed on regular U2OS cells 24h post-infection with JΔNI-Lhx2 and JΔβββ4-Lhx2, this time using M.O.I. 1. Consistently with previous results, even in this case we got the positive signal for Lhx2 protein on U2OS cells infected with both viral vectors (Fig. 20).

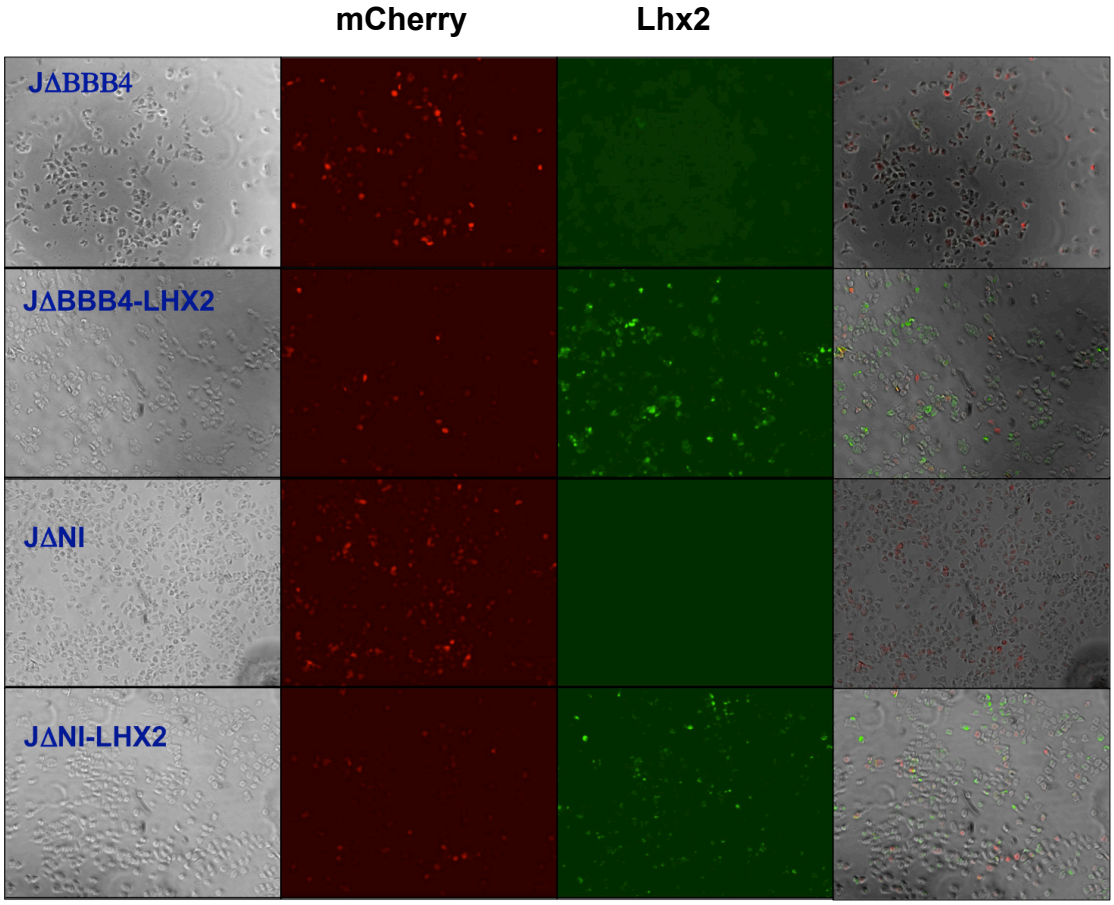


Fig. 20: *Immunofluorescence staining of Lhx2 on U2OS infected cells, 24h post-infection with JΔβββ4, JΔβββ4-Lhx2, JΔNI, JΔNI-Lhx2 at MOI1. Lhx2 positive U2OS cells were detected upon infection with both JΔNI-Lhx2 and JΔβββ4-Lhx2 viruses, even though this latter produced a stronger signal, vouching for a major transgene expression*

Moreover, from this analysis we can undoubtedly infer that both these vectors are not able to form plaques in U2OS gathering that no mutation potentially reverting the viral phenotype occurred, keeping both viruses replicative-incompetent in infected non-complementing cell-lines.

As discussed above, U2OS cells can partially complement for the deletion of ICP0. To assess if this endogenous complementation could affect the levels of transgene, another cell type must be used for infection. Hence, Mouse Embryonic Fibroblasts (MEFs) were

infected at M.O.I. 10; 24h post-infection, cell lysates were collected and analyzed through western blot. Unexpectedly, only cells infected with JΔNI-Lhx2 revealed the clear band of Lhx2 protein. Again, this result enhances that JΔβββ4-based virus is dramatically more toxic then JΔNI-based vectors, at least *in vitro*. Actually, according to their morphology, as soon as 24h after the infection, MEFs infected with JΔβββ4-based viruses looked visibly suffering if compared to those infected with the JΔNI-based vectors.

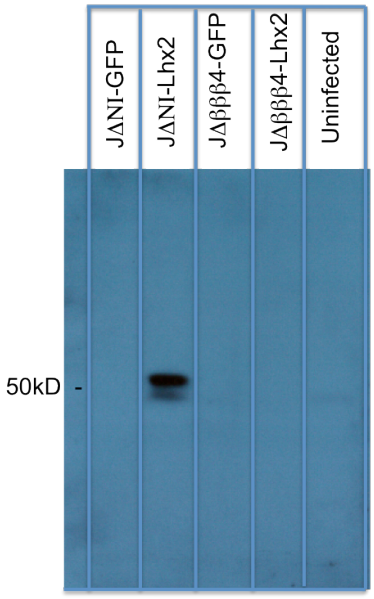


Fig. 21: *Western Blot analysis on mouse embryonic fibroblasts lysate,s 24h upon infection at MOI 10 with JΔNI-GFP, JΔNI-Lhx2, JΔβββ4-GFP and JΔβββ4-Lhx2. 20 μg of protein lysates were loaded on each lane. Only the lysate of MEF infected with JΔNI-Lhx2 revealed the band of Lhx2 protein. The absence of any band for JΔβββ4-Lhx2 infected MEF is probably due to the high toxicity elicited by this vector on fibroblasts.*

Both these viruses were used to test their infectivity and transgene expression on mouse Embryonic Stem Cells (mESCs). mESCs were initially cultured on MEF-inactivated feeder layer to facilitate the maintaining of their indifferentiated state and then, 3 days before infection, moved on a feeder-free culture to minimize MEF contaminations (Fig. 22)

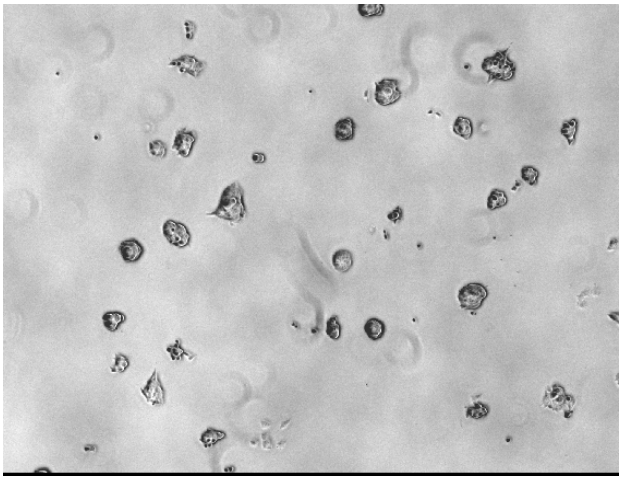


Fig. 22: *Feeder-free culture of mouse embryonic stem cells showing healthy and indifferentiated colonies*

24h after infection, cells were fixed and stained by immunofluorescence against Lhx2. Again, Lhx2 expression looks higher on mESCs infected with JΔβββ4-Lhx2 virus, even though we can detect Lhx2-positive cells also upon infection with the JΔNI-Lhx2 vector (Fig. 23). Interestingly, uninfected cells showed few Lhx2-positive cells, confirming the very early role of this transcription factor.

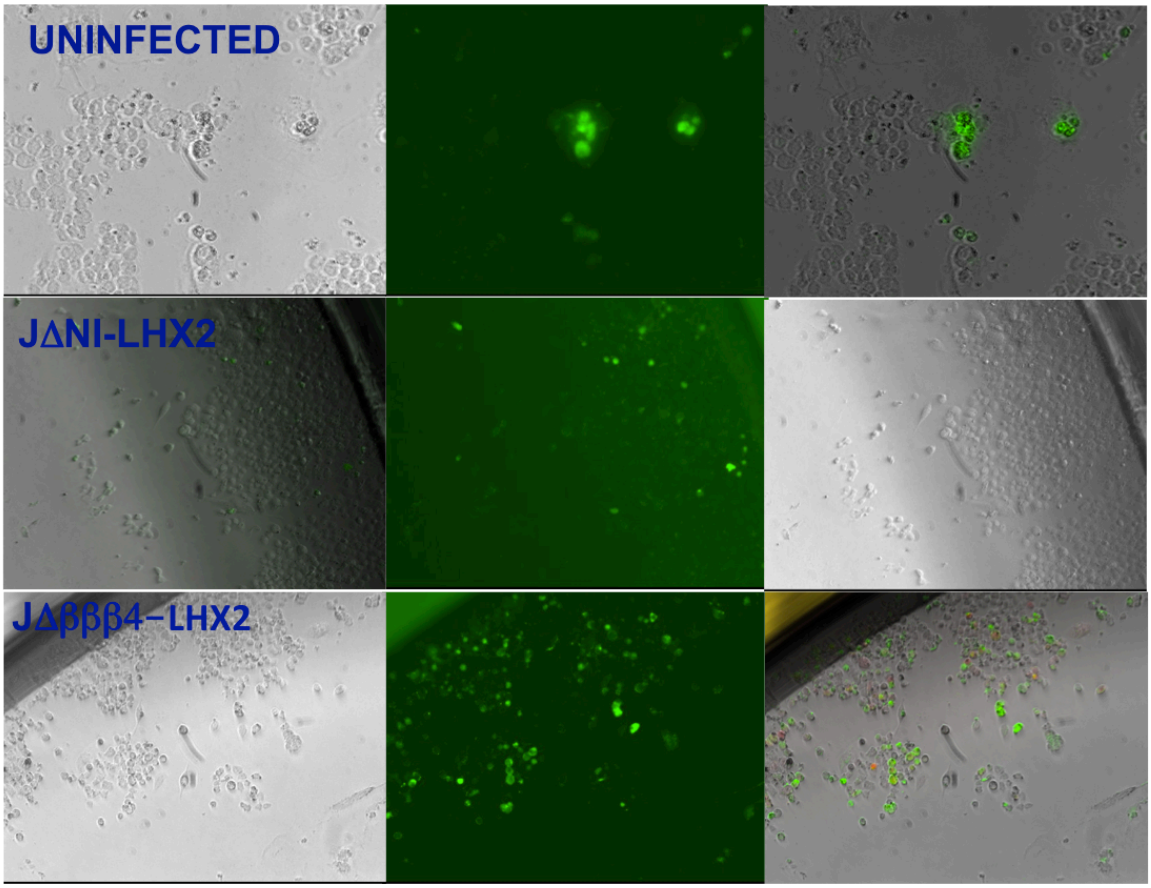


Fig. 23: *Immunofluorescence staining of Lhx2 on mouse embryonic stem cells infected with JΔNI-Lhx2 and JΔβββ4-Lhx2. Uninfected cells showed rare Lhx2-positive cells (upper row, picture took at higher magnificence) confirming the early role of this* transcription factor.

To assess whether these two viral backbones may exert toxicity on mESCs, we let them to aggregate on embryoid bodies after infection. mESCs were infected in suspension at M.O.I. 10 by rotating the tube at 37C. Uninfected cells were exposed at the same conditions to serve as negative control. After 3h of viral exposure, mESCs were pelleted down and the virus was removed. Cells were then resuspended on mESC medium without LIF (to trigger the differentiation program) and plated on 96mw plate (round wells) opportunely coated with Pluronic F127 (Sigma) to avoid the attachment on well surface. Cells were allowed to hang (37°C, 5% CO_2), kept undisturbed for five days; at day 5, pictures were taken at fluorescence microscope (Fig. 24).

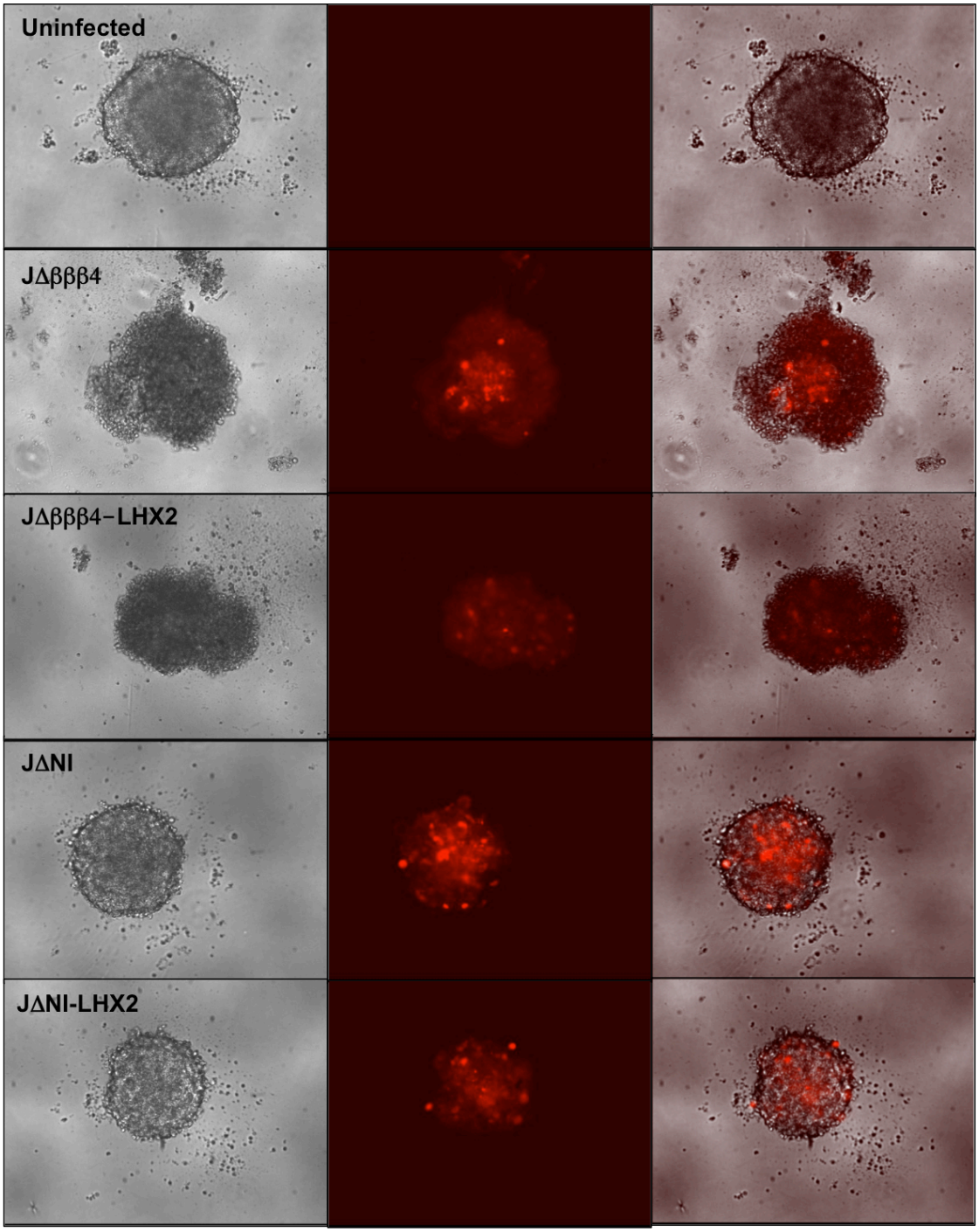


Fig. 24: *Embryoid bodies (EBs) formation 5 days upon infection of mESCs (MOI10) with JΔβββ4, JΔβββ4-Lhx2, JΔNI, JΔNI-Lhx2 viruses. JΔβββ4-infection couldn't allow the generation of EBs, killing mESCs before during the first days upon infection*

From these images we can clearly point out how JΔβββ4-based viruses are particularly toxic for mESCs, strongly affecting cellular health and inhibiting embryoid bodies formation. On the other hand, mESCs infected with JΔNI-based viruses gave rise to healthy and growing embryoid bodies, showing several mCherry-positive cells 5 days after infection and even later, meaning that this backbone is dramatically less toxic then

its previous version, at least concerning mESCs.

3.5 in vitro infection of primary cultures of neurons and astrocytes from JΔβββ4- and JΔNI-based HSV-1 vectors

The final in vitro investigation was carried out on primary neuronal and astroglial cell cultures derived from P1 mice pups, since the target of this work is indeed the cellular population residing in central nervous system. This experiment held a crucial relevance for our purposes because we didn't have any indication about JΔβββ4- and JΔNI-based HSV-1 viral effects on cells of CNS.

Distinct primary cell cultures of neurons and astrocytes were made from the same P1 mice brood. Five days after cell plating, both neurons and astrocytes were infected at M.O.I. 1, 10 and 50 with JΔβββ4-eGFP and JΔNI-eGFP viruses to check infectivity and transgene expression in living cells. Likewise it has been done for Lhx2 cloning, eGFP gene has been inserted into both HSV-1 recombinant BAC-DNAs through LR-reaction, inserting the transgene into the Gateway cassette between UL3 and UL4 with EF1α promoter to drive its expression (Miyagawa et al., unpublished data).

24h post-infection, several neurons and astrocytes infected with both vectors were mCherry-positive, even at the lowest M.O.I.; many but not all these cells showed eGFP expression, even though the intensity profile of eGFP emission appeared lower compared to mCherry. To increase the eGFP signal, 72h post-infection primary neurons and astrocytes were fixed and respectively fluorescence immunostained for MAP2 (neuronal marker) or GFAP (astrocyte marker) and co-labeled with an antibody against the eGFP protein. From this assay we could clearly detect eGFP expression in both neurons and astrocytes 3 days after infection with either JΔβββ4- and JΔNI-derived vectors, suggesting that both of them could actually infect rodent neurons and astrocytes with a quite good transgene expression during the early infection stages (Fig. 25).

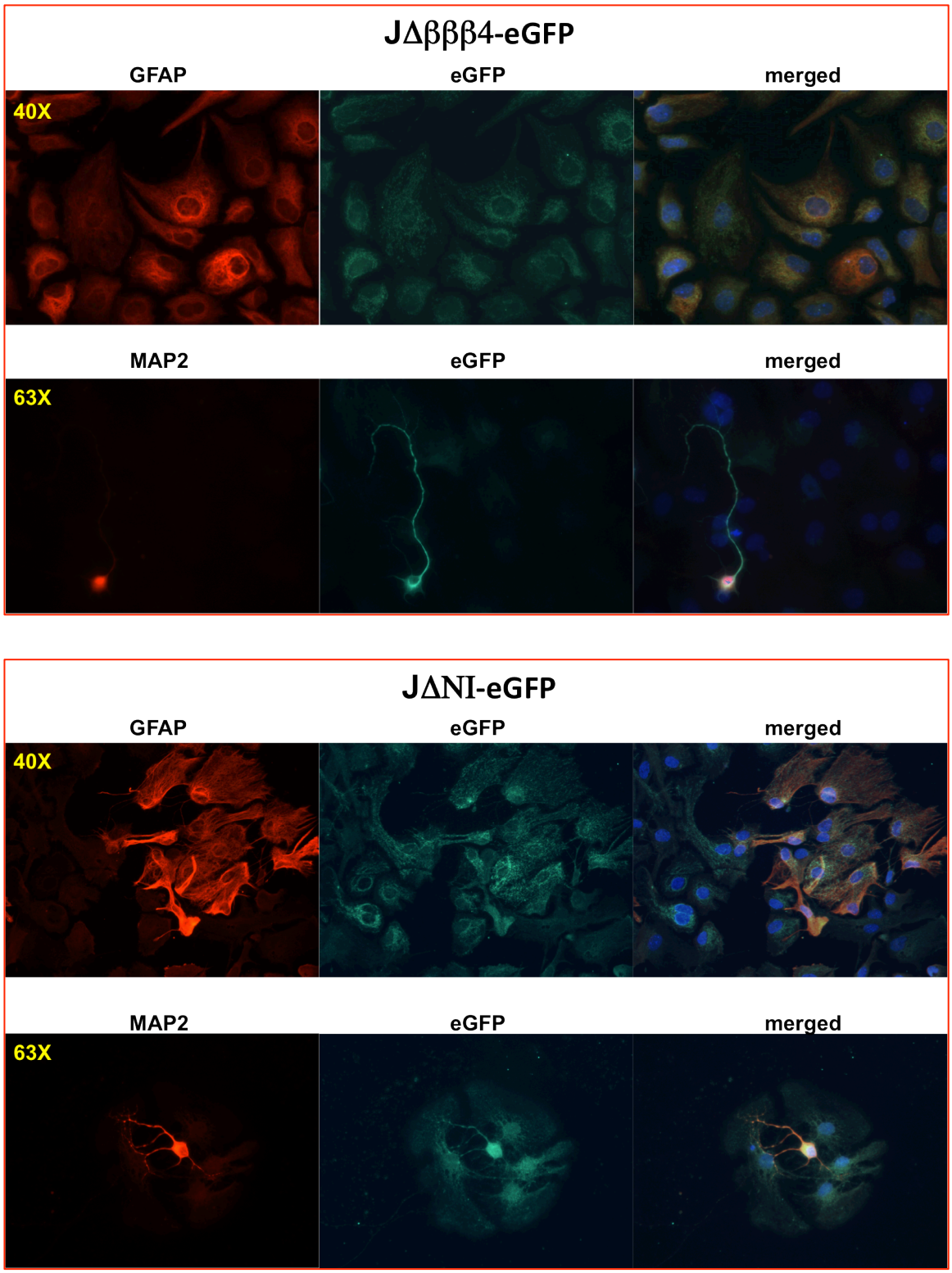


Fig 25: Immunofluorescence staining of *primary cultures of neurons and astrocytes, 72h upon infection with JΔβββ4-eGFP and JΔNI-eGFP viruses. Both MAP2-positive neurons and GFAP-positive astrocytes colocalize with eGFP viral protein.*

3.6 In vivo inoculation of HSV-1 based vectors

The encouraging results got in vitro turned our attention on in vivo administration of the two viral vectors.

The viral preparation was directly injected in the hippocampus of adult rats through a single stereotaxical inoculation; anesthetized animals were sacrificed by decapitation and brain tissue was collected and frozen immediately.

36 rats were included for these in vivo studies.

We decided to split the animals in 2 indipendent groups:

1) “Naïve group”, which included 15 rats that didn’t receive any treatment before inoculation of JΔβββ4 and JΔNI viruses. This group was constituted to assess whether HSV-1-based vectors are able to exert their infectivity even in vivo and, most importantly, if the infection might be accompanied by relevant toxicity. The animals of this group were subdivided as following:

5 rats inoculated with just the vehicle solution (PBS+10%glycerol)

5 rats inoculated with $1x10^6$ pfu in 2 μl of PBS+10%glycerol of JΔβββ4 vector

5 rats inoculated with $1x10^6$ pfu in 2 μl of PBS+10%glycerol of JΔNI vector

These animals were sacrificied at day 12 post-infection (Fig. 26a).

2) “Pilocarpine group”, including animal models of epilepsy previously treated with the proconvulsivant drug pilocarpine. With this group we wanted to investigate whether Lhx2 overexpression mediated by JΔβββ4-Lhx2 and JΔNI-Lhx2 vectors could influence the ratio neurons:astrocytes and have a disease-modifying effect changing the severity and the number of seizures per day.

20 animals were subjected to pilocarpine treatment; unfortunately, 7 of them died during status epilepticus, so we eventually got 3 animals for vector type (including relative controls). Animals belonging to this group were sacrificied 28 days post infection. During the last week, they were monitored 24/24h to assay the number and the severity of seizures (Fig 26b).

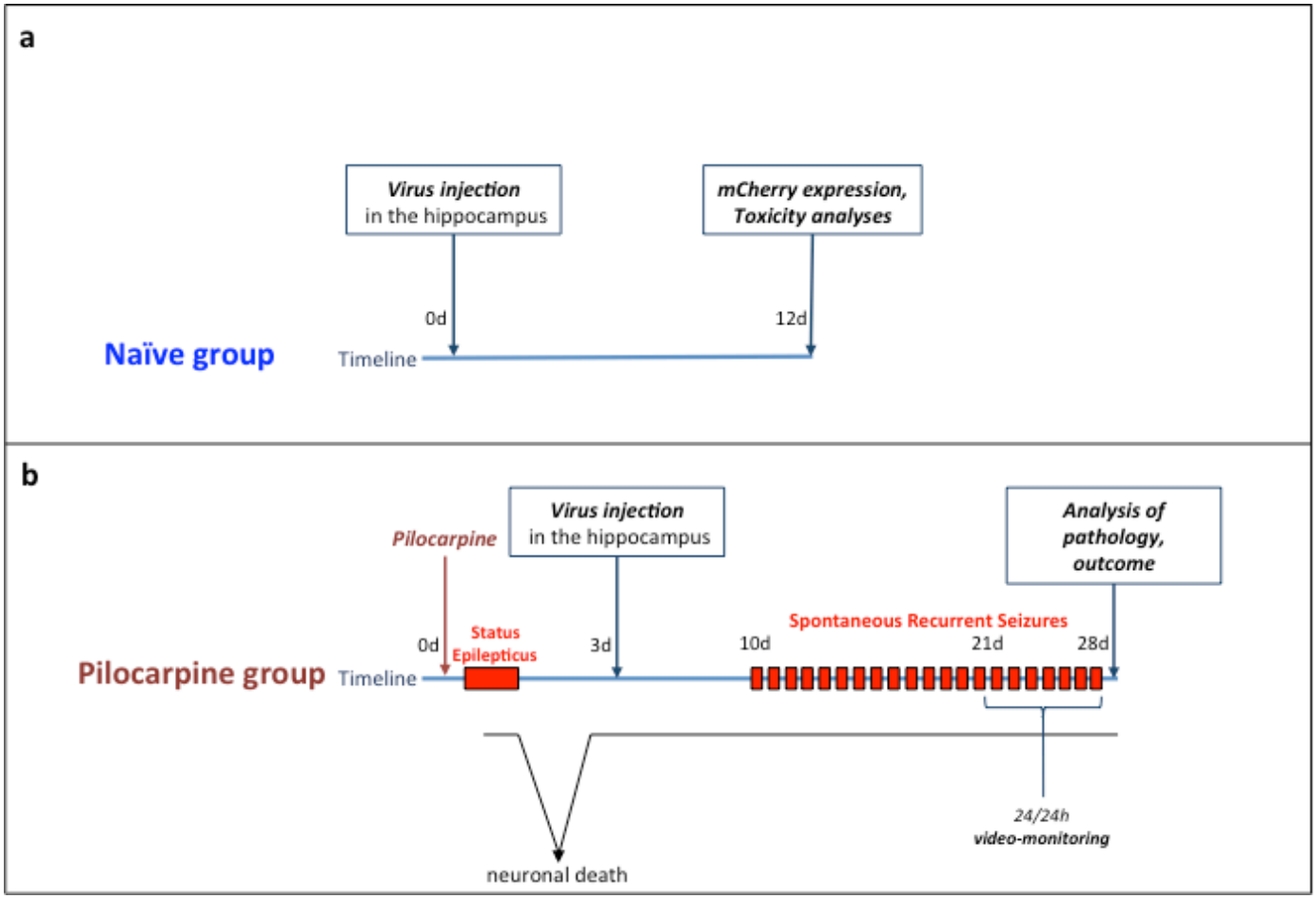
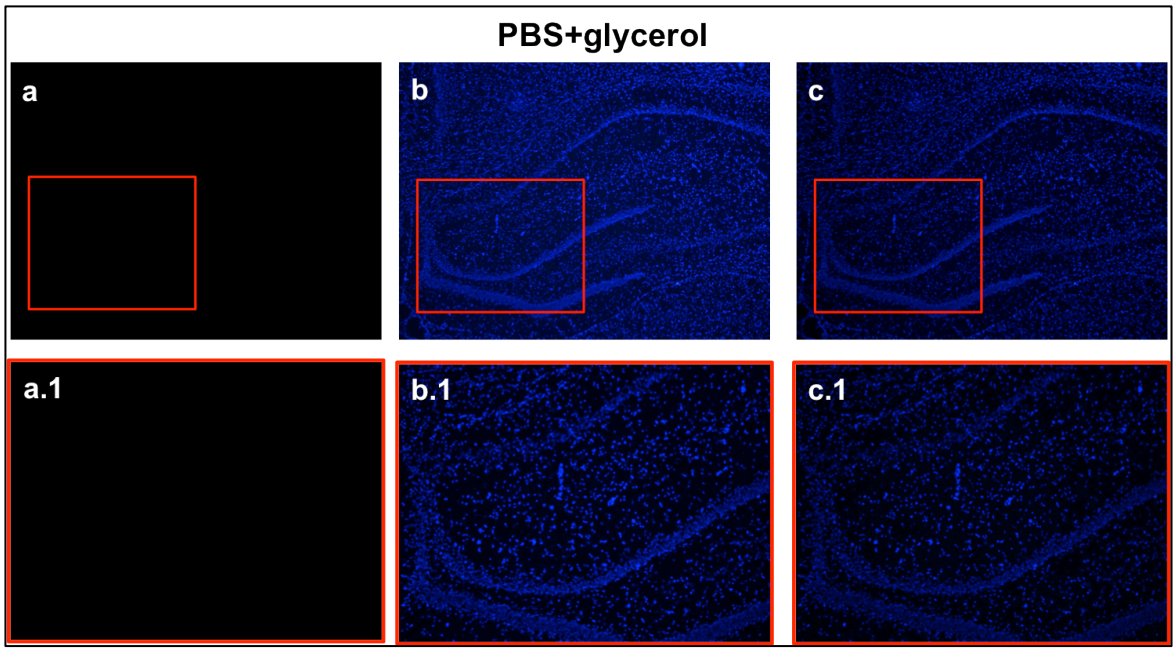
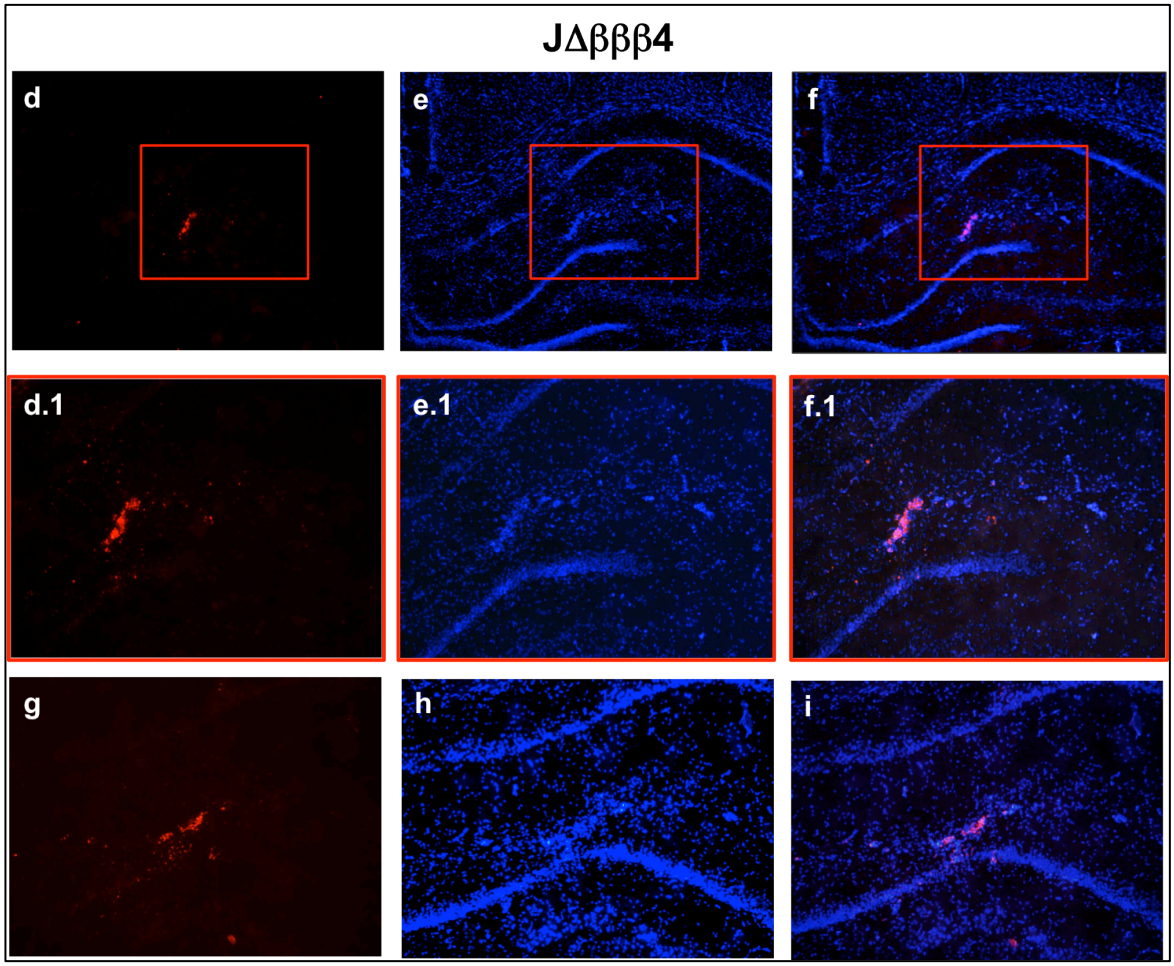


Fig. 26: *Schematic diagram of in vivo experiments in both naïve (a) and pilocarpine (b) groups.*

Ex vivo experiments were conducted on 20μm brain slices were, cut by criostatic microtome.

First analysis was aimed to disclose the presence of viral infection into hippocampus of naïve animal group. Since every viral vector has been engineered to constitutively express mCherry upon infection, with fluorescence microscope we sought the presence of red fluorescence protein on hippocampus of infected animals. Interestingly, we could detect red signal in both JΔNI- and JΔβββ4-injected brains (Fig. 27).


PBS+glycerol
a
b
c
a.1
b.1
c.1


JΔβββ4
d
e
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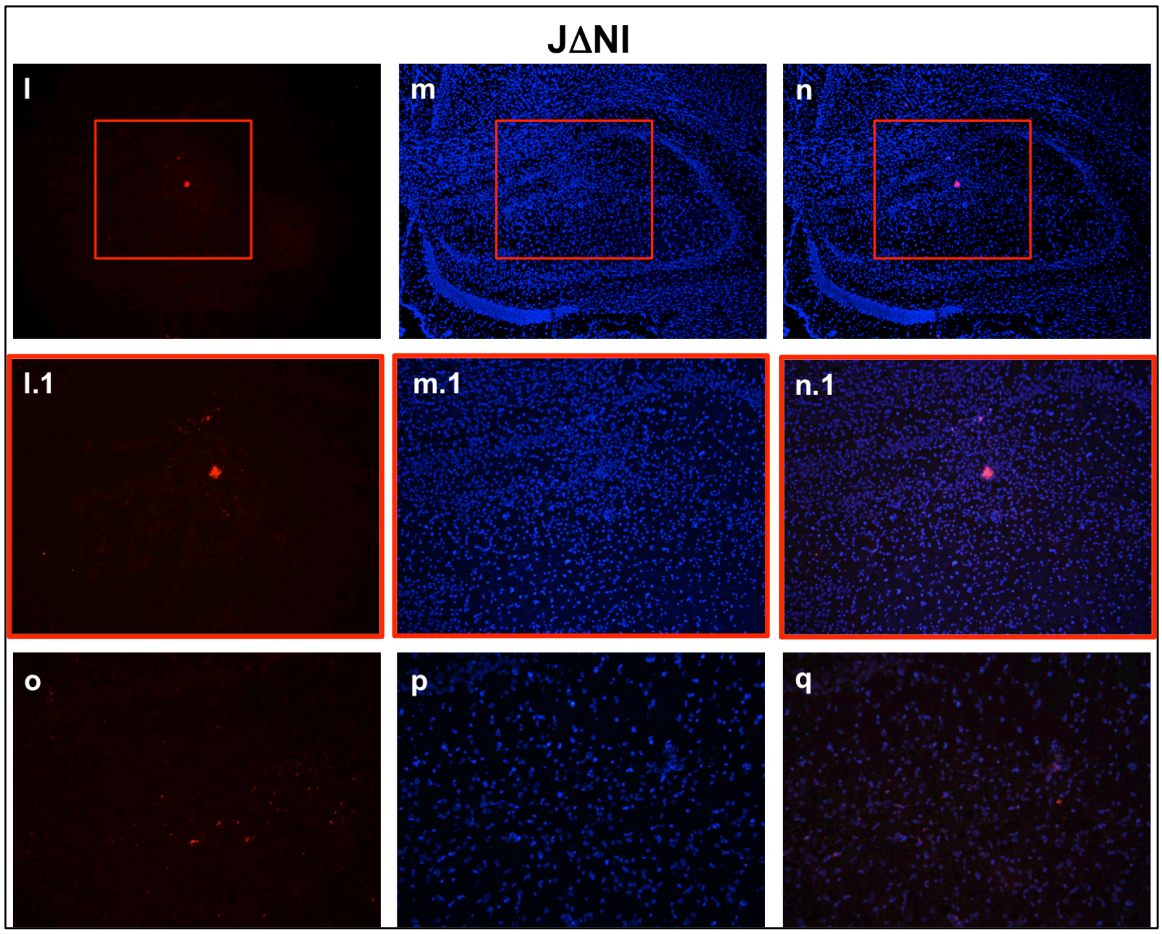

Fig. 27: *20 μm coronal frozen sections brain slices of naïve group of animals, counterstained with DAPI. First panel (a-c.1) shows the brain of a sham-animal injected with the vehicle solution (PBS+10%glycerol). Second panel (d-i) shows the brain of an animal injected with JΔβββ4 virus: mCherry signal is detected around the injection site (d-f, for higher magnification see d.1-f.1); moving to a more posterior level of the injection site (g-i) the expression of mCherry is still strong. Last panel (l-q) shows the brain of an animal injected with JΔNI virus. mCherry expression is detectable in the injection site (i-n) even though is not considerably extended (for higher magnification see i.1-n.1); some red spots are visible in a more posterior level (o-q).*

3.7 Naïve group: hematoxilin-eosin staining of coronal brain slices

To evaluate from a macroscopic point of view the ongoing damage evoked by viral injection, brain slices of naïve animals were stained with hematoxylin and eosin. Beside its usefulness to determine the integrity of the tissue, this staining give us some important indications regarding the accumulation and infiltration by cells of the immune system. The first evidence we could find out from these assay is the absence of either a strong immune response or extended tissue damage in proximity of the viral inoculation site. Even in this case we can detect some differences between the behavior of the two HSV-

1 vectors: JΔβββ4-based virus evoked a bigger infiltration of ectopic cells around the spot of injection, if compared to his counterpart JΔNI virus. This result is consistent with the previous in vitro data, confirming the differential toxicity exerted by the two vectors even though it seems to be restricted to the inoculation site (Fig. 28).

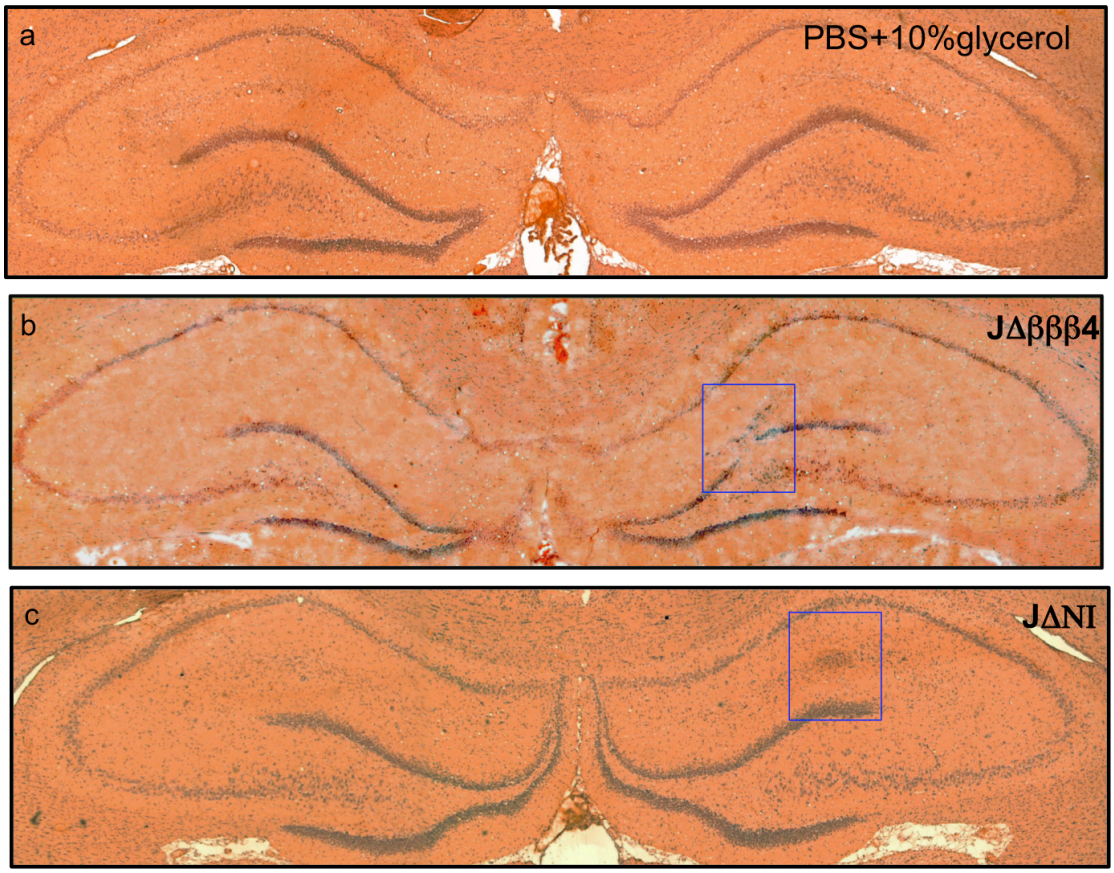

Fig. 28: *Hematoxylin-eosin staining of coronal frozen brain slice of naïve group of animals. Both hippocampi have been included in these pictures. a) sham animal, injected with the vehicle. b) JΔβββ4-virus injected brain. c) JΔNI-virus injected brain.*

3.8 Naïve group: Fluro-Jade staining of coronal brain slices

To investigate the eventual neuronal degeneration in the injected brains, we further stained the brain slices with Fluoro Jade, a fluorochrome particularly useful for our purposes since it labels degenerating neurons in ex vivo samples of central nervous system. However, we couldn't detect any positive signal 12 days post infection on slice brain of naïve group. We could just observe some positive cells in proximity of the injection site on the cortex surface, due certainly to mechanic injury caused by the glass needle (Fig. 29). We could then assess that after 12 days from infection, both viruses

don’t cause any neuronal degeneration.

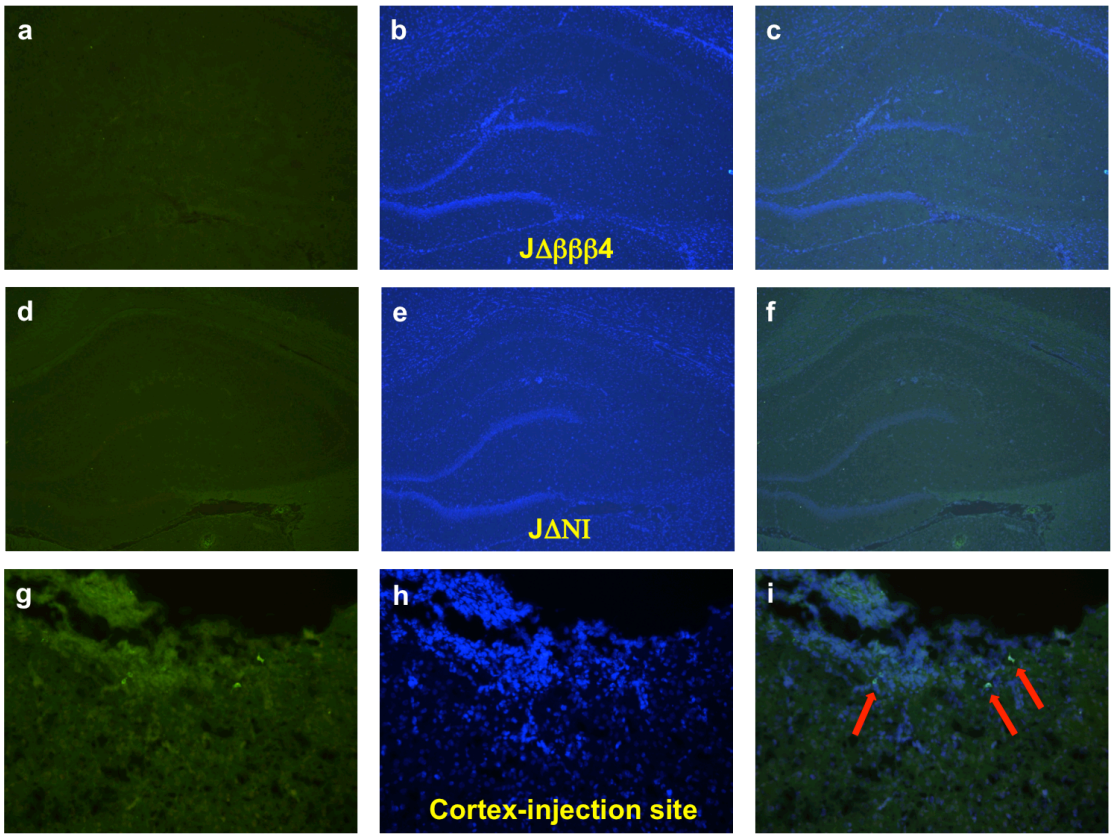

Fig. 29: *Fluoro Jade staining of JΔβββ4 (a-c) and JΔNI (d-f) injected brains. No positive cells were detected after infection with both viruses 12 days after infection. We can see just some positive cell on cortical surface (g-i), due to the mechanical damage caused by the entry of the needle.*

3.9 Effects on SE-Induced Astrocytosis and outcome of pathology

Once was stated that both JΔβββ4 and JΔNI viruses can efficiently infect cells of CNS upon injection in rat hippocampus, our attention turned on the effective target of this study, the animal models of epilepsy. Both the HSV-1 based vectors were tested on the pilocarpine SE–induced astrogliosis and epileptogenesis. In this epilepsy model, the pharmacologically evoked status epilepticus produces intense neuronal damage and, after a latent period of about 2 weeks, spontaneous seizures occur (Pitkanen et al., 2006). In the present experimental series, pilocarpine (370 mg/kg i.p.) rapidly induced a robust convulsive SE that was interrupted after 2 hours through administration of the anticonvulsant diazepam (10 mg/kg i.p.). According to Racine seizures scale observation (Racine, 1972), the severity of SE in the different animals was indistinguishable. This drug treatment caused damage in several brain areas: in particular hippocampal damage closely resembled human hippocampal sclerosis and was invariably remarkable

(Lehmann et al., 2000; Poirier et al., 2000; Paradiso et al., 2009).

Three days after SE, these lesioned animals were randomly assigned to five groups: the first two group were injected with the JΔNI-LHX2 and JΔβββ4-Lhx2, respectively; other two groups were injected with the relative control vectors (JΔβββ4 and JΔNI); and, finally, the last group was not treated at all. Since no significant differences were observed between these latter three groups in any of the parameters examined, they have been pooled together for subsequent analysis and collectively termed ‘‘control’’.

Viral vectors were stereotactically injected into the rat brain (a area between the molecular layer of DG and the radial layer of CA1 of the right hippocampus), three days after SE. At this time point, the pilocarpine-lesioned brain reacted to the injury by a strong increasing of cell proliferation, in an attempt to repair the neuronal loss. However, most of these cells differentiate into reactive astrocytes, giving rise to an epileptiform tissue and, consequently, eliciting epilepsy.

Animals were killed 28 days post SE (25 days after viral injection). During the last week, they were accurately video-monitored 24/24h to assay the number and the severity of the seizures for each subgroup (Fig. 26b).

To evaluate the pathology, the various experimental subgroups were compared to assess eventual differences in astrocytes:neurons ratio by cell staining with GFAP (astrocytic marker) and NeuroTrace (neuronal marker). The density of GFAP-positive cells in the entire hippocampus was significantly increased in pilocarpine-treated animals compared with naïve controls, an indication of reactive astrocytosis (Garzillo and Mello, 2002). Interestingly, in the subgroup of animals treated with both the vectors expressing Lhx2, the density of GFAP-positive cells appeared to be decreased in comparison with the control, as shown in Fig. 30. An intriguing data emerging from this analysis is the tangible differences in astrocytic density between the hippocampi injected with JΔNI-Lhx2 or JΔβββ4-Lhx2: for these latter, the astrocyte number is indeed much smaller than the JΔNI-Lhx2 subgroup in particular in the radial layer of CA1, a region mostly affected by astrocytosis after pilocarpine treatment (Becq et al., 2005; Parent et al., 1997; Parent et al., 2006). In the DG, instead, the difference with control in the number of astrocytes seemed smaller, probably because that area it’s too far from the injection site.

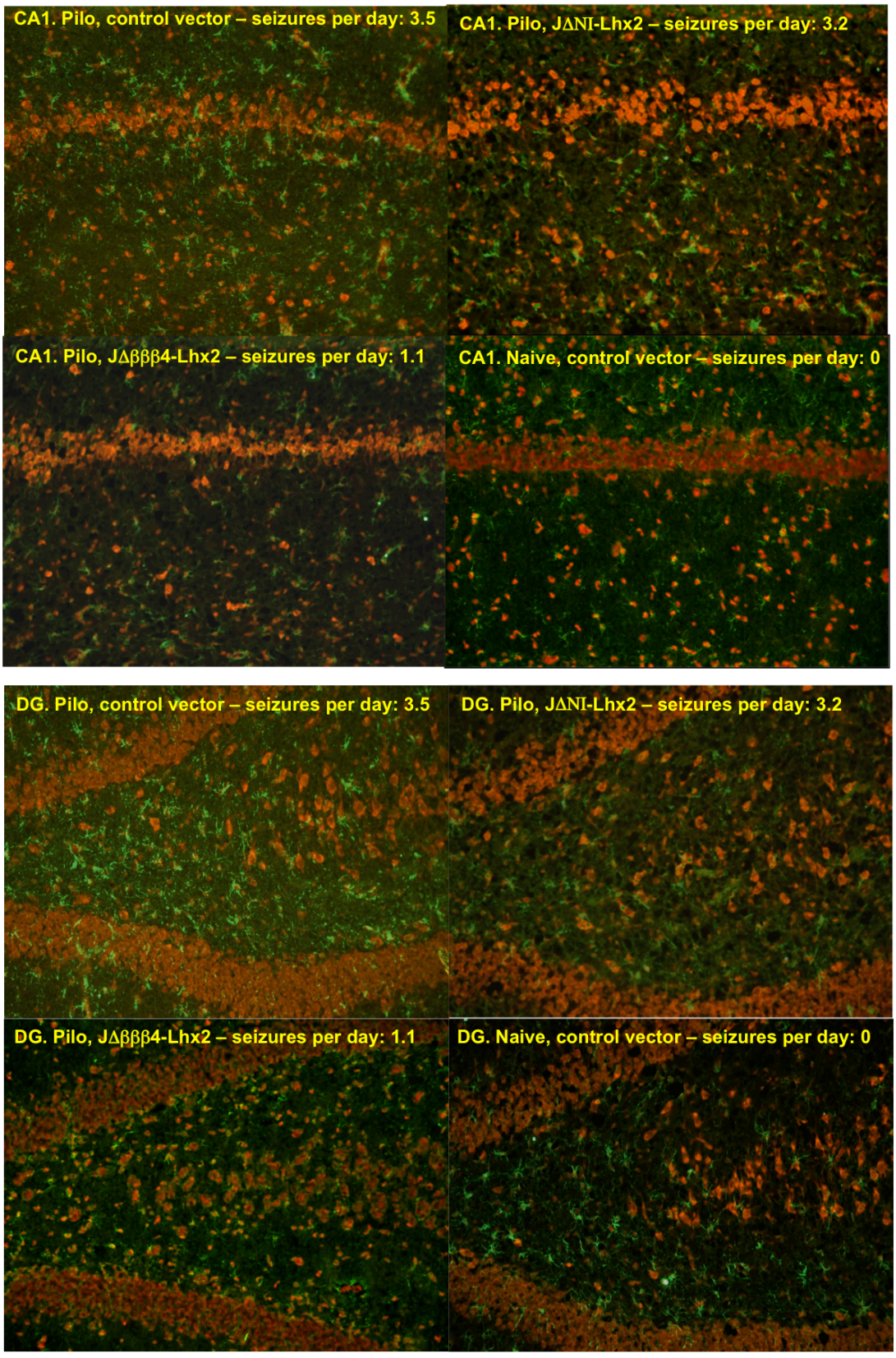

Fig. 30: *Immunofluorescence staining of astrocytes (GFAP, green) and neurons (NeuroTrace, red), on coronal frozen brain slices of pilocarpine-group of animals, 25 days upon viral injection. Upper panel shows the CA1 region (where injection has been performed), lower panel the dentate gyrus.*

The decreased reactive astrocytosis may imply a reduced hyperexcitability of the brain

and, consequently, a lower susceptibility to develop seizures. We then finally went to investigate if this beneficial effect could ameliorate the outcome of the pathology in terms of SRSs. We performed behavioral analyses during the last week of life of all the subgroup of animals, when the occurrence, severity, and duration of spontaneous recurrant seizures (SRSs) were recorded. As expected, all non vector-injected pilocarpine-treated rats exhibited, on average, about 3 spontaneous generalized seizures per day. Administration of the control vectors did not modify this pattern. Even the animals treated with the JΔNI-Lhx2 vector displayed a similar SRSs pattern. In contrast, rats injected with the JΔβββ4-Lhx2 vector showed an important improvement, with an average reduction of the seizures in the measure of 65% (Table 4)

	Control	**JΔNI-Lhx2**	**JΔβββ4-Lhx2**
Seizures per day (average)	3,5	3,2	1,1

Table 4: *Average of seizures per day of pilocarpine group of animals.*

Thus, the hippocampal injection of JΔβββ4-Lhx2 vector after the establishment of hippocampal sclerosis by pilocarpine treatment might provide both astrocytosis decrease and reduction of SRSs.

4. DISCUSSION

Epilepsy is most commonly treated with anti-epileptic drugs (AEDs). However, the pharmacological treatment is not a cure for epilepsy, but it just aims to try stop seizures. None of the AEDs developed up today can indeed prevent the progression of symptomatic epilepsies resulting from a known brain insult (i.e. head trauma, prolonged febrile seizures, brain infection, brain tumors, episode of status epilepticus, stroke). Moreover, AEDs have been demonstrated partially or totally ineffective to control seizures for more then 30% of patients; last option for the pharmacoresistant patient is usually the surgical resection of the epileptic focus, operation that could distress the person even more.

It is therefore necessary to identify novel strategies to meet this relevant clinical demand, preventing the development of disease or, at least, improving the clinical picture for millions of patients worldwide affected from this invalidating pathology.

Last years have been marked by a fluctuating but, after all, increased enthusiasm around gene therapy, an emerging new field of medical research that ambitiously promises to cure a wide range of untreatable diseases, including epilepsy. Novel approaches of gene therapy are constantly emerging, prompted by encouraging results from a variety of pre-clinical and clinical studies. Several strategies have been explored to achieve the highest efficiency for gene transfer while minimizing side effects, but the challenge to set up final tools for an efficient and safe gene delivery is still open. The most employed approach for gene delivery to the target cells take advantage of genetically engineered non-toxic viral vectors. To date, the systems which best couple high efficiency at transducing cells with transgene long-term expression are based on retroviral and lentiviral vectors. However, although these vectors can be considered highly useful tools for genetic analysis and pre-clinical studies, the hazardous drawbacks deriving from viral DNA insertion into the host genome are seriously limiting the access to clinical trials.

Thus far, gene therapy attempts to treat symptomatic epilepsies took advantage of recombinant adeno-associated or herpes simplex viruses. These latter strongly burst into the gene therapy scene by offering unique features that support their development as the best candidate viral vectors especially for targeting the nervous system. HSV is a highly infectious, naturally neurotropic virus with the largest cloning capacity among all other

viruses, able to establish life-long latency in neurons without any integration into the host genome, thus eliminating every possibility of insertional mutagenesis. HSV vectors could become the first choice for future approaches of gene therapy if residual drawbacks that still limit their use (such as toxicity and level/duration of transgene expression) will be overcome.

Riding this wave, we addressed our study on novel vectors based on Herpes Simplex Virus-1 for epilepsy treatment. In this work I have shown data on in vitro and in vivo testing of the first and the second generation of two HSV-1 recombinant vectors both generated by Dr. Yoshitaka Miyagawa in Glorioso's lab at University of Pittsburgh (unpublished data). Through recombinational cloning, I inserted the gene encoding for the transcription factor Lhx2 into both backbones (kindly shared by Dr. Miyagawa).

The decision to pick this gene for epilepsy gene therapy relies upon the characteristics of the Lhx2 protein, involved in the regulation of a wide range of processes from early embryogenesis to adult tissue homeostasis, including neural patterning of the central nervous system. Although its essential role as a "selector gene" for cerebralcortical fate during the very early stages of embryogenesis has been extensively demonstrated, recent experimental evidences have extended this view to a "novel" function for Lhx2 in the modulation of active neurogenesis in the post-natal central nervous system. In particular, Lhx2 could ensure a proper neurogenesis by inhibiting astrogliogenesis selectively in the hippocampus, preventing premature astrocyte formation (Subramanian et al., 2011). Because epileptogenesis is associated with loss of neurons and reactive astrogliogenesis, the key roles played by Lhx2 in neural specification of the adult hippocampus make it appealing for a gene therapy approach addressed to antiepileptogenic treatments. This was the rational of this thesis: targeting Lhx2 gene in specific hippocampal regions during brain remodelling after evoked status epilepticus might change the astrocytes-to-neurons ratio by increasing newborn neurons and not reactive glial cells. This intervention could potentially ameliorate the outcome of the disease, possibly preventing the aberrant transformation of injured brain tissue after an epileptogenic insult. Time of intervention is critical, since the goal is to interfere with the complex molecular and cellular modifications entailing the plastic reorganization of lesioned brain architecture during the time between the precipitating event and the first spontaneous seizure. In this seizure-free time, the injured brain reacts to the loss of pyramidal neurons by increasing the production of newborn cells in an attempt to restore the pre-existing cellular network. However, not all the newborn neurons will properly

integrate in the pre-existing circuits: some of them might adopt an ectopic localization, potentially giving rise to a new aberrant network triggering spontaneous discharges. Moreover, accumulating evidence suggest that a central role in the epileptiform transformation may be played by astrocytes. While astrocytes are essential to maintain the homeostasis of healthy brain tissue through diverse mechanisms, high proliferating reactive astrocytes usually take place near degenerating neurons upon severe brain injury. The altered pattern of gene expression and morphology of these newborn glial cells might lead to abnormal brain signals, promoting seizure development.

The aim of this work was to test the infection effectiveness of the novel Herpes-Simplex Virus 1-based vectors, to investigate about their toxicity and transgene expression through in vitro and in vivo assays and, further, to evaluate the outcome of the pathology after Lhx2 delivery in the hippocampus of animal models of epilepsy with HSV-1 based vectors.

Both the HSV-1 vectors used in this work have been engineered to be replication-incompetent by deletion of immediate early genes essential to replication. The first version, named JΔβββ4, is deleted for the immediate early genes ICP4 and ICP47; the other IE genes ICP0, ICP22 and ICP27 have been converted to β genes, driven from promoters of early genes. Thus, this vector does not express any viral functions but can be produced in complementing U2OS cells engineered to express ICP4 *in trans*. However, since a leaky expression of the βICP0 has been detected in human fibroblasts infected with the JΔβββ4 virus along with cytotoxicity, from this backbone the second generation vector, named JΔNI, has been derived by deletion of the ICP0 IE gene (Miyagawa et al., unpublished data).

To establish which, between the JΔβββ4 and JΔNI backbones, best meets our need, we decided to test both of them for in vitro and in vivo studies.

As expected, all in vitro experiments confirmed that cells infected with the JΔβββ4 vectors resulted in greater transgene expression if compared to JΔNI. However, this differential transgene expression was also correlated with highest toxicity of JΔβββ4 vector, highlighting how ICP0 function can strongly make the difference even when placed under the control of a weaker promoter. Nevertheless, even though the lack of ICP0 in JΔNI vectors significantly reduces the toxicity compared to its parental version, the effects on transgene expression are not detrimental: all cells infected in vitro with the JΔNI virus can indeed express detectable levels of transgene without showing strong signs of toxicity.

These results where consistent even upon infection of primary cultures of murine neurons and astrocytes, both cultures efficiently infected by the JΔβββ4 and the JΔNI vector along with a good transgene expression.

Encouraged by promising in vitro results, we decided to test these novel HSV-1 based vectors also in vivo. Even if several efforts have been made to find a good strategy to cross the blood-brain barrier after non-invasive peripheral administration of the vectors in the blood, these approaches still present several drawbacks that do not allow replacement of the "classical" stereotactical injection into the brain.

JΔβββ4 and JΔNI were then directly inoculated in the right hippocampus of rats, divided in two groups: the first one (naïve group) was established to assess the efficiency of in vivo infection and the possible toxicity evoked by the two viral backbones, the second one (pilocarpine group) was designed to get indications about potential effects of hippocampal Lhx2 delivery in on animal models of epilepsy.

Since both JΔβββ4 and JΔNI viral vectors have been engineered to express mCherry in infected cells, I could detect red signal in both JΔNI- and JΔβββ4-injected brains, in confirmation that are both able to efficiently infect CNS cells not only in vitro but also in vivo. As expected, the JΔβββ4 virus elicited a more extended infection or, at least, a stronger mCherry expression upon infection, confirming the central importance of ICP0 also in vivo.

Interestingly, even though the surgical procedure for injection could per se induce an immune cellular response with infiltration of blood cells into the brain through breaking of the blood-brain barrier, we did not detect a strong response mediated by host immune system nor a relevant cellular toxicity evoked by the two vectors 12 days after viral injection.

Finally, we investigated the consequences of Lhx2 gene delivery through JΔβββ4 and JΔNI on the lesioned hippocampi of pilocarpine-treated rats. Interestingly, Lhx2 delivery with both backbones seemed to be effective in decreasing the density of reactive astrocytes, usually substantially enhanced upon induced status epilepticus as shown by the lesioned-uninfected control brain. The most evident data resulting from these analyses is the substantial differences in astrocytosis between hippocampi injected with the JΔNI-Lhx2 and those injected with the JΔβββ4-Lhx2: these latter displayed a strong reduction in astrocyte number. The decreased reactive astrocytosis may imply a reduced hyperexcitability of the brain and, consequently, a lower susceptibility to seizures.

These ex vivo data were strengthened by analysis of the outcome of the pathology in terms of spontaneous recurrent seizures. Even though animals injected with the JΔNI-Lhx2 vector displayed a similar SRSs pattern of control subgroup, rats injected with the JΔβββ4-Lhx2 vector displayed a significant improvement of the clinical picture, undergoing an dramatic reduction of SRSs (about 65% compared to the control group).

In conclusion, the JΔβββ4 and JΔNI-based vectors may be useful tools for differential purposes: while for in vitro applications the JΔNI vector has been demonstrated to be the best compromise between transgene expression and low toxicity effects, for in vivo gene transfer it result almost ineffective; in contrast the JΔβββ4 vector has exactly the opposite features, too toxic for in vitro approaches but much more effective for in vivo gene delivery. Moreover, the in vivo data from this thesis confirmed our hypothesis on the potential therapeutic effects of Lhx2 protein delivered into the hippocampus of an animal model of epilepsy before SRSs, supporting this approach for the prevention of epilepsy.

This thesis reported initial data of an ongoing study. Several new generation vectors have been already generated to further improve transgene expression and to completely remove any residual toxicity. These new vectors could represent the “final tools” for gene transfer both in vitro and in vivo, in particular in the central nervous system, allowing the efficient delivery of several candidate genes we want to test for gene therapy of epilepsy, first of all Lhx2.

5. TABLE OF ARTICLES

During my PhD program I contributed to other projects. One of these is in press (has been just published on line); two other have been submitted. Here below I'm attaching the abstract of these papers.

5.1 Increased excitability in tat-transgenic mice: role of tat in HIV-related neurological disorders

Neurobiol Dis. 2013 Feb 27. [Epub ahead of print]

Increased excitability in tat-transgenic mice: role of tat in HIV-related neurological disorders.

Zucchini S, Pittaluga A, Brocca-Cofano E, Summa M, Fabris M, Michele RD, Bonaccorsi A, Busatto G, Barbanti-Brodano G, Altavilla G, Verlengia G, Cifelli P, Corallini A, Caputo A, Simonato M.

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Abstract

HIV-1 associated neurocognitive disorders (HAND) are a major complication of HIV-1 infection. The mechanism(s) underlying HAND are not completely understood but, based on in vitro studies, the HIV-1 Tat protein may play an important role. In this study, the effect of prolonged exposure to endogenously produced Tat in the brain was investigated using a tat-transgenic (TT) mouse model constitutively expressing the HIV-1 tat gene. We found that stimulus-evoked glutamate exocytosis in the hippocampus and cortex was significantly increased in TT as compared with wild-type control (CC) mice, while GABA exocytosis was unchanged in the hippocampus and decreased in the cortex. This suggests that Tat generates a latent hyper-excitability state, which favor the detrimental effects of neurotoxic and/or excitotoxic agents. To challenge this idea, TT mice were tested for susceptibility to kainate-induced seizures and neurodegeneration, and found to exhibit significantly greater responses to the convulsant agent than CC mice. These results support the concept that constitutive expression of tat in the brain generates a latent excitatory state, which may increase the negative effects of damaging insults. These events may play a key role in the development of HAND.

http://www.sciencedirect.com/science/article/pii/S0969996113000661

5.2 Bradykinin B_2 receptors increase hippocampal excitability and susceptibility to seizures in mice

Bradykinin B_2 receptors increase hippocampal excitability and susceptibility to seizures in mice

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Abstract

Bradykinin (BK) and its receptors (B1 and B2) may exert a role in the pathophysiology of certain CNS diseases, including epilepsy. In healthy tissues, B2 receptors are constitutively and widely expressed and B1 receptors are absent or expressed at very low levels, but both receptors, particularly B1, are up-regulated under many pathological conditions. Available data support the notion that up-regulation of B1 receptors in brain areas like amygdala, hippocampus and enthorinal cortex favors the development and maintenance of an epileptic condition. The role of B2 receptors, instead, is still unclear. In this study, we used two different models to investigate the susceptibility to seizures of B1 knockout (KO) and B2 KO mice. We found that B1KO are more susceptible to seizures compared with wild-type (WT) mice, and that this depends on B2 receptors, in that i) B2 receptors are overexpressed in limbic areas of B1 KO mice, including the hippocampus and the piriform cortex; ii) hippocampal slices prepared from B1 KO mice are more excitable than those prepared form WT controls, and this phenomenon is B2 receptor-dependent, being abolished by B2 antagonists; iii) kainate seizure severity is attenuated by pretreatment with a non-peptide B2 antagonist in WT and (more effectively) in B1 KO mice. These data highlight the possibility that B2 receptors may have a role in the responsiveness to epileptogenic insults and/or in the early period of epileptogenesis, that is, in the onset of the molecular and cellular events that lead to the transformation of a normal brain into epileptic.

5.3 Changes in the sensitivity of $GABA_A$ current rundown to drug treatments in a model of temporal lobe epilepsy

Changes in the sensitivity of $GABA_A$ current rundown to drug treatments in a model of temporal lobe epilepsy

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Abstract

The pharmacological treatment of temporal lobe epilepsy (TLE), the most common epileptic syndrome in adults, is still unsatisfactory, as one third of the patients are or become refractory to antiepileptic agents. It is thought that the disease may undergo a progressive evolution, which may affect the sensitivity to drugs. TLE has been shown to be associated with a dysfunction of the inhibitory signaling mediated by $GABA_A$ receptors. In particular, the repetitive activation of $GABA_A$ receptors produces a use-dependent decrease (rundown) of the evoked currents (I_{GABA}), which is markedly enhanced in the hippocampus and cortex of drug-resistant TLE patients. This phenomenon has been also observed in the pilocarpine model of TLE, where the increased I_{GABA} rundown is observed in the hippocampus at the time of the first spontaneous seizure, then extends to the cortex and remains constant in the chronic phase of the disease. Here, we examined the pharmacological modulation of I_{GABA} in the natural history of experimental TLE. We focused on the antiepileptic agent levetiracetam and on the neurotrophin BDNF, which were previously reported to attenuate TLE-induced increased rundown in the chronic human tissue. In the pilocarpine model, BDNF displayed a paramount effect, decreasing rundown in the hippocampus at the time of the first seizure, as well as in the hippocampus and cortex in the chronic period. In contrast, levetiracetam did not affect rundown in the hippocampus, but attenuated it in the cortex. Interestingly, this effect of levetiracetam was also observed on the still unaltered rundown observed in the cortex at the time of the first spontaneous seizure. These data suggest that the sensitivity of $GABA_A$ receptors to pharmacological interventions undergoes changes during the natural history of TLE, implicating that the timing of treatment may highly affect the therapeutic outcome.

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