UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE
IN MEDICINA MOLECOLARE

Curriculum in Neuroscience and Experimental Neurology

COMBINATORIAL GENE THERAPY FOR EPILEPSY

DoS: Professor Michele Simonato

Second Supervisor: Professor Dimitri Michael Kullmann

Tesi di DOTTORATO DI RICERCA di Stefano Cattaneo
Matr. 013919
Ciclo di dottorato XXXIV
SSD BIO/14

Anno Accademico 2021/2022

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

Il/la sottoscritto/*I* *Stefano Cattaneo*

Matricola / *registration number* *013919*

nato a/ *born at* *Mariano Comense (CO)*

il/*on* *30/07/1992*

autore della tesi di Dottorato di ricerca dal titolo / *author of the PhD Thesis titled Combinatorial gene therapy for epilepsy*

NON AUTORIZZA la Consultazione della tesi per 12 mesi /DOES NOT AUTHORIZE *the public release of the thesis for* 12 *months*

a partire dalla data di conseguimento del titolo e precisamente */ from the PhD thesis date, specifically*

Dal / *from* 08/04/2022 Al / *to* 08/04/2023

Poiché /*because*:

□ l'intera ricerca o parti di essa sono potenzialmente soggette a brevettabilità/ *The whole project or part of it might be subject to patentability;*

□ ci sono parti di tesi che sono già state sottoposte a un editore o sono in attesa di pubblicazione/ *Parts of the thesis have been or are being submitted to a publisher or are in press;*

E' fatto divieto di riprodurre, in tutto o in parte, quanto in essa contenuto / *Copyright the contents of the thesis in whole or in part is forbidden*

Data /Date 28/03/2022

Firma /Signature .....

DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.

All the results presented here were obtained by myself, except for:

1. Western blot experiments, ELISA assay, and lentiviral production were performed by Dott.ssa Barbara Bettegazzi, PhD, Ospedale San Raffaele, Milan, Italy.
2. Calcium measurement experiment were performed by Dott.ssa Barbara Bettegazzi in collaboration with Prof. Franca Codazzi, Ospedale San Raffaele, Milan, Italy.
3. JΔNI8 viral vector production was performed in collaboration with Dr. Gianluca Verlengia, PhD, Ospedale San Raffaele, Milan, Italy.
4. NGS Sequencing and analysis were performed by Dr.ssa Anna Sofia Tascini at the Center for Omics Science (Ospedale San Raffaele, Milan, Italy).

All sources of information are acknowledged by means of reference.

Some of the work contained in the present thesis have been previously published.

Part of the text of the introduction (p.35-48) were taken from:

1. Cattaneo, S. et al.; NPY and Gene Therapy for Epilepsy: How, When,.. and Y. Frontiers in Molecular Neuroscience (2021).

Result of "HSV-1 based amplicon vectors do not cause any electrophysiological alteration", Figure 16, figure legend, and relative methods were taken from:

2. Soukupová, M. et al.; Improvement of HSV-1 based amplicon vectors for a safe and long-lasting gene therapy in non-replicating cells. Molecular Therapy - Methods and Clinical Development (2021)

ACKNOWLEDGMENTS

I thank Dr.ssa Anna Fabiano and Dr. Alessio Cantore (San Raffaele Telethon Institute for Gene Therapy, Milan) for the Lentiviral titration.

I thank Bioviron® company for sharing the amplicon vector.

I thank Professor Flavia Valtorta for kindly providing TKO mice.

I thank Dr. Stefano Taverna for the help with the electrophysiology experiments.

I thank the Center for Omics Science and the Advanced Light and Electron Microscopy BioImaging Center (ALEMBIC, San Raffaele Scientific Institute) for the technical support.

ABSTRACT

Epilepsy is a neurological disease characterized by a persistent predisposition to generate seizures, that affects about 1% of the world population. About 30% of epileptic patients are drug-resistant, thus refractory to currently available anti-epileptic drugs (AEDs). Less than 10% of these drug-resistant patients are eligible for resective brain surgery, often due to generalized or multiple epileptic foci, or due to proximity of the epileptic focus to eloquent brain areas. Therefore, gene therapy may represent a doable approach for the unmet medical need of these patients.

Neuropeptide Y (NPY) can act as an endogenous anticonvulsant, in particular by activating its receptor Y2 which mediates an anti-epileptic effect. NPY expression is increased both in rodent and human hippocampal sections from temporal lobe epilepsy surgical samples, despite the strong loss of hilar GABAergic NPY+ interneurons. Therefore, NPY-based gene therapy may represent a novel approach for the treatment of focal epilepsies. Ideally, however, such vectors should contain multiple elements (at least NPY and Y2Rs driven by appropriate promoters).

In the past, great advancements in the field of viral vectors based on HSV-1 have been made by our laboratory. We therefore aimed at combining the potential of HSV vectors to accommodate large payloads with the complexity of the NPY system to create an "ideal" combinatorial therapeutic cassette. However, residual concerns on the safety and translatability of our new generation HSV-1 based vectors (named JΔNI8) let us first characterize their electrophysiological properties in primary neuronal culture, to assess both safety and efficacy profiles. Surprisingly and disappointingly, we show that mutations in the envelope glycoprotein B (gB), which is responsible for viral entry and cell fusion, might arise during viral vector production. In turn, mutated gB can increase firing frequency while reducing both input resistance and resting membrane potential of transduced neurons. Altogether, these data suggest that careful evaluation of envelope glycoproteins is needed to develop safe HSV-1 replication-defective vectors for the treatment of CNS disorders. We, therefore, decided to move to LV vectors, a more robustly characterized platform despite a more limited packaging capacity compared with HSV vectors.

To potentiate the protective effect of NPY, we developed a combinatorial gene therapy approach based on the expression of NPY together with its receptor (Y2). Since Y2

receptors act mainly pre-synaptically to reduce glutamate release by lowering Ca2+ influx, transgenes expression was driven by the minimal CamKII promoter, thereby biasing their expression in excitatory neurons. We characterized the ability of our lentiviral vectors to express NPY and its functional Y2 receptor in hippocampal neurons and mouse brains. Telemetry video-EEG monitoring was then used to assess the effect of the therapeutic genes on the epileptic phenotype of a genetic mouse model of epilepsy.

We found that the combined expression of NPY and Y2 is sufficient to reduce both the frequency and duration of seizures in the Synapsin triple-KO epilepsy model. These data further strengthen the hypothesis that strategies aimed at the delivery of NPY and Y2 may be successful for the treatment of epilepsy, particularly for pharmaco-resistant and genetic forms of the disease.

TABLE OF CONTENTS

Chapter I - ACRONYMS AND ABBREVIATIONS

Abbreviation	Name
aa	amino acid
AAV	Adeno associated virus
ACSF	Artificial cerebrospinal fluid
ASDs	Antiseizure drug
AHP	Afterhyperpolarization
ALD	Adrenoleukodystrophy
ALS	Amyotrophic lateral sclerosis
AmP	Aminopeptidase P
AMPA	α-amino-3- hydroxy-5-methyl-4-isoxazole propionic acid
ANT-DBS	Anterior nucleus of the thalamus - Deep brain stimulation
AP	Action potential
APV	DL-2-amino-5-phosphonopentanoic acid
ASD	Antiseizures drug
AV	Adenovirus
BAC	Bacterial artificial chromosome
bp	base pair
CA	*Cornu Ammonis*
CAG	CAG promoter
CBA	CBA promoter
CD	Canavan disease
CLN	Neuronal ceroid lipofuscinoses
CMV	Cytomegalovirus
CNS	Central nervous system
COSR	Center for Omics Science
CPON	C-terminal flanking peptide of neuropeptide-Y
cPPT	Central polypurine tract
DCV	Dense core vesicles
DG	*Dentate gyrus*
DIV	Days in vitro
DNA	Deoxyribonucleic acid
DP4	Dipeptidyl peptidase IV
E	Early gene
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay

EMA	European Medicines Agency
EPSP	Excitatory post synaptic potential
FDA	Food and Drug Administration
GABA	γ-aminobutyric acid
GAERS	Genetic Absence Epileptic Rats from Strasbourg
GAT-1	Sodium- and chloride-dependent GABA transporter 1
gB	Glycoprotein B
GCL	Granule cell layer
GEFS+	Generalized epilepsy with febrile seizures-plus
GFAP	Glial fibrillar acidic protein
EGFP	Enhanced Green fluorescent protein
GGE	Genetic generalized epilepsy
GLUT1DS	Glucose transport type 1 deficiency syndrome
GM1/2	Gangliosidosis
GPCR	G protein-coupled receptors
HD	Huntington disease
HEK293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICC	Immunocytochemistry
ICP	Infected cell protein
IE	Immediate early gene
IGV	Integrative Genome Viewer
IHC	Immunohistochemistry
ILAE	International league against epilepsy
IML	inner molecular layer
INS	Insertion
IRES	Internal ribosome entry site
IRL	Internal repeat long
IRS	Internal repeat short
ITR	Internal terminal repeat
JΔNI8	Joint deleted no immediate 8
JOINT	Herpes simplex virus joint region
KA	Kainic acid
kb	kilobase
KD	Krabbe disease
KO	Knockout

L	Late gene
LTR	Long Terminal Repeats
LV	Lentivirus
MAPK	mitogen-activated protein kinase
MES	maximal electroshock
MFS	Mossy fiber sprouting
miRNA	microRNA
MLD	Metachromatic Leukodystrophy
MPS	Mucopolysaccharidoses
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MSA	Multiple system atrophy
MW	Molecular weight
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NGS	Next generation sequencing
NMDA	N-methyl-d-aspartic acid
NPY	Neuropeptide Y
NSE	Neuron-specific enolase
OML	Outer molecular layer
PAM	peptidyl-glycin-α-amidating monooxygenase
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PFU	plague forming unit
PKU	Phenylketonuria
PND	Postnatal day
PNS	Peripheral nervous system
PP	pancreatic peptide
PRV	Pseudorabies virus
PTZ	pentylenetetrazol
PV	Parvalbumin
PYY	peptide YY
Rin	Input resistance
RMP	Resting membrane potential
RNA	Ribonucleic Acid
RRE	Rev Response Element
RT	Room temperature
RTT	Rett syndrome

SCN1A	Sodium voltage-gated channel alpha subunit 1
SE	Status epilepticus
shRNA	short hairpin RNA
siRNA	short interfering RNA
SMA	Spinal muscular atrophy
SNPs	Single nucleotide polymorphism
SRS	Spontaneous recurrent seizures
STXBP1	Syntaxin binding protein 1
SUDEP	Sudden unexpected death in epilepsy
SV2A	Synaptic vesicle glycoprotein 2A
SYNI	Synapsin I
TALENs	Transcription activator-like effector nucleases
TBI	Traumatic brain injury
TKO	Synapsin triple KO
TLE	Temporal lobe epilepsy
TRL	Terminal repeat long
TRS	Terminal repeat short
TTX	Tetrodotoxin
TU	Transducing unit
UL	Unique long
US	Unique short
VNS	Vagus nerve stimulation
VSV-G	Vesicular stomatitis virus G glycoprotein
WB	Western blot
YRs	Neuropeptide Y receptor
ZFNs	zinc finger nucleases
Ψ	Psi sequence

Chapter II - LIST OF FIGURES AND TABLES

Chapter III - INTRODUCTION

Epilepsy

More than 4.000 years have now passed from the first Assyrian and Babylonian reports on epilepsy. Through the ages, patients were poorly considered as such, but rather as possessed or human beings punished by any sort of divine figure. It was only during the 18th and 19th centuries, when medicine made advancements on a solid scientific base, that "sufferers" became recognized as patients (Magiorkinis et al, 2014). Unfortunately, despite a long time has passed, people that suffer from epilepsy are still strongly exposed to stigma and prejudice. Indeed, epilepsy not only damages health but also disrupts many aspects of life, imposing physical, psychological and social burdens on individuals.

Approximately 2.4 million new cases of epilepsy are diagnosed every year, with an estimation of over 70 million people affected worldwide. The incidence follows a bimodal distribution with the highest risk in the young and older population (Beghi & Giussani, 2018). Among neurological disorders, epilepsy is the first in the young's population (<40 years) and the third in the older one (Thijs *et al*, 2019; Feigin *et al*, 2020).

Regarding prevalence, a recent meta-analysis comprising 197 studies showed an approximated value of 0.6 - 0.7% which is not influenced by age, sex or study quality. Nonetheless, a higher prevalence is found in low- and middle-income countries (Fiest et al, 2017).

In Europe alone, more than 6 million people live with epilepsy and more than 300,000 new cases are diagnosed each year. Despite significant advances in knowledge and development of therapies, treatments are still unsatisfactory because of severe adverse effects and the fact that about 30% of the patients are drug-resistant, i.e., have inadequate seizure control with pharmacological therapy (Kwan & Brodie, 2009; Kwan et al, 2010). This condition is associated with high rates of depression, suicide, accidents, and social exclusion. Ultimately, patients with drug resistant epilepsy have a yearly mortality of 0.5-1% due to sudden unexpected death in epilepsy (SUDEP), which mostly affects young adults from 20 to 40 years old (Tomson *et al*, 2005).

Needless to say, the development of treatments for drug-resistant seizures and for epilepsy syndromes with few or poor treatment options; the improvement of tolerability of treatments; the identification of disease-modifying therapeutic strategies that prevent

or attenuate the progression of epilepsy; the development of treatments capable to prevent or ameliorate the common comorbidities that contribute to disability in people with epilepsy, like cognitive impairment, anxiety, and depression are all strong unmet medical needs that should be primarily taken in consideration by neuroscience researchers.

Epilepsy and seizures: not simple entities to define

It is clear to all the experts in the field that epilepsy is a complex and heterogenous disease that must be considered not as a single entity but rather as a multiplicity of disorders with many different facets. Intense efforts have been made by many organizations and in the last century the International League Against Epilepsy (ILAE) regularly provided news and updates on the classification and terminology used to define and diagnose this complex disease (Fisher *et al*, 2005). In any case, the main symptom of epilepsy is the spontaneous occurrence of seizures which have been defined as:

> *"transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain".*

Therefore, a general definition of epilepsy is:

> *"a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure".*

However, population-based studies demonstrated that nearly everyone may have at least one seizure during life in particular circumstances. More specifically, we all face a lifetime risk to experience a seizure of about 8-10%, but the chance of suffering from epilepsy goes down to 3%. Indeed, after the occurrence of the first unprovoked seizure, only 30-50% will recur; and after a second unprovoked seizure, only 70-80% will recur (Pohlmann-Eden *et al*, 2006).

Thus, in more recent years the definition of epilepsy has been refined adding more specific clinical parameters. Based on these, the disease can be diagnosed if any of the following criteria is satisfied (Fisher *et al*, 2014):

- *"At least two unprovoked (or reflex) seizures occurring >24 h apart".*
- *"One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years".*
- *"Diagnosis of an epilepsy syndrome".*

"Epilepsy is considered to be resolved for individuals who had an age-dependent epilepsy syndrome but are now past the applicable age or those who have remained seizure-free for the last 10 years, with no seizure medicines for the last 5 years".

Thus, looking at these parameters with renewed eyes, the diagnosis of epilepsy moved from "occurrence of at least one epileptic seizure" to a wider concept of probability of having seizures taking into account the propensity of individuals in developing seizures over time.

In this view, an important concept that must be introduced is the seizure threshold, a dynamic concept that changes over time referring to a theoretical line above which a person can develop a seizure. Epileptogenic abnormalities, which can be either structural, metabolic, genetic, or combinations of these, account for oscillations of the threshold. In normal individuals, these abnormalities may be marginally present but remain well separated from the seizure threshold, such that spontaneous seizures do not occur. In a person with epilepsy, epileptogenic abnormalities might be more pronounced or might lower the seizure threshold. Thus, the combination of abnormalities fluctuating in time might occasionally overcome the threshold leading to spontaneous seizures (Engel Jr. *et al*, 2013).

Classification of epilepsies and seizures

As for the definition of *epilepsy* itself, the classification of seizures is constantly updated based on the scientific progress. Properly classifying seizures and epilepsy is highly important for orienting the therapeutic approach.

Since current knowledge is still insufficient to provide a strong, scientifically based classification, in 2017 the ILAE revised the former operational classification into a new one based on three levels (Fisher *et al*, 2017).

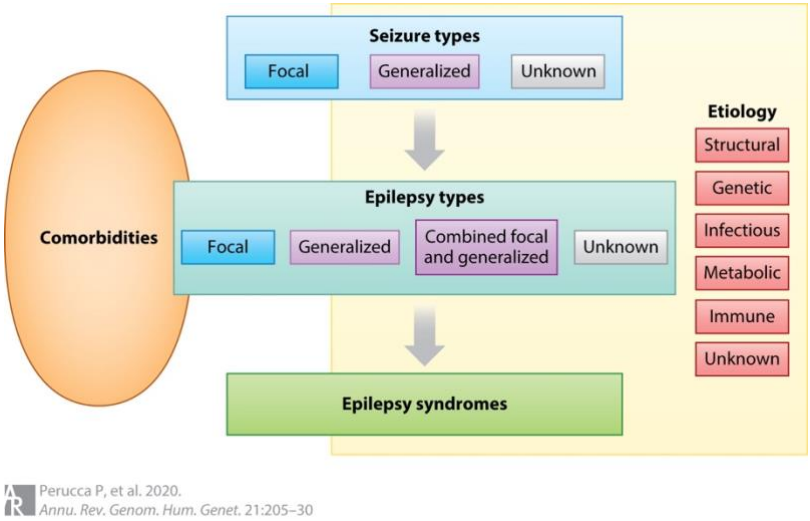

Figure 1 – International League Against Epilepsy 2017 classification of the epilepsies
Adapted from (Perucca et al*, 2020)*

As shown in Figure 1, the first level consists of defining seizure types, which can be generally categorized based on the onset. A generalized onset seizure involves the whole brain from the beginning of the manifestation while a focal onset seizure involves a limited part of the brain. If the onset cannot be identified in the initial manifestation, the seizure is then classified as unknown onset. As shown in Figure 2, categorization of seizures can be expanded adding other phenotypic features. These include preservation of awareness, presence or absence of motor behavior, type of motor signs. Note that the classification can be more or less refined depending on the experience of the person who is classifying the seizure (Fisher *et al*, 2017).

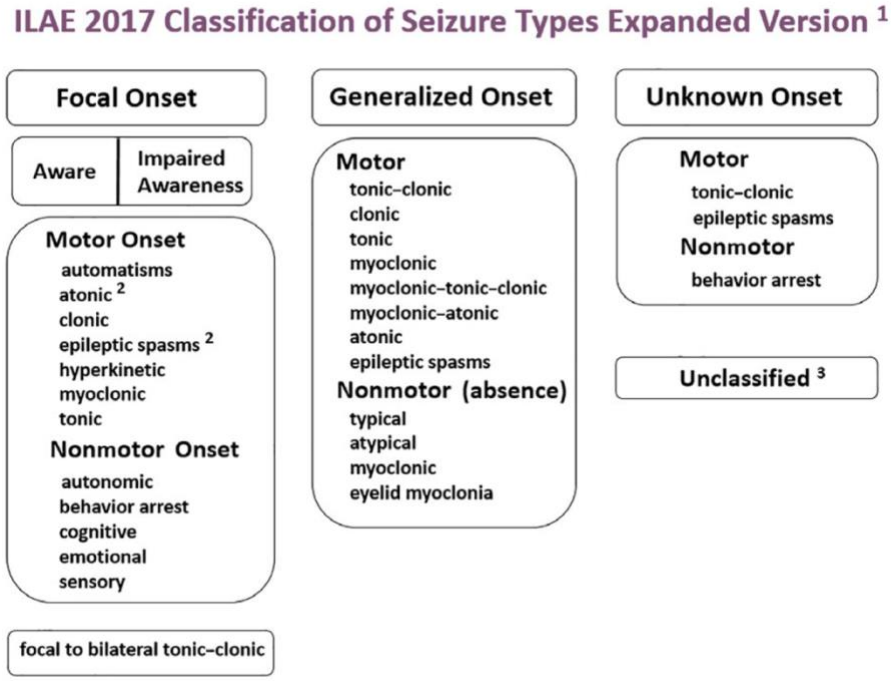

Figure 2 – International League Against Epilepsy 2017 operational classification of seizure types.
Adapted from (Fisher et al*, 2017)*

The second classification level is the epilepsy type. This level reflects that on seizure classification, categorizing epilepsy types as: focal, generalized, and unknown while adding the new group of combined focal and generalized epilepsy which reflects the existence of both types of seizures in the same patient.

The third level is the possible identification of an epilepsy syndrome, which often has an age-dependent presentation and a specific range of comorbidities. According to an ILAE report discussing the currently proposed definition (Wirrell *et al*, 2021) an epilepsy syndrome can be classified as *"a characteristic cluster of clinical and EEG features, often supported by specific etiological findings"*.

As mentioned above, people with epilepsy not only have seizures, but also face a wide number of comorbidities. It is important to include such comorbidities in the diagnosis as a fundamental part of the disease. Finally, this classification scheme emphasizes the importance of possibly identifying the etiology of the disease, which may be structural, genetic, infectious, metabolic, immune, or unknown. We will discuss this aspect in the following section.

Etiology

Back in 1975, a cause for epilepsy could be identified in only about 25% of the cases (Figure 3). Identifiable etiologies were stroke, head trauma, brain tumor, or brain infection; other cases (the large majority) were classified as "idiopathic", i.e., without cause (Hauser & Kurland, 1975). In the following years, some forms of epilepsy could be referred to autoimmune mechanisms (Quek *et al*, 2012) and the increased sensitivity of magnetic resonance imaging (MRI) allowed the identification of subtle lesions responsible of focal forms. However, the major advancement was to be able to overcome the term *idiopathic.* Most of the cases described as *idiopathic* in 1975 can now be classified into the complex group of genetic epilepsies. Indeed, the incredibly great advancement in gene sequencing technology coupled with refined epidemiological studies revealed that genetic factors are at the basis in these epilepsies (Corey *et al*, 2011).

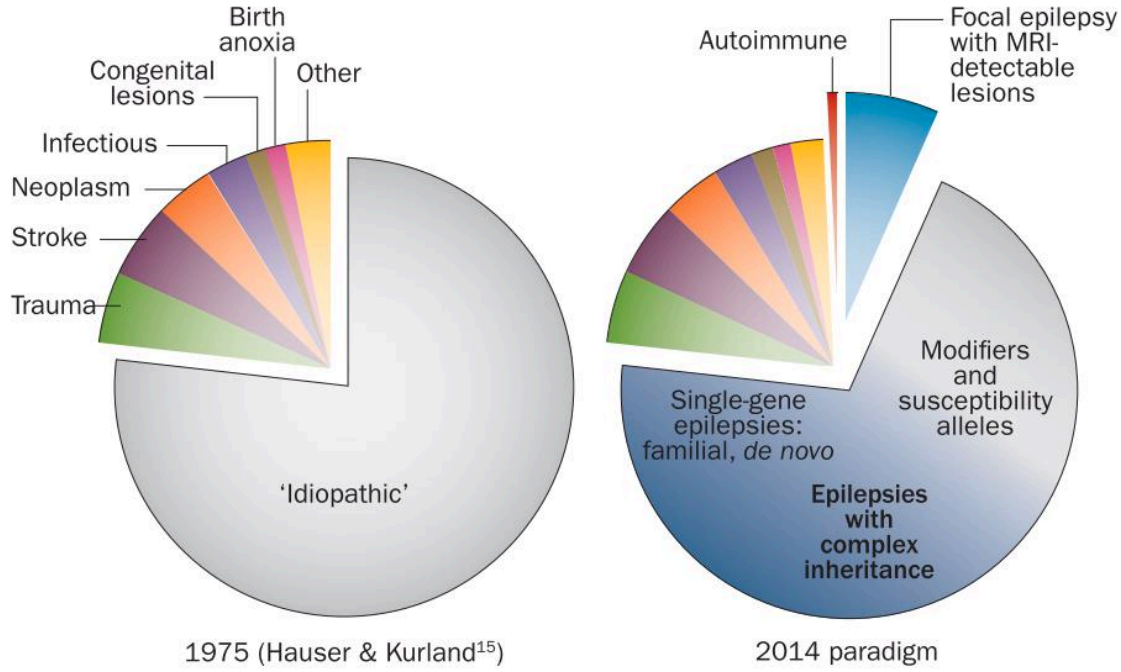

Figure 3 – Advances in understanding the causes of epilepsy
Adapted from (Thomas & Berkovic, 2014) License number: 5237160198194

A first group of genetic epilepsies comprises monogenic syndromes. Single gene, Mendelian inherited syndromes, in which seizures are the sole manifestation in most or all affected individuals are rare, and relatively rare are also cases in which seizures are occasional or comorbid features of the disease. Although more than one hundred single-gene causes of epilepsy have been identified, there is not a single gene that account for

more than 1% of total cases (Helbig & Ellis, 2020). More common in this group are de novo single-gene epilepsies, such as Generalized Epilepsy with Febrile seizures-plus (GEFS+) (Bonanni *et al*, 2004) and Dravet syndrome (Lopez-Santiago & Isom, 2019).

A second group is characterized by complex inheritance, for example oligogenic or polygenic mutations or inheritance of multiple susceptibility genes, with little or null environmental influence. This group is generally referred to as Genetic Generalized Epilepsy (GGE) and accounts for 15-20% of all the epilepsies (Jallon & Latour, 2005). GGE includes syndromes such as childhood absence epilepsy and juvenile myoclonic epilepsy.

In addition, genetic factors can also affect epilepsies that are categorized as acquired. For example, family history data suggest that a strong genetic contributes to post-stroke epilepsy (Eriksson *et al*, 2019). The presence of modifiers and susceptibility genes can increase the risk of suffering from these types of seizures and future studies are therefore needed to understand the underlying genetic vulnerability.

Current treatments for epilepsy

The first therapeutic option for epilepsy is the use of anti-seizure drugs, commonly known as antiseizure drugs (ASDs). These drugs are usually used as monotherapy and act at the level of the neuronal circuitry to decrease hyperexcitability by enhancing GABAergic transmission or by reducing excitation through the modulation of sodium, potassium or calcium conductance (Figure 4) (Löscher & Schmidt, 2011; Löscher & Rogawski, 2012; Hanaya & Arita, 2016). It is important to underline that ASDs cannot be considered disease-modifying agents because, at best, they can only guarantee a relief from the cardinal symptom, i.e., seizures, but do not modify comorbidities or the natural history of the disease. Moreover, as these drugs are administered systemically, they are also responsible for adverse effects due to actions on brain regions that are not directly affected by the pathology or by systemic effects of ASDs (Eddy *et al*, 2011).

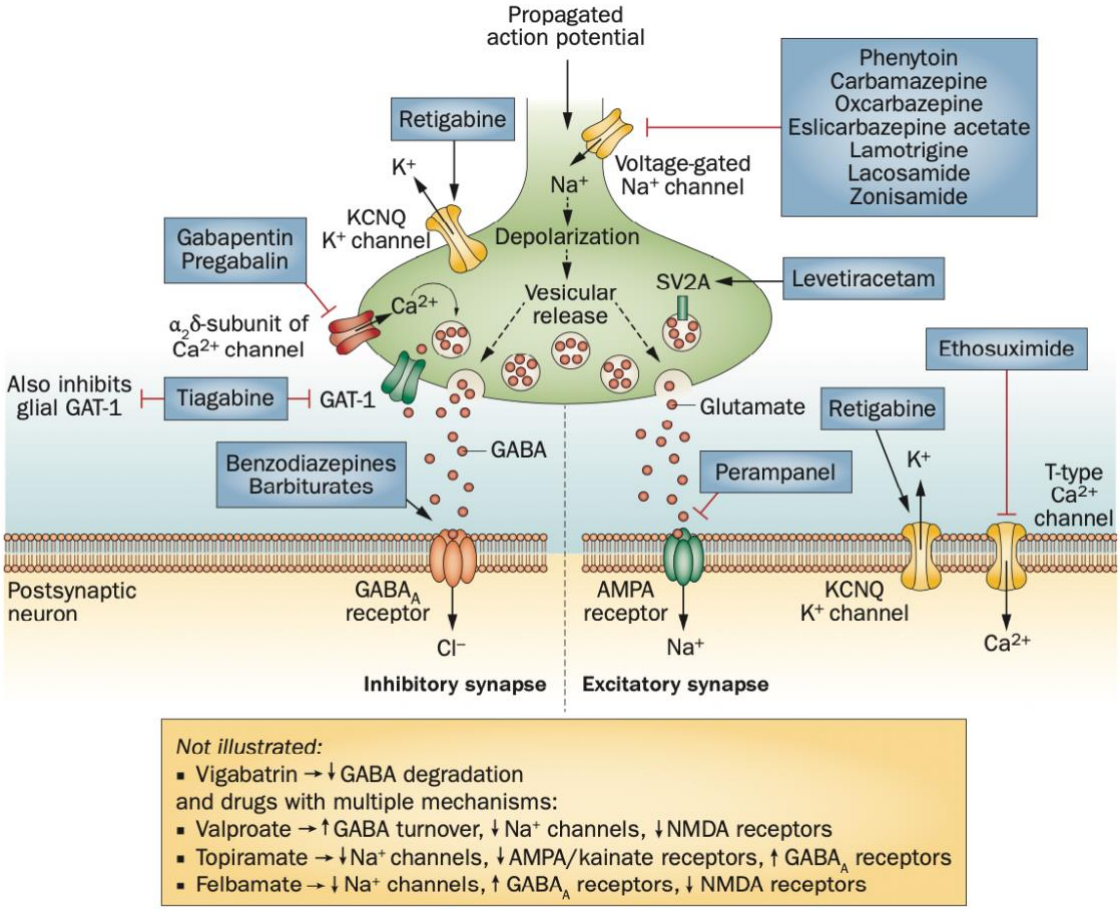

Figure 4 – Mechanism of action of antiseizures drugs.
Left half of the image represent effects primarily on inhibitory synapses. Right half shows mechanisms of action on excitatory terminals. Abbreviations: AMPA, α-amino-3- hydroxy-5-methyl-4-isoxazole propionic acid; GABA, γ-aminobutyric acid; GAT-1, sodium- and chloride-dependent GABA transporter 1; SV2A, synaptic vesicle glycoprotein 2A.
Adapted from (Löscher & Schmidt, 2012). License number:5237160507497

Drug resistance epilepsy and alternative treatments

In spite of significant advances in knowledge and development of therapies, more than 30% of the patients are drug-resistant where drug-resistance definition proposed by ILAE is as follow (Kwan, 2011; Tang *et al*, 2017):

> *"Failure of adequate trials of two tolerated and appropriately chosen and used ASD schedules (whether as monotherapy or in combination) to achieve sustained seizure freedom"*

The lack of alternatives for refractory epilepsy poses a big clinical problem. Hence, the need for innovative and new ways of treating patients is urgent (Simonato *et al*, 2013; Kullmann *et al*, 2014; Ingusci *et al*, 2019a). The first treatment option for this subgroup of patients is surgery. For individuals with single and identifiable seizure foci, resection of that specific portion of the brain could ameliorate symptoms and guarantee seizure-freedom in approximately 65% of the cases (West *et al*, 2019). However, despite its advantages, surgery is only applicable to a minority of patients because the epileptic focus might reside within eloquent area of the brain (Thijs *et al*, 2019). In addition, epilepsy surgery is a costly procedure that can be run exclusively in highly specialized centers, and therefore the majority of patients who may get benefit do not have access to it.

Other therapeutic options include deep brain stimulation of the anterior nucleus of the thalamus (ANT-DBS), vagus nerve stimulation (VNS), and closed-loop responsive neurostimulation of the epileptogenic zone. However, these therapies are considered essentially palliative, as seizure-freedom is acquired in a minority of the individuals and for a relative short time after treatment (Ryvlin *et al*, 2021).

ANT-DBS consists in implantable devices located in the anterior nucleus of thalamus. Even if the precise mechanisms of action of ANT-DBS remains elusive, chronic stimulation of this nucleus is thought to reinforce GABAergic transmission in the hippocampus, thereby increasing seizure threshold.

Differently from ANT-DBS, the close-loop responsive neurostimulation consists in implanting electrodes intracranially in the zone(s) of the suspected epileptic foci. The system provides continuous EEG monitoring and, upon detection of personalized patterns of ictal discharges, delivers short bursts of high frequency electrical stimulation to abort a potential seizure.

Another option for the treatment of drug-resistant patients that cannot undergo surgery is VNS. The technique consists in the implant of a stimulator device whose electrodes are surgically wrapped around the left vagus nerve. The device may act as a sort of pacemaker that controls hyperactive networks. The most recent closed-loop VNS devices automatically trigger stimulation and acts as neuromodulator which might decrease severity and duration of seizures or decrease post-ictal symptoms more than preventing seizures. However, only a few individuals achieve complete freedom from seizures and complications after surgery are still a concern (Giordano *et al*, 2017).

Finally, it has been shown in children that switching to a ketogenic diet, that has a high (90%) fat and low protein and carbohydrate content, can decrease seizure frequency. Unfortunately, it is very difficult to remain compliant to this diet (D'Andrea Meira *et al*, 2019).

Pre-clinical models of epilepsy

As discussed in the previous section, there is an urgent demand to address the unmet clinical needs of drug-resistant epilepsy and epileptic syndromes with few or no therapeutic options. Unfortunately, many of the ASDs introduced in the clinics in the past decades failed to deliver (i.e., the percentage of drug-resistance patients did not significantly change), most likely because of limited predictivity of available animal models and of the gap of knowledge on the pathophysiology of most human epilepsies (Löscher & Schmidt, 2011). Historically, seizure models such as the maximal electroshock (MES), the pentylenetetrazol (PTZ) test, and the electrical kindling model have been extensively used for screening (Simonato *et al*, 2014). However, acutely evoked seizures in a normal brain are likely different from spontaneous seizures in a chronically epileptic brain.

Indeed, induced animal models are subdivided in two main groups, acute seizures model and chronic models. The first group consists of models in which no evidence of persisting changes in seizures threshold nor of occurrence of spontaneous seizures is observed. Often, these models include exposure to provoking stimuli such as chemoconvulsants, electrical stimulation, hypoxia, or hyperthermia. These models have the advantage of medium-high throughput for screening purposes, but also have the limitation of identifying only restricted anti-seizures drugs, because the underlying pathophysiological mechanisms of epilepsy are not modeled (French *et al*, 2013).

On the other hand, we have chronic models of epilepsy that typically include post-insult (trauma, stroke, status epilepticus, SE) or genetic models (see below). What these models have in common is the occurrence of an epileptogenic process, i.e., the development and the re-modelling of a tissue that became capable of spontaneously generating seizures in mid/long-term studies (Pitkänen *et al*, 2013). Given the obvious disadvantage of not being suitable for high-throughput screening, their main advantages are that they better represent the human condition and represent a better approach to

model the mechanisms underlying epilepsy development and spontaneous seizures. On the other side, a specific inciting insult (e.g., stroke, chemically induced SE) might not produce the identical alterations of another, raising the need of validating therapeutic approaches in several models before proceeding to clinical translation.

Chronic models of epilepsy

Among chronic models, the most used are the post-SE models of epilepsy. This category is characterized by a stimulus, that can be either electrical or chemical, which gives rise to SE and triggers epileptogenesis. Reflecting what happens in human, SE is followed by a latency period in which no seizures occur, and by the subsequent appearance of spontaneous recurrent seizures (SRS) mostly originating from the limbic system. Kainic acid and pilocarpine are the most commonly employed chemoconvulsants (Lévesque *et al*, 2016), administered either systemically or intracerebrally. Pilocarpine produces SE by activating the muscarinic receptor M1 and is usually administered systemically (Curia *et al*, 2008). Kainic acid, a glutamate analogue, triggers excitotoxicity by activating KA receptor and can be administered peripherally or delivered through intracerebral injection in either the hippocampus or in the amygdala (Henshall, 2017).

A different, commonly employed category of chronic model is the traumatic brain injury (TBI), which is based on a mechanical insult that can trigger disease development like in the corresponding human etiology (Thompson *et al*, 2005).

Genetic models of epilepsy

As described above, more than one hundred epileptogenic single-gene mutations have been identified in the past few decades. Most of these have been identified due to their huge impact on brain function. For the most, they affect genes encoding ion channels. However, the most modern sequencing techniques allowed to identify many other epileptogenic genes that encode proteins with other roles. These include proteins involved in synaptic vesicle recycling, such as Synapsin I (SYNI) or Syntaxin binding protein 1 (STXBP1) (Dhindsa *et al*, 2015), protein synthesis (Lam *et al*, 2016), basic metabolic functions like glucose transport (Brockmann *et al*, 2001). Of note, many of these newly identified genes cause epileptic phenotypes that overlap with autism, intellectual disability or other neurodevelopmental disorders. In addition, intragenic variability

should be considered. For example, there are cases in which a missense mutation or deletion on different domains of the same gene can lead to either loss or gain of function, resulting in different phenotypic features.

These animal models can be instrumental for understanding the consequences of specific mutations. A successful example of this has been obtained for patients with glucose transport type 1 deficiency syndrome (GLUT1DS). These patients can rely on ketogenic diet to provide ketone bodies as energy sources for neurons, obtaining a good control of seizures (Fujii *et al*, 2016).

Whereas models of acquired epilepsy generally display a high number of seizures, making the effects of test therapies relatively easily measurable, genetic models are often either too severe (i.e., seizures may be fatal (Zeng *et al*, 2011)) or too mild (i.e., seizures may be too rare (Greco *et al*, 2013; Singh *et al*, 2008)). However, genetic models may very adequately recapitulate a human genetic disease and, therefore, provide a reliable representation of the pathological mechanisms underlying the development of seizures. Altogether, it is advisable to use both induced and genetic models in order to maximize the odds of developing effective new treatments.

Gene therapy for the central nervous system

As discussed above, the treatment of drug-resistant forms of epilepsy is largely unsatisfactory, posing the issue of identifying new, effective alternatives to those currently available. In this context, gene therapy is now emerging as a doable approach. Gene therapy can be viewed as a heterogenous discipline. In principle, the concept is easy: “Gene therapy is a technique that modifies a person’s genes to treat or cure a disease” (FDA Research, 2020). However, designing and implementing such a technique is far from being easy, because the process exploits several disciplines from basic biology to technological development, and clinical practice. That having said, the potential is enormous, with the promise of developing new therapies not only for rare genetic but also for common diseases (Naldini, 2015; Kullmann *et al*, 2014).

In the most general sense, gene therapy encompasses all the many different approaches that aim at modifying or manipulating the expression of genes, altering the biological properties of living cells for therapeutic use. It can be divided into two major groups: ex-vivo and in-vivo gene therapy. The first involves engineering the patient’s cells in-vitro

and then re-infusing or implanting them directly into the affected tissue. The in-vivo approach is based on the direct gene transfer in the diseased tissue by using viral or non-viral vectors.

To date, the main method for in-vivo gene therapy approaches is the use of viral vectors, and this will be the focus of the present thesis. For such an approach to be successful, there are many features to consider in order to optimize the natural ability of viruses to transfer genetic material into target cells. From a historical point of view, the first gene therapy attempts led to adverse events that prevented the possibility of entering clinical trials for about a decade (Hacein-Bey-Abina *et al*, 2003; Raper *et al*, 2003). Today, many (but not all) questions regarding the safety of gene therapy approaches have been addressed. Based on the number of clinical trials currently underway and planned, it can be expected that several new gene therapy approaches will become available soon (FDA Commissioner, 2021). Needless to say, this will require a detailed characterization of the short- and long-term effects of each approach, the safety, and the pharmacokinetics of each viral platform.

Classically, drug development attempts are made to identify new therapies for diseases affecting a large proportion of the population. In the case of gene therapy, there has been a paradigm shift, both because of the very nature of this approach and because of the initial need to treat diseases with very low incidence. Indeed, rare genetic diseases proved particularly suited for early trials of gene therapy because the underlying genetic problem is (at least partially) known.

There are sixty-one gene therapy trials currently underway for the treatment of rare genetic diseases affecting only the CNS (Jensen *et al*, 2021) (Figure 5). In addition, many other trials have been attempted or are currently ongoing for more common diseases such as Parkinson's (Axelsen & Woldbye).

TABLE 1 | Overview of clinical gene therapy trials for groups of rare genetic diseases affecting the brain and spinal cord found on https://clinicaltrials.gov 1st of May 2021.

Group	Disease	Prevalence per 100,000	Number of Trials	Clinical Trial Phase Reached
Neurodegenerative Disorders	Spinal Muscular Atrophy (SMA)	10	9	Gene therapy market approval by FDA and EMA (Zolgensma®)
	Multiple System Atrophy (MSA)	2	1	Gene therapy trial planned, not yet recruiting
	Amyotrophic Lateral Sclerosis (ALS)	5	1	Compassionate-use study in two patients
	Huntington's Disease (HD)	3	1	Phase 1/2 trial
Lysosomal Storage Diseases	Batten Disease (CLN, Neuronal Ceroid Lipofuscinoses)	2–4	6	Phase 1/2 trials and LTFU
	Krabbe Disease (KD)	1	2	Phase 1/2 trials
	Metachromatic Leukodystrophy (MLD)	1–2	6	*Ex vivo* autologous hematopoietic stem cell gene therapy approved by EMA (Libmeldy®)
	Mucopolysaccharidoses (MPS)	4	19	Phase 2/3 trials
Neurometabolic Disorders	Canavan Disease (CD)	<16	3	Phase 1/2 trials
	X-linked Adrenoleukodystrophy (ALD)	7	5	Phase 2/3 trial
	Phenylketonuria (PKU)	10	3	Phase 1/2 trials
	Gangliosidoses (GM1/2)	1	5	Phase 1/2 trials

Figure 5 – Overview of clinical gene therapy trials for rare genetic disease.
Modified from (Jensen et al*, 2021)*

Gene therapy for epilepsy

Concerning epilepsy, several syndromes fall within the group of rare diseases. These include the Dravet syndrome (severe myoclonic epilepsy in infancy) which affects 2.5 in 100,000 children (Lopez-Santiago & Isom, 2019), the Lennox-Gastaut syndrome (15 in 100,000)(Asadi-Pooya, 2018), West syndrome (infantile spasms; 8 in 100,000)(Pavone *et al*, 2020), and Angelman syndrome (5-8 in 100,000) ((Buiting *et al*, 2016). These are, for the most, drug-resistant epilepsies with onset in the first months or years of life. Hence, intervening employing genetic approaches seems rationale and promising. Although the genetic background can be heterogeneous in each of these diseases, alterations in a single gene is often responsible for the majority of cases. For example, the lost-of-function mutation in the SCN1A gene results in voltage-gated sodium channels with a non-functional NaV1.1 subunit primarily in Parvalbumin (PV) GABAergic neurons and leads to hyperexcitability and seizures associated with a high risk of sudden infant death in Dravet syndrome (Samanta, 2020). Gene therapy approaches aimed at correcting this gene may therefore be attempted. For example, preclinical results, presented in a scientific poster entitled "*A GABA-Selective AAV Vector-Based Approach to Up-Regulate Endogenous Scn1a Expression Reverses Key Phenotypes in a Mouse Model of Dravet Syndrome*" during the 2019 Annual Meeting of the American Society of Gene & Cell Therapy, report that a single injection of an AAV vector (ETX101) mediating increased production of functional copies of SCN1A specifically in Parvalbumin GABAergic interneurons lead to decreased seizure frequency and severity

as well as lower mortality in a Dravet mouse model. In addition, genome editing approaches have also been explored. CRISPR/Cas9-based gene therapy triggering SCN1A transcription in inhibitory neurons ameliorated seizures in Dravet mice (Colasante *et al*, 2020).

The great effort and the pioneering studies on pre-clinical models of rare genetic epileptic syndromes paved the way for gene therapy approaches. However, gene therapy is now trying to target general disease mechanisms underlying seizure development more than single mutated gene (Jensen *et al*, 2021). In this regard, anti-seizures approaches are underway targeting hyperexcitability, a common pathophysiological trait of epilepsy, rather than the primary cause. In particular, a clinical trial involving intracerebral inoculation of viral vectors mediating focal overexpression of engineered Kv1.1 potassium channels (NCT04601974 (Snowball *et al*, 2019)) is ongoing, while promising strategies based on the inhibitory neuropeptide Y and its antiepileptic receptor Y2 (AAV1-NPY-IRES-Y2 (Szczygieł *et al*, 2020) have been developed.

These approaches can be offered to patients with drug-resistant focal epilepsies selected for surgical resection. Viral injection would be then confined in a specific brain area, such that transgene over-expression would be restrained within the epileptogenic lesion only. This would abolish or lower the risk of affecting healthy brain tissue and, therefore, the risk of unpredictable side effects. In addition, should the treatment not prove effective or well-tolerated, patients would undergo resective surgery as originally planned.

Types of gene therapy

There are three main classes of gene therapy approaches: gene addition, gene suppression and gene editing. Of these, the most straightforward form is gene addition, which has the goal to transfer a therapeutic gene into defective cells and express it long-term and at a high enough level to restore the lost physiological function. Gene suppression instead aims at "switching off" a gene that acquired a gain of function. To this aim, RNA interfering strategies are usually employed. Last, both gene addition and suppression can be achieved through currently available gene editing tools such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the CRISPR/Cas systems. In more general term, however, gene editing comprise also

approaches that span from adding any desired modification to the genome of a cell to epigenetic manipulation (Lubroth *et al*, 2021).

Key elements of the gene transfer

When we think about gene therapy we are dealing with a series of parameters and factors that are important for the choice of a strategy. First of all, **transgenes**, the element that will be transferred, which include a whole variety of possibilities such as protein coding genes, DNA encoding short hairpin RNAs (shRNA), small interfering RNAs (siRNA), microRNAs (miRNA), or antisense RNAs. In order to efficiently transfer the desired transgenes, these should be engineered in an **expression cassette**, a system that is usually built up with a promoter and additional transcriptional elements. This system should be compact enough to be introduced into the delivery vehicle and to be functional once in the cell.

Vector represents the vehicle used to deliver the expression cassette that contains the transgene(s). Various types of vectors can be employed, and the choice depends on multiple factors, primarily the route of administration. For *ex vivo* administration, in which the cells to be treated are isolated, the possibilities are much wider as one can use non-viral transfection agents, such as chemical systems based on calcium phosphate, electroporation, lipofection or nanoparticles, all systems that are quite effective ex vivo, but much more difficult to use in vivo.

For *in vivo* delivery, instead, we rely on the biological systems that have evolved over millions of years precisely to enter tissues and cells and transfer their genes, i.e., the viruses.

Viral-mediated gene transfer in neuroscience

Viral-mediated gene transfer approaches are the most promising in the field of neuroscience, especially for in vivo approaches (Nectow & Nestler, 2020). To properly select the best viral vector platform there are some essential key principles to analyze. In this section I will summarize these universal principles and apply them to the most promising viral vectors currently in use.

First, we must define **viral packaging limit** and **payload**. The first denotes how much nucleic acid a viral particle can carry, comprising both the therapeutic cassette and the

original viral genome sequence that cannot be removed for the proper function of the viral particles. Consequently, the payload is the length and type (depending on the Baltimore classification[1] of the employed virus (Baltimore, 1971)) of genomic material that can be successfully packaged into a particle. This is an important limitation because exciding the packaging capacity brings to a lower yield during viral vector preparation or even to the impossibility to produce viral particles. Indeed, in vivo studies in rodents demonstrate that, to achieve sufficient expression in a high number of targeted cells within a given brain region, injection of very large numbers of particles is often required, i.e., high titres of viral vectors are needed (Chan *et al*, 2017; Szczygieł *et al*, 2020; Soukupová *et al*, 2021).

A second issue is the **delivery method**, which can be via local or systemic injections. Local administration is usually performed by stereotaxic surgery, confining the delivery in a specific brain area of the CNS or in the periphery, whereas systemic administration is usually via the bloodstream (e.g., tail vein injection). When translated to currently ongoing clinical trials in humans the route of administration can be further expanded and comprise intracerebral, intracisterna magna, intracerebroventricular, intrathecal, and intravenous. However, not all viruses can be delivered in all ways and anatomical barriers must be considered (Piguet *et al*, 2021).

Third, **tropism**, i.e., the ability of a virus to infect a specific cell type(s). Indeed, to obtain a productive infection a virus must successfully attach, entry and express its gene(s) in the targeted cell. Although some viruses have a natural neurotropism, genetic engineering and the process of "pseudotyping" brought other viral vectors into the neuroscience field (Kato *et al*, 2014). For example, pseudotyping also conferred new features to AAV vectors, such as an enhanced spreading, non-neuronal tropism or the ability to cross the blood-brain barrier (Domenger & Grimm, 2019).

---

[1] Established in 1971 by David Baltimore, the homonymous Classification subdivides viruses in seven groups accordingly to the nature of their genome and replication cycle.

Group I (dsDNA, double stranded DNA virus),

Group II (ssDNA, single stranded DNA virus),

Group III (dsDNA, double stranded RNA virus),

Group IV (ssRNA+, single positive-stranded RNA virus),

Group V (ssRNA-, single negative-stranded RNA virus),

Group VI (ssRNA RT, single stranded RNA with retro-transcriptional activity),

Group VII (dsDNA RT, double stranded DNA with retro-transcriptional activity).

Similarly, **access** is defined as the ability of a virus to enter a cell type and express its gene product(s). This feature is obviously strictly dependent on the attachment and entry process, but it is also strongly related to the construction of the expression cassette. An example is the use of specific promoter or - for animal models only - the use of molecular logic operations such as Cre or Flp recombinase to activate or inhibit expression (Atasoy *et al*, 2008; Fenno *et al*, 2014).

Infectivity and toxicity are closely related but different entities. The first refers to how efficiently a virus infects a cell, while the latter refers to how harmful the virus can be for the cell. Hence, the ideal viral vector should be highly infective and nominally not toxic. It should also be taken into account that all cells possess a "defensive system" to recognize foreign proteins and nucleic acids that in extreme cases can directly induce apoptosis or release of pro-inflammatory cytokines. Moreover, subtle changes can also be present such as transcriptional alteration (He *et al*, 2019) or fusogenic events (McCarthy *et al*, 2009) that can strongly alter cell physiology following viral infection. Indeed, many of the vectors that will be described in the following section are highly defective and devoted of many viral genes which are only delivered *in trans*; however, care must be used to avoid the restoring of wild type gene or the carry-over of replication competent viruses.

A last issue to consider are the **transgene expression dynamics**, defined as the time course of onset and persistence of transgene expression, where onset is defined as the peak of maximal expression of the transgene and persistence is the duration of the detectable overexpression (Soukupová *et al*, 2021).

Altogether, these principles play a fundamental role in determining the choice of the vector when designing a gene therapy approach, and they will be more specifically analyzed for the most promising viral vector platforms in the following sections.

Adeno-Associated Viral vectors

Adeno-Associated Viruses (AAV) are defective *parvoviruses* that are not able to replicate independently but depend on a helper virus, which is usually an adenovirus or a herpesvirus. In the absence of a helper, they establish a latent infection with frequent integration of their DNA into that of the host cell (apparently in a specific region of chromosome 19). From here the viral DNA can be excised and induced to replicate when

the cell is superinfected by a helper. Together, their apathogenicity, the fact that they induce a weak immune response, their broad tissue specificity give them a huge potential for the application in the gene therapy field. They are one of the smallest proteinaceous viruses, with a capsid diameter of about 20 nm that contains a genome of about 4.7 Kb belonging to the Baltimore Classification II (ssDNA). The AAV genome only contains two genes, *rev* and *cap,* which are included into two 145bp *internal terminal repeats* (ITR) (Ingusci *et al*, 2019b).

When constructing an AAV transfer plasmid, transgenes of interest are placed between the ITR. The first generations of AAV-based viral vectors were prepared using a helper virus, resulting in potentially contaminated stocks (Conway *et al*, 1999). However, this hurdle was easily overcome by providing AAV *rep* and *cap* genes *in trans* by a first plasmid, and the helper genes (such as E4, E2a and VA from Adenovirus) with a second "helper" plasmid. Finally, the plasmids are transfected into E1 complementing Hek293 cells to produce AAV particles. Notably, with the removal of *rep* from the vector genome, AAV vectors lose their integration-specific ability, increasing their potential for CNS gene therapy approaches.

Tacking advantage of the possibility to provide *rep* and *cap* by a separate plasmid, it has been possible to exploit another feature of AAVs, i.e., their broad number of serotypes. Indeed, the set of proteins expressed on the surface is different among different serotypes of wt AAVs, which broadens the spectrum of cellular receptors they can recognize. Combining genes of different serotype allowed scientists to modify and tune the tropism according to the specific need (Zinn & Vandenberghe, 2014).

The very limited payload capacity is a major issue for AAV based vectors. Owing to the small viral packaging limit, AAV vectors can accommodate less than 4.5kb of exogenous payload. Although different strategies are under investigation to resolve this issue, so far they all converge on the idea of splitting the gene(s) of interest into two or three different AAV vectors (Maddalena *et al*, 2018). Technically, these strategies lay either (1) on the innate AAV ability to undergo a genomic intermolecular recombination that can give rise to head-to-tail DNA concatamerization (Yang *et al*, 1999) resulting in the reconstitution of the desired transgene, or (2) on the implementation of Intein in frame with the transgene, i.e. of a protein splicing system, which, once has taken place, is able to reconstitute the original protein in the target cell (Tornabene *et al*, 2019).

Altogether, AAVs are probably the most promising tools for delivery of therapeutic genes in the CNS, due to their good safety profile and transduction efficiency. Indeed, two AAV drugs have already been approved for clinical use by the FDA in the context of CNS disorder. The first is LUXTURNA, an AAV indicated for the treatment of patients with confirmed biallelic RPE65 mutation-associated retinal dystrophy. The second is ZOLGENSMA for the treatment of type I Spinal Muscular Atropy. In addition, a previous platform was licensed from EMA, a gene therapy approach for the treatment familial lipoprotein lipase deficiency (GLYBERA), that was approved in 2012 but unfortunately recently retired due to its high cost and the small patient population.

In spite of these successful stories, careful monitoring and general attention are still mandatory, because recent in vitro and in vivo studies showed a relatively high frequency of integration (ranging from 1% to 3%) in the liver, and most of these integrations have been demonstrated to cause rearrangements in the host genome (Dalwadi *et al*, 2021; Koblan *et al*, 2021). However, these concerns relate to gene delivery by systemic administration of AAV particles at a relatively high dose, while in case of brain-directed delivery no similar rearrangements are expected not have been reported so far.

Lentiviral vectors

Lentiviruses belongs to the Retroviridae family of viruses. They are enveloped viruses of about 80-100 nm containing a positive single-stranded RNA genome (ssRNA) of about 8-10 Kb (Baltimore Classification VI). To date, lentiviral vectors are more widely used and more developed than their predecessors, the γ-retroviruses. Like the latter, they are integrative and guarantee stability in transgene expression. They have a simple genome, composed of only 9 genes, which makes them much easier to engineer than larger vectors (such as Herpes Simplex Virus (HSV)). There are 3 structural genes, gag, pol, and env, coding respectively for capsid proteins, enzymes (Polymerase, Retrotranscriptase, and Integrase), and envelope proteins. In addition, Human Immunodeficiency Virus (HIV) has additional regulatory and accessory genes, including tat and rev, which made engineering slightly more complex than for AAV vectors. Several generations have been created during the development of these vectors. In the context of this thesis, we will specifically focus on the latest, third generation (Blömer *et al*, 1996).

Concerning tropism, lentiviruses infect a few cell types, typically macrophages or lymphocytes, and therefore, in principle, have little utility in the field of CNS. However, hybrid vectors with pantropic envelopes were generated using proteins such as Vesicular stomatitis virus G glycoprotein (VSV-G). Nowadays, variants expressing rabies virus glycoproteins have also been engineered to allow neuron-specific retrograde gene transfer (Kato *et al*, 2014). The transfer vector was constructed by eliminating all sequences that are not essential for the correct production (genome packaging) and infectious phase. To date, these constructs retain only:

- The central polypurine tract (cPPT), a recognition site for proviral DNA synthesis that increases transduction efficiency and transgene expression.
- The Psi (Ψ) sequence, that is essential for genome packaging in the nucleocapsid.
- The Rev Response Element (RRE) sequence, to which the Rev protein binds.
- Two Long Terminal Repeats (LTR, at 3' and 5') that delimit the entire viral genome. In particular, the LTR at 5' acts as an RNA Pol II promoter. Thus, the transcript that will consist of the viral genome begins at the beginning of R, is capped, and proceeds through U5 and the rest of the provirus. Third-generation vectors use a hybrid 5' LTR with a constitutive promoter such as CMV. This engineering has been optimized to make the vectors capable of self-inactivation by deletion of the LTR at the 5' once the transfer vector is integrated. At the other end, the addition of a poly-A tract just after the R sequence terminates the viral genome (Ingusci *et al*, 2019b). To produce the viral particles, however, the structural proteins of the virus are needed and are expressed *in trans* in the packaging cells. Three plasmids containing the gag and pol proteins (1st plasmid), the REV protein (2nd plasmid), and the VSV.G envelope (3rd plasmid) are used for this purpose.

The lentiviral platform has been extensively used and approved for clinical trial in several ex vivo gene therapy studies for diseases not involving the CNS (Tucci *et al*, 2021). However, strategies exist in which gene-corrected hematopoietic stem or progenitor cells are exploited as cell vehicles to deliver therapeutic molecules into the central nervous system. Indeed, EMA recently approved Libmeldy®, an ex vivo gene therapy with lentivirus vector-transduced autologous CD34-positive stem cells, for treatment of metachromatic leukodystrophy (Jensen *et al*, 2021).

Finally, the LV ability to integrate the genome in the host cell implies the risk of insertional mutagenesis. Therefore, non-integrating LV may be useful especially in non-dividing cells, in which losing expression after cell division is irrelevant. Therefore, in the last decade lentiviral platform have been engineered with a defective integrase giving the possibility of safer gene transfer approaches for the CNS (Banasik & McCray, 2010; Snowball *et al*, 2019).

Herpes Simplex Virus-1 based vectors

Herpes viruses are a large family of DNA viruses (Herpes-viridae) that can infect both humans and several animal species. They belong to the first class of the Baltimore Classification. Viruses belonging to this family are classified in 8 different subtypes from HSV-1 to HSV-8. Herpesviruses have been also grouped into three subfamilies: Alphaherpesviruses (α), Betaherpesviruses (β) and Gammaherpesviruses (γ), which differ according to genome structure, tissue tropism, cytopathology and the site of latent infection. Up to date, amplicons, replication-defective and replication competent vectors have been developed based on HSV-1. All these three platforms have been obtained by modifying the genome, which consists of a linear molecule of a double-stranded DNA, about 152Kb long. Being one of the largest and most complex viruses able to infect animals, genetic engineering has always been a challenge for scientists (Artusi *et al*, 2018).

Due to the high complexity of their genome and safety concern, their use is not yet common. Thus, HSV-1 based viral vectors have not been applied as extensively as LV or AAV vectors. In the following sections, I will mainly focus on the HSV-1 biology and the work that has been performed so far to develop replication-defective and amplicon vectors.

HSV-1 genome overview

The HSV-1 genome is composed of two unique regions, one long and one short, termed UL and US (Unique Long and Unique Short; Figure 6A). These regions are flanked and separated by inverted repeat sequences: two internal repeated sequences, called IRL and IRS (Internal Repeats long and short), which separate UL and US and give rise to the so called “JOINT” region; two terminal repeated sequences placed at the

5' and 3' end of the genome, named TRL and TRS (Terminal Repeats long and short), that interact together and are responsible for the genome circularization (Macdonald *et al*, 2012). The HSV-1 genome encodes for at least 84 genes, a few of which are present in duplicate (notably ICP0 and ICP4). Among these 84 genes, 38 are "essential" for viral replication and 46 are "accessory" or non-essential for a productive virus replication cycle (Manservigi *et al*, 2010). Altogether, HSV genes code for the many proteins that build the viral particles, which are composed by four main elements: envelope, tegument, capsid, and core.

The manipulation of different classes of HSV-1 viral genes has led to the creation of three types of HSV-based vectors: amplicons, replication-defective vectors and replication-competent vectors (Glorioso, 2014).

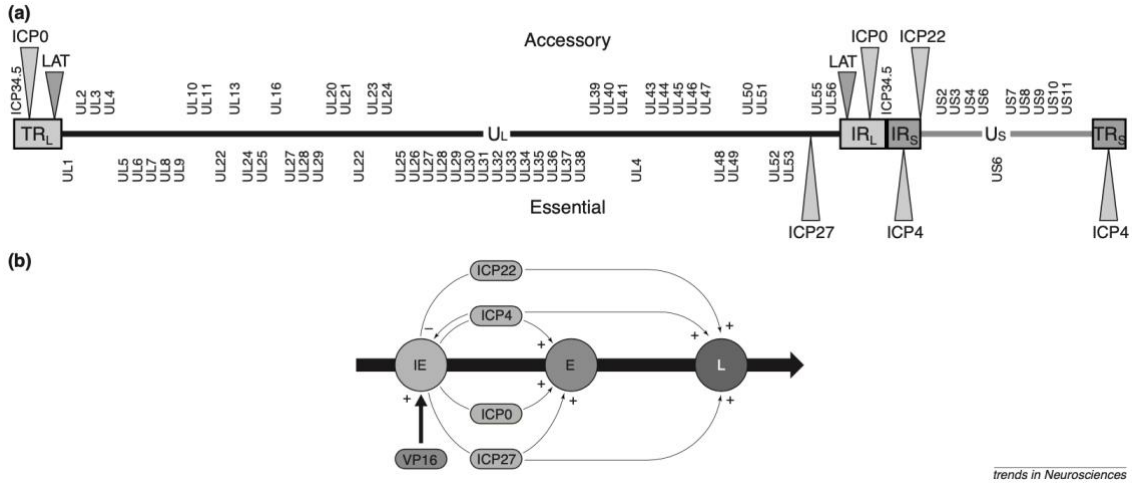

Figure 6 – HSV-1 genome organization and gene expression regulation
(a) Representation of the 152kb dsDNA HSV-1 genome (linearized). Genome is subdivided in two unique regions, unique long (U_L) and unique short (U_S). There regions are separated by inverted terminal repeats (TR_L, IR_L, IR_S, and TR_S). Upper portion represents the location of accessory gene, which can be deleted without affecting viral replication. Lower part shows essential genes, which are required for viral replication. ICP4, ICP27, ICP22, ICP0, and LAT locus locations are highlighted by triangles. (b) Temporal cascade of HSV gene expression. Genes are divided in three main groups, Immediate-Early (IE), Early (E) and Late (L) genes. Their regulation follows a cascade of expression and activation that starts when VP16 (a protein of the tegumentum) is release into the host cell. VP16 activates IE gene such as ICP0, ICP4, ICP22 and ICP27 which in turn promote the expression of E and L genes. Adapted from (Simonato et al*, 2000). License number:5237170757190.*

Replication-competent vectors

Since the main use of this viral platform is confined to anti-tumor therapy, where they act as oncolytic vectors, this class will be not extensively analyzed in the frame of this

thesis. Briefly, replication-competent vectors are obtained by the deletion of non-essential genes or from their mutation, in order to make them conditionally active, e.g., responsive to drugs. In this way, the virus will not be able to complete the lytic cycle unless in permissive cellular environments, such as that of the tumor cells. However, the removal of one or more non-essential genes significantly reduces pathogenicity raising the possibility to use this platform as a therapeutic tool for humans (Argnani *et al*, 2005).

Replication-defective vectors

The study of HSV productive cycle is fundamental for the proper molecular engineering of replication-defective vectors. This cycle is characterized by a temporally regulated cascade of gene expression (Figure 6B). In particular, this cascade is organized in three major groups of genes: Immediate Early (IE), Early (E) and Late (L) that encode for α, β, γ proteins respectively. The system is self-regulated: IE genes are first activated when the virus genome enters the cell, by an α-trans-inducing factor (VP16) contained in the tegument. Expression of α proteins, in turn, activates the promoters of the Early non-structural genes that will activate the synthesis of the late structural ones. The switching on of these late genes will deregulate the expression of IE and E genes.

IE genes are essential for the establishment of a lytic reproductive cycle, but also to avoid innate immune responses, to block cell division, and to prevent host cell apoptosis and epigenetic repression of viral genes. Thus, engineered HSV-1 vectors are obtained by functional deletion of the IE genes. In this way, virions are unable to start a productive lytic replication cycle and present remarkably reduced cytotoxicity (Krisky *et al*, 1998; Knipe, 2015).

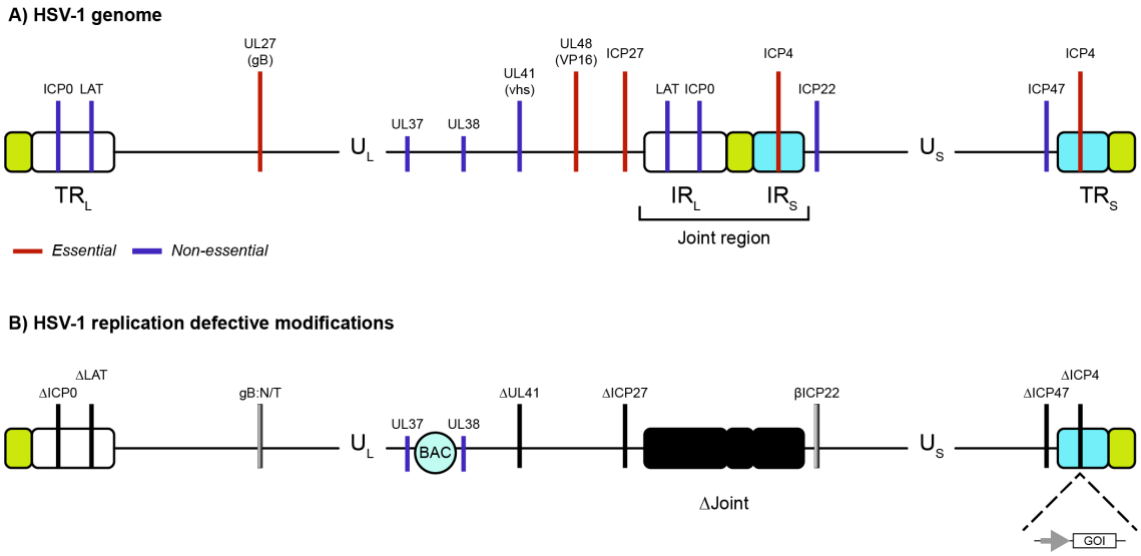

Figure 7 – HSV-1 genome and replication-defective viral backbone
(A) Representation of the HSV-1 genome highlighting the location of the essential (red) and non-essential (blue) genes that have been modified during sequential generation of replication-defective viral vectors. TR_L, IR_L, IR_S, and TR_S represents inverted terminal repeats of the unique short (U_S) and long (U_L) regions. (B) Diagram of advanced replication-defective viral backbone employed in the present thesis (JΔNI8). Note the Bacterial Artificial Chromosome (BAC) sequences inserted between UL37 and UL38. Modified from (Artusi et al*, 2018)*

The IE genes encode for five infected cell proteins (ICP): ICP0, ICP4, ICP22, ICP27 and ICP47. The first two vector backbones where obtained by deletion of ICP4 and ICP27 (Shepard & DeLuca, 1991; Wu *et al*, 1996). In both cases, vectors were able to establish a long-term expression without being replicative but were highly cytotoxic due to the residual presence of ICP0 in transduced cells. In both cases, viruses were produced using complementing cells expressing ICP4 and ICP27 in trans during the production.

The consequent deletion of ICP0 gave rise to a new generation of vectors that were devoid of toxicity but displayed short-term expression of the transgene (Samaniego *et al*, 1997). Indeed, ICP0 has been demonstrated to maintain HSV genes expression by reducing heterochromatin formation (Ferenczy & DeLuca, 2011; Boutell & Everett, 2013). Thereafter, the challenge was to obtain sustained, long-lasting expression in viral vectors deleted for ICP0. To overcome this hurdle, an insulator sequence (CTRL) present in the latency-associated locus was used to shield transgene expression (Bloom *et al*, 2010). This new vector did not display any sign of toxicity in several non-neuronal cells in vitro (Miyagawa *et al*, 2015). In addition, and unexpectedly, an expression cassette inserted in the second copy of the ICP4 gene in the remaining repeat bordering Us, created originally only to monitor viral replication trough the IE genes deletion variants,

displayed a robust expression only in neuronal cells. Although a clear explanation of this observation is not yet known, a possible explanation may be that the ICP4 locus is flanked on each side by a cluster of CTCF-binding motifs (Harkness *et al*, 2014; Bloom *et al*, 2010). In other words, sustained neuron-specific expression of transgene was reached both in vitro and in vivo using viral vectors deleted for ICP4, ICP27 and ICP0 when the expression cassette was inserted in the ICP4 locus contained in the TRS (Verlengia *et al*, 2017). Subsequently, a further modification that further enhanced the long-lasting expression of the transgenes was the deletion of the UL41 gene encoding for the virion host shutoff (VHS) protein (Miyagawa *et al*, 2017).

The two remaining ICP genes, ICP47 and ICP22, have been also modified. In particular, ICP47 is the only ICP gene that is not responsible for the subsequent activation of E genes, because it encodes a protein that interferes with immune recognition of virus-infected cells (Hill, 1995). In this case, the deletion was limited to the promoter and start codon of the ICP47.

ICP22 deletion further improved the safety profile but drastically impaired virus growth in complementing cells. Instead, removal of the VP16-responsive TAATGARAT motif from the ICP22 promoter/enhancer proved to be preferrable over a complete gene deletion. This modification changed the gene expression kinetics to that of an early gene, remaining silent in target cells but active in the post-IE stages of vector replication in ICP4/ICP27-complementing cells and thereby allowing robust vector production (Samaniego *et al*, 1997).

More recently, in order to overcome the risk of using only the ICP4 locus to insert the expression cassette, the viral backbone was further engineered with specific cellular anti-silencing elements at the transgene promoter level cloned outside the ICP4 locus. Several anti-silencing elements were evaluated and A2UCOE was found to be the most effective in increasing neuronal transgene expression both in vitro and in vivo (Han *et al*, 2018).

In the frame of this thesis, we employed the replication-defective Joint deleted No Immediate 8 (JΔNI8) vector which is deleted of the Joint Region, the ICP0 and the ICP27 immediate-early genes and owns many of the features described so far Figure 7B. The ICP4 immediate-early gene is truncated trough the insertion of a gateway cassette, which offers the opportunity to easily insert genes of interest. Last, a hyperactive glycoprotein

B (gB) allele, D285N/A549T (gB N/T) has been obtained through repeated passages, resulting in increased transduction efficiency (Uchida *et al*, 2010).

Amplicon vectors

Amplicons are the latest, promising class of HSV-1 based vector. These vectors retain only two elements of the HSV-1 genome, the viral origin of replication (ori) and the capsid packaging sequence (pac), the other part consisting of a concatemer repetition of the transgene expression cassette (Figure 8) (Epstein, 2009). The advantages of amplicons include the possibility of inserting up to 150 kb of exogenous DNA and the highly reduced toxicity and risk of reactivation, due to the complete absence of all viral genes. The disadvantage is that amplicon propagation is difficult, because no cell line exist that can complement all viral proteins.

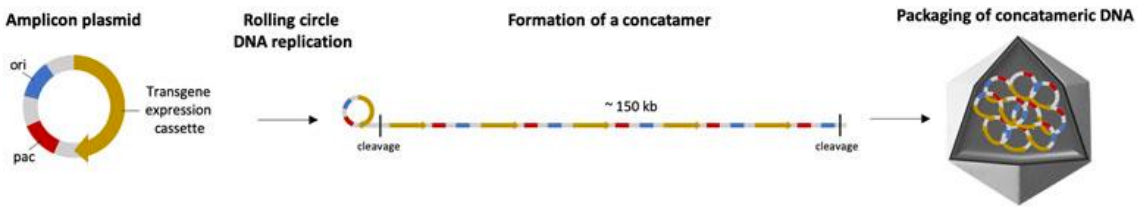

Figure 8 – Schematic representation of an amplicon vector
Amplicon transfer plasmid contains viral DNA replication origin (ori, blue), packaging signal (pac, red), and transgene expression cassette (yellow) sequences. During replication in permissive cells, transfer plasmid is replicated as head-to-tail concatemers. Once DNA reaches approximately 150kb, it is cleaved and packaged in HSV particles. Adapted from (Ingusci et al*, 2019b)*

Therefore, the first generation of amplicon vectors was propagated by using an HSV-1 helper virus. Although this method allowed the successful production of virus stocks, batches had a significant contamination with the helper virus of about 1% (Pechan *et al*, 1996). To improve the safety of amplicons, a method based on bacterial artificial chromosome (BAC) has been developed. In this case, the “helper virus” function is carried by a BAC containing all the viral genes but lacking essential genes (such as ICP27 or ICP4) and the *pac* signal sequence (i.e. packaging-defective genomes). Moreover, the artificial chromosome has been adapted with an addition of a "stuffer" sequences into the ICP0 locus to increase its size to 178 kb. Thus, the oversized BAC is cloned into partially complementing cells (in which ICP4/ICP27 are express *in trans*) together with the amplicon plasmid. This method allows the generation of stocks free of helper particles

(Saeki *et al*, 2001). An alternative approach was developed using the LaL helper virus, in which the viral genome carries a packaging sequence floxed between two loxP site and can be removed once the particles infect a Cre-expressing production cell line. This system resulted in a lower, but still present, helper contamination of about 0.05–0.5% (Zaupa *et al*, 2003). More recently, stocks completely free of helper particles have been developed (Laimbacher & Fraefel, 2012).

As noted, the main advantage of amplicon vector is a payload virtually equal to the viral packaging capacity. Hence, the unique feature of this viral vector is to potentially accommodate complete gene loci, introns as well as regulatory sequences, to obtain highly tissue-specific expression or expression cassettes coding for complex machineries, for example the one needed to perform precise genome editing. Furthermore, the versatility of amplicons resides in the fact that the amplicon plasmids containing the therapeutic genes are amplified trough a rolling circle-like mechanism, generating long concatemers composed of many repeats of the gene(s) of interest (Boehmer & Lehman, 1997). Hence, since HSV-1 tends to always reach the packaging limit of about150-kbp, the genome size of the viral particles that will be carried over is directly dependent on the transgene length. For example, a 10kbp transgene expression cassette will be packed approximately as a 15-fold repetition in the final particle (Kwong & Frenkel, 1984), greatly increasing its potential to obtain a high expression in the target cell.

Despite their great potential, because of their difficult propagation and production, only a few studies have been focused on their use for gene therapy (Cuchet *et al*, 2007; Falcicchia *et al*, 2016).

NPY and gene therapy for epilepsy: How, When,… and Y

In this thesis, we characterized different viral vector platforms with the aim of developing a gene therapy strategy based on the combined expression of a ligand (NPY) and its receptor (Y2R) specifically in excitatory neurons. This will in principle potentiate an auto-inhibitory feedback that decrease glutamate release, but not GABA release by interneurons. Finally, we tested our viral platform in a genetic model of epilepsy.

Part of the text and figures included in the following chapters (pag. 36-48) have been previously published in Cattaneo et al. 2021.

NPY discovery, evolution and function

NPY is a 36-aminoacid peptide described for the first time in 1982 that shares high homology with two others family members, the pancreatic peptide (PP) and the peptide YY (PYY). The NPY gene has evolved from an orthologue NPY-like system that regulates energy homeostasis in invertebrates, acting on growth and reproduction (De Jong-Brink *et al*, 2001; Gershkovich MM, Groß VE, Kaiser A, 2019; Kooijman & Troost, 2007). The NPY genes probably originated through a chromosome quadruplication event that took place during evolution at the jawed vertebrate appearance (Larhammar & Salaneck, 2004).

NPY has a widespread expression throughout the CNS and peripheral nervous system (PNS) and it is typically co-released with other signaling molecules. Its actions are mediated by a remarkably broad repertoire of receptor subtypes, each activating specific signaling pathways in different tissues and cellular sub-regions (Dumont *et al*, 1992; Wai *et al*, 2004; Leblanc *et al*, 1987; Elfvin *et al*, 1997; Keast, 1991; Cerdá-Reverter & Larhammar, 2000).

The effects of NPY range from cell proliferation to the control of energy metabolism, pain and, most interestingly in the frame of this thesis, neuronal activity (Kuo *et al*, 2007; Tilan & Kitlinska, 2016). NPY is involved in cardiovascular, respiratory, and metabolic diseases as a paracrine hormone, and, as a neuromodulator, it is strongly associated with different neurologic disorders (Vezzani & Sperk, 2004; Atanasova & Reznikov, 2018; Pedrazzini *et al*, 2003).

In the CNS, NPY acts at cellular level by modulating excitability and neurogenesis. Moreover, it exerts modulatory effects in the regulation of food intake, stress response, and pain perception. It is expressed in multiple areas of the brain, from the neocortex to the posterior root of spinal nerves, predominantly in GABAergic interneurons, but also in catecholaminergic neurons, e.g., in the brainstem and in hypothalamic nuclei (Benarroch, 2009; Chronwall *et al*, 1985; de Quidt & Emson, 1986; Silva *et al*, 2005a). In the mesial temporal lobe, NPY is extensively expressed in different subnuclei of the amygdala, where it exerts an anxiolytic effect (Tasan *et al*, 2010; Wood *et al*, 2016), and in the hippocampus, where it exerts an inhibitory action on excitatory synaptic

transmission by reducing the release of glutamate (Colmers *et al*, 1985; Greber *et al*, 1994; Klapstein & Colmers, 1992; Mcquiston & Colmers, 1996).

Gene structure, peptide trafficking, processing and release

The human NPY gene (~8 kbps) is located on chromosome 7p15. It encompasses several regulatory elements at the transcription start site, and the full length mRNA is 551 bp long (Minth *et al*, 1984). After translation in the endoplasmic reticulum, NPY is directed to the secretory pathway as a prepro-peptide upon signal peptide truncation. Subsequentially, while trafficking inside dense core vesicles (DCVs), the full coding sequence of prepro-NPY is sequentially split into three fragments.

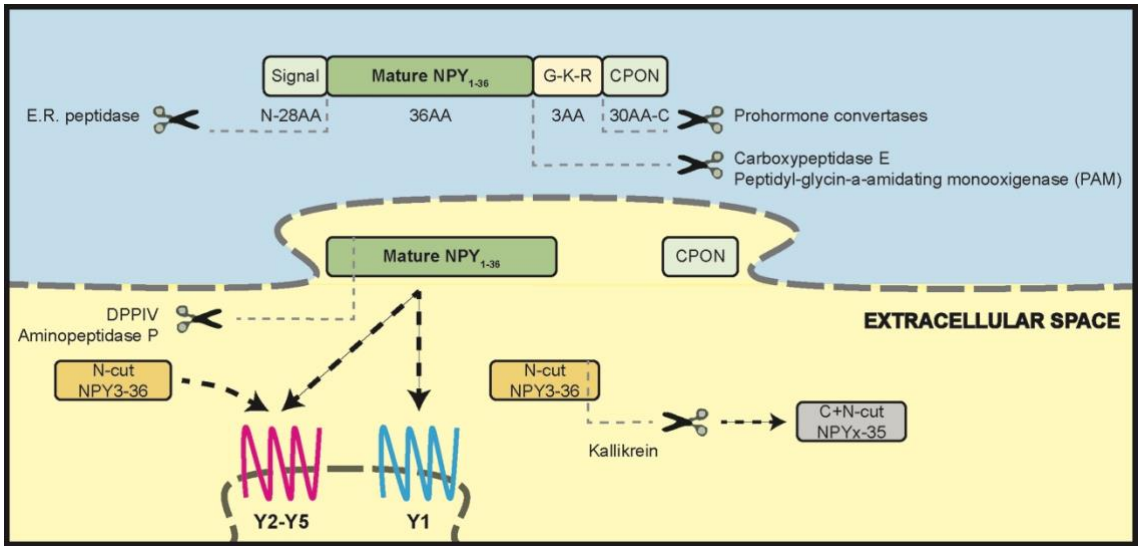

Figure 9 – Schematic representation of NPY intracellular processing and extracellular metabolism
Adapted from (Cattaneo et al*, 2021)*

As shown in Figure 9, endoplasmic reticulum peptidases mediate the cleavage of the N-terminus 28-amino acid (aa) signaling peptide. Subsequently, the mature 36 aa, 4.2 kDa peptide (NPY_{1-36}) is cleaved at the C-terminal at a glycine-lysine-arginine (G-K-R) site, crucial for CPON cleavage by pro-hormone convertases and for the amidation of the mature NPY, performed by carboxypeptidase E and peptidyl-glycin-α-amidating monooxygenase (PAM). Lastly, the C-terminal processing of NPY_{1-36} results in a 30-aa C-terminal flanking peptide of neuropeptide-Y (CPON). The CPON structure is highly conserved during evolution (Cerdá-Reverter & Larhammar, 2000). It has been suggested that it may play a role in epilepsy control, but current data do not confirm this hypothesis (Soud *et al*, 2019).

As a final step, NPY and CPON are packaged in DCVs and released upon calcium influx. Even if it is classically assumed that release would require a long, high frequency firing rate (van den Pol, 2012; Lundberg *et al*, 1986), this idea has been recently questioned by evidence that physiological synaptic activity is sufficient to mediate NPY release by hippocampal neurons (Li *et al*, 2017).

Once the peptide is released in the extracellular space, mature NPY can bind to its receptors and activate signal transduction (Walther *et al*, 2011) or be metabolized, either close or far away from its release site, in the cerebrospinal fluid or in the blood. Proteolytic processing either at the N-terminal or C-terminal portion of NPY, dependent upon a number of peptidases with compartment-dependent concentration and activity, can alter receptor binding affinity (Wagner *et al*, 2015; Allen *et al*, 1987).

Most commonly, NPY N-terminal processing by dipeptidyl peptidase IV (DP4) leads to the formation of NPY_{3-36}, which is subsequently cleaved at the C-terminal yielding an inactive fragment. Alternatively, with a relative lower efficiency, aminopeptidase P (AmP) produces NPY_{2-36} (Abid *et al*, 2009). Both NPY_{3-36} and NPY_{2-36} display a decreased affinity for Y1 receptors, therefore preferentially binding to other (Y2 and Y5) receptor subtypes (Grandt *et al*, 1996; Yang *et al*, 2018; Hubers *et al*, 2018).

After inactivation, other plasmatic peptidases catalyze metabolism to smaller fragments (Satoh *et al*, 1999).

NPY receptors

The NPY system is not only multi-ligand, as described above, but also multi-receptor, and this makes it a complex target for therapeutic applications. Five different NPY receptors are expressed in mammals: Y1, Y2, Y4, Y5 and y6. The latter is a pseudogene in humans and other primates and is missing also in the rat genome (Larhammar & Salaneck, 2004). Even if structurally different, NPY receptors (YRs) can respond to the same ligands (Larhammar & Salaneck, 2004). The Y4R binds preferably PP, and Y2Rs bind NPY_{1-36}, NPY_{3-36}, and NPY_{2-36} with similar affinity (Lindner *et al*, 2008). All the NPY receptors are G protein-coupled receptors (GPCRs) with seven transmembrane domains, acting preferentially via hetero-trimeric Gi/o proteins (Michel *et al*, 1998). They can trigger several intracellular responses, including inhibition of adenylyl cyclase, regulation of potassium and calcium channels and activation of the mitogen-activated

protein kinase (MAPK) cascade in some cell types (Howell *et al*, 2005; Lu *et al*, 2010; Thiriet *et al*, 2011; Shimada *et al*, 2012).

High levels of NPY binding can be found in the cortex, hippocampus, amygdala, striatum and cerebellum (Dumont *et al*, 1993). Specific binding to Y1 receptors can be visualized in different layers of the cortex, in the CA1 and CA3 stratum radiatum and oriens and in the dentate gyrus of the hippocampus, in the amygdala, striatum, cerebellum and, at lower levels, in some thalamic, hypothalamic and brainstem nuclei (Dumont *et al*, 1990, 1993; Aicher *et al*, 1991; Cabrele & Beck-Sickinger, 2000; Kopp *et al*, 2002). Y1Rs are mainly localized post-synaptically in the dendrites and soma (Kopp *et al*, 2002) of neurons of the hippocampus, striatum, and cortex (Wahlestedt *et al*, 1986; Caberlotto, 1997; Kopp *et al*, 2002). However, some studies also suggest a pre-synaptic localization (Brumovsky *et al*, 2002; Colmers *et al*, 1987, 1988; Flood & Morley, 1989; Glass *et al*, 2002; Kopp *et al*, 2002; Pickel *et al*, 1998; Li *et al*, 2017; Stanić *et al*, 2006). Albeit NPY and Y1R scarcely co-localize (Stanić *et al*, 2011), the presence of Y1R on the cell soma of NPY-containing hilar interneurons and cultured hippocampal neurons is suggestive of a possible role of these receptors in an autoinhibitory feedback (St-Pierre *et al*, 2000; Paredes *et al*, 2003). Regarding Y1Rs function, their main role is regulation feeding behaviors and energy homeostasis (Baldock *et al*, 2007; Nguyen *et al*, 2012). Y1R-mediated antidepressant and anxiolytic effects have been described in rodents (Wahlestedt *et al*, 1993; Verma *et al*, 2012), while their role in epilepsy remains controversial (see below).

Among all YRs, the Y2R is the most abundant (Dumont *et al*, 1998). Y2Rs are expressed in many brain regions, including the hippocampus, thalamus, hypothalamus and cortex; in the peripheral nervous system, Y2Rs are found in parasympathetic, sympathetic and sensory neurons; finally, they are also present in the intestine and in certain blood vessels (Stjernquist & Owman, 1990; Wahlestedt *et al*, 1986; Gehlert *et al*, 1992; Dumont *et al*, 1993; Rettenbacher & Reubi, 2001). In the hippocampus, Y2 receptors are particularly enriched in the CA1 and CA3 areas, respectively in the pyramidal cell layer and in the stratum radiatum (Colmers *et al*, 1987, 1988, 1991; Monnet *et al*, 1992). If compared with Y1Rs, Y2Rs expression is often complementary. For example, high levels of Y2Rs are detectable in the stratum oriens and radiatum of CA1-CA3, where Y1 receptor levels are relatively low, the opposite being true in the DG

molecular layer (Stanić *et al*, 2011). Y2Rs are highly expressed in the terminal regions of the mossy fibers and Schaffer collaterals (Jacques *et al*, 1997), where they act pre-synaptically by inhibiting calcium-mediated neurotransmitter release (Klapstein & Colmers, 1993). Indeed, NPY and a Y2R selective agonist inhibit evoked EPSPs on CA1 pyramidal cells. Conversely, bath application of a Y2R selective antagonist is able to block the inhibitory action of NPY on glutamate release (El Bahh *et al*, 2002).

Y2Rs are expressed by both GABAergic and glutamatergic terminals (Stanić *et al*, 2006, 2011) and may therefore inhibit the release of both neurotransmitters, GABA and glutamate, in particular under chronic epileptic conditions (Martire *et al*, 1993; Greber *et al*, 1994; Klapstein & Colmers, 1997; Vezzani & Sperk, 2004; Silva *et al*, 2005b). However, the Y2Rs strong immunoreactivity found at Schaffer collateral terminals in the CA1 of the hippocampus, together with the strong up-regulation of the receptors on mossy fiber terminals in epileptic condition (see following section) makes Y2Rs an interesting target in epilepsy (Vezzani & Sperk, 2004), in particular, a suitable target for gene therapy. Moreover, thanks to the high affinity for its ligand, there is the possibility of modulating receptor signaling by NPY volume transmission (Dum *et al*, 2017). Interestingly, Y2Rs display desensitization (Ziffert *et al*, 2020a) but can undergo arrestin beta3-dependent and independent internalization only when exposed to high concentrations of agonist (Walther *et al*, 2011; Lundell *et al*, 2011). The low rate of Y2R internalization may depend on the presence of a N-terminal extracellular domain rich in acidic/anionic residues (Parker *et al*, 2001; Gicquiaux *et al*, 2002).

Y5Rs are mainly found in the pyramidal cell layer of CA2 in the hippocampus, with lower concentrations in the hilar region of the dentate gyrus and in CA3. They participate in the modulation of hippocampal excitability and display anticonvulsant effects (Dumont *et al*, 1998; Guo *et al*, 2002; Gerald *et al*, 1996). They are also found in the hypothalamus where, together with Y1Rs, contribute to the regulation of food intake and energy homeostasis (Criscione *et al*, 1998; Nanobashvili *et al*, 2004; Woldbye *et al*, 1997). Finally, Y5R KO mice display a reduced NPY-mediated inhibition of glutamatergic synaptic transmission and are therefore more susceptible to kainate-induced seizure mortality (Marsh *et al*, 1999; Baraban, 2004).

NPY and epilepsy

Despite the strong loss of hilar GABAergic interneurons both in rodent and human sections from temporal lobe epilepsy (TLE) surgical samples (Sperk *et al*, 1992; Furtinger *et al*, 2001), NPY expression in the hippocampus is increased in epilepsy. One of the reasons for this is that the excitatory granule cells, which in the epileptic hippocampus give rise to mossy fiber sprouting (MFS[2]), have been demonstrated to ectopically produce and release NPY (Figure 10 (Mathern *et al*, 1995; McCarthy *et al*, 1998).

A shift toward higher Y2 receptor density is also evident in patients with hippocampal sclerosis, another common pathological trait of TLE. This phenomenon is observed in CA1, CA3, in the hilar region and in the inner molecular layer of the hippocampus (Furtinger *et al*, 2001). Y2R persistent up-regulation in the epileptic hippocampus could be justified by recent evidence showing that Y1, but not Y2, receptors are rapidly internalized and recycled after binding to their ligand (Ziffert *et al*, 2020a, 2020b). Moreover, Y2R knockout mice are totally insensitive to the anti-epileptic actions of NPY, both in vitro and in vivo (Woldbye *et al*, 2005) suggesting that a combined NPY and Y2Rs up-regulation may function as an endogenous anti-epileptic mechanism (El Bahh *et al*, 2005).

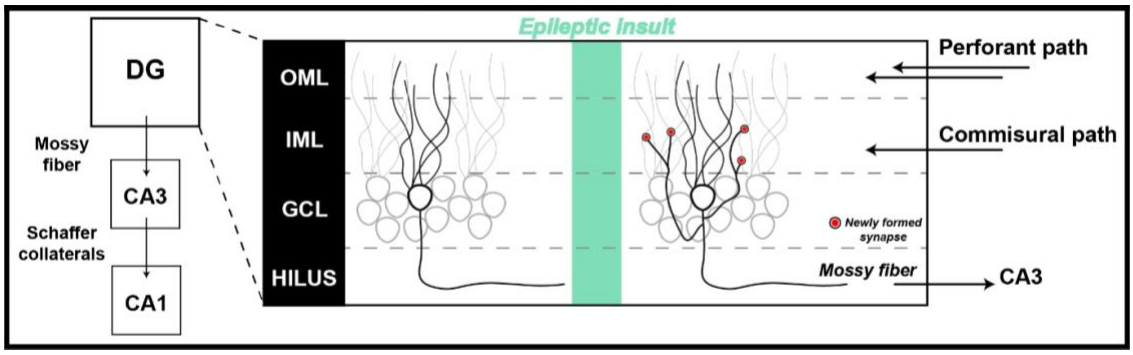

Figure 10 – Neuropeptide Y potential role in the epileptic hippocampal network.
Illustration of hippocampal formation rearrangements after an epileptic insult. Red dots represent synapses newly formed by the mossy fiber sprouting in the inner molecular layer that contain NPY and pre-synaptic Y2 receptors. DG: dentate gyrus; CA3/CA1: Cornu Ammonis; OML: outer molecular layer; IML: inner molecular layer; GCL: granule cell layer. Adapted from (Cattaneo et al, 2021)

[2] *Mossy fiber sprouting (MFS) is the aberrant sprouting of granular axons that recurrently innervate granule cell dendrites in the molecular layer generating an auto-excitatory loop (Fig. 10). Although it is commonly accepted as a marker of temporal lobe epilepsy, its pathophysiological role is still controversial (Cavarsan* et al*, 2018).*

Concerning Y1 regulation, it has been demonstrated an opposite effect with respect to Y2Rs. Indeed, Y1 receptor mRNA and specific binding to the receptor actually decrease in both kindled rats (Gobbi *et al*, 1998) and in intra-hippocampal kainate-treated mice (O'Loughlin *et al*, 2014). Consistently, a reduced density of Y1Rs has been also demonstrated in human patients with hippocampal sclerosis, indicating a reduced expression of the receptor or a loss of Y1R-expressing neurons (Furtinger *et al*, 2001; Kofler *et al*, 1997). Of note, the hypothesis that Y1Rs are rapidly internalized after binding to NPY (Ziffert *et al*, 2020a, 2020b) suggests a possible mechanism underlying the loss of Y1R immunoreactivity after an epileptic insult. Further experiments have to be performed to clarify this mechanism. However, administration of Y1R antagonists produces antiepileptic effects in animal models of epilepsy (Vezzani *et al*, 2000; Gariboldi *et al*, 1998) and Y1 KO mice display reduced mortality rate upon NPY administration (Lin *et al*, 2006). Thus, the reduced density and signaling of Y1Rs may be interpreted as an antiepileptic adaptive mechanism. It cannot be excluded, however, that this adaptive downregulation is linked to epilepsy-induced depressive or anxious behavior, described in patients and in animal models (Vrinda et al, 2017; Zanirati et al, 2018; Yilmazer-Hanke et al, 2016). Conversely, pharmacological activation of Y5Rs has been reported to exert antiseizure effects (Woldbye et al, 1997) and the decreased level of Y5Rs in epilepsy models (Bregola et al, 2000) might represent a maladaptive mechanism.

A further layer of complexity in the NPY system is the evidence that expression levels of NPY-related genes may strongly vary across species, with rats having higher expression of both NPY and Y2 compared to mice (Károly N, Dobó E & and Mihály, 2015; Nadler et al, 2007). This discrepancy may produce confusion when comparing data from different species and considering potential therapeutic target. For example, differences between rodents and humans have been found at the electrophysiological level. In human slices, prepared from surgically resected hippocampi of drug-resistant patients, NPY application reduces both lateral perforant path-evoked excitatory response in granule cells (Patrylo *et al*, 1999) and currents evoked by medial perforant path stimulation (Ledri *et al*, 2015). Conversely, experiments on hippocampal slices from an animal model of epilepsy (pilocarpine-treated rats) show that NPY does not affect the response of granule cells to perforant path stimulation but reversibly inhibits recurrent

synaptic transmission of mossy fibers on granule cells themselves (Tu *et al*, 2005). Hence, the precise mechanism of action of the NPY system on the epileptic network has not been completely clarified.

However, a clear effect of NPY in inhibiting epileptiform activity on human hippocampal sections challenged with [0] Mg^{2+}/4-amino-piridine has been demonstrated (Wickham *et al*, 2019). Moreover, it has been shown that the effect of NPY administration can be abolished by pre-treatment with a specific Y2 receptor antagonist (Ledri *et al*, 2015; Tu *et al*, 2005; Wickham *et al*, 2019) further corroborating the idea that the anti-epileptic effect is predominantly mediated by Y2Rs.

In line with the evidence presented so far, our working hypothesis is that an epileptic insult in the brain can cause a synchronous activation of granule cells that fail to inhibit the propagation of excitation from the entorhinal cortex to the hippocampus. It is then tempting to speculate that an adaptive compensatory mechanism might arise. Hence, granule cells, after losing their target hilar inhibitory neurons, sprout their axons to the molecular layer. This process of MFS increases excitability but, at the same time, produces synapses containing both NPY and Y2R at the presynaptic level (Figure 10). The combination of NPY and Y2Rs expression in turn may provide a negative feedback that, activated upon high frequency stimulation, reduce the overall hyperactivity of the local neuronal network. This hypothesis is also in line with the discrepancies that have been observed between mice and rats, with the latter showing higher recurrent mossy fiber sprouting and displaying higher levels of NPY and Y2 immunoreactivity coupled with a stronger inhibitory effect upon NPY application (Tu *et al*, 2005).

Taken together, these data suggest a significant involvement of NPY in the epileptogenic process, supporting the idea that both pharmacological and genetic approaches targeting the NPY system may represent effective strategies for the treatment of epilepsy.

Exploiting NPY in gene therapy

In the last two decades, multiple pre-clinical studies have been devoted to the development of gene therapy products investigating the potential effects of the NPY system in the treatments of epilepsy. Among all the approaches designed to modulate

NPY, those predominantly employed were based on AAV vectors directly infused in the epileptogenic areas (Table 1).

First author (year)	Species	Model of epilepsy	Vector	Time of vector delivery	Transgene
Richichi et al. (2004)	WT rats	Intrahippocampal and intracerebroventricular kainic acid; Kindling	rAAV2_NSE-NPY; rAAV1/2-NSE-NPY	Before seizure onset	Human pre-pro-NPY
Lin et al. (2006)	WT mice; Y1 -/- and Y2 -/- mice	Systemic kainic acid	rAAV1/2_NSE-NPY	Before seizure onset	Human NPY cDNA
Foti et al. (2007)	WT rats	Intraperitoneal kainic acid	rAAV2_CBA-NPY; rAAV2_CBA-NPY13-36	Before seizure onset	Full length and NPY13-36 (Species not specified)
Sørensen et al. (2008)	WT rats	None	rAAV1/2_NSE-NPY	N/A	Human pre-pro-NPY
Noè et al. (2008)	WT rats	Electrically induced status epilepticus	rAAV1/2_CBA-NPY	After seizure onset	Human pre-pro-NPY
Sørensen et al. (2009)	WT rats	Kindling	rAAV1/2_NSE-NPY	Before seizure onset	Human pre-pro-NPY
Noè et al. (2010)	WT rats	Intrahippocampal kainic acid	rAAV1_CBA-NPY; rAAV1/2_CBA-NPY	Before seizure onset	Human pre-pro-NPY
Woldbye et al. (2010)	WT rats	Kindling; Subcutaneous kainic acid	rAAV1/2_NSE-NPY; rAAV1/2_NSE-Y2	Before seizure onset	Human pre-pro-NPY Full length mouse Y2 receptor
Gøtzsche et al. (2012)	WT rats	Subcutanous kainic acid	rAAV1/2_NSE-Y5; rAAV1/2_NSE-NPY	Before seizure onset	Human pre-pro-NPY Full length mouse Y5 receptor
Olesen et al. (2012b)	WT mice	Subcutaneous kainic acid	rAAV1/2_NSE-Y1	After seizure onset	Full length mouse Y1 receptor
Olesen et al. (2012a)	WT mice	Subcutaneous kainic acid	rAAV1/2_NSE-Y5	After seizure onset	Full length mouse Y5 receptor
Dong et al. (2013)	WT rats	Intrahippocampal kainic acid	rAAV1/2_CMV-NPY	Before seizure onset	Full length NPY (species not specified)
Zhang et al. (2013)	WT rats	Intracerebroventricular kainic acid	rAAV1/2_NPY (unknown promoter)	Before seizure onset	Not specified
Nikitidou Ledri et al. (2016)	WT rats	Intrahippocampal kainic acid	rAAV1/2_NSE-NPY; rAAV1/2_NSE-Y2	Before seizure onset	Human pre-pro-NPY Full length mouse Y2 receptor
Powell et al. (2018)	GAERS (Genetic Absence Epilepsy Rats)	Genetic model of absence epilesy	rAAV1/2_NSE-NPY	N/A	Human pre-pro-NPY
Melin et al. (2019)	WT rats	Intrahippocampal kainic acid	rAAV1_CAG-NPY/Y2	Before seizure onset	Human pre-pro-NPY Human Y2 receptor

Table 1 – Comparison of different gene therapy strategies designed to modulate the NPY system, based on the use of recombinant adeno-associated vectors.
Adapted from (Cattaneo et al, 2021)

Early gene therapy attempts explored the anti-seizure potential of NPY overexpression mediated by bilateral AAV vector injections in the hippocampus (Richichi *et al*, 2004) in a rat acute seizures model (intra-hippocampal KA). Notably, these authors compared the effects of two different AAV serotypes. They found that the NPY expression mediated by the neuron-specific enolase promoter (pNSE) using an AAV serotype 2 (AAV2) was predominantly confined in hilar interneurons. Conversely, the identical expression cassette delivered through the chimeric serotype AAV1/2 displayed a more widespread expression in diverse subtypes of neurons, also conferring a more robust protection from epileptogenesis and chronic seizures. These data were later confirmed by Foti et al. (2007) with the over-expression of NPY or NPY_{3-36} in the piriform cortex in the rat kainate model

of epilepsy. Another early study demonstrated that AAV-mediated NPY delivery in Y2 but not in Y1 knockout mice did not result in reduction of seizures (Lin *et al*, 2006). Taken together, data confirm that activation of Y2Rs was essential for the anti-epileptic effect of NPY. More recently, an AAV1/2 expressing-NPY vector has been used in a genetic model of epilepsy. The vector was infused into the thalamus or somatosensory cortex of a rat model of genetic generalized epilepsy (GAERS, Genetic Absence Epileptic Rats from Strasbourg), resulting in reduced seizure activity, in particular when injected in the thalamus (Powell *et al*, 2018).

In these studies (with the exception of those on GAERS) NPY delivery was performed before epilepsy onset, in a scenario that is obviously non-reproducible in real patients. To overcome this limitation and to test whether AAV-mediated NPY overexpression could also suppress spontaneous recurrent seizures, Noé et al. (2008) tested the effect of hippocampal injection of an AAV1 vector after the establishment of epilepsy in rats (Noè *et al*, 2008). Interestingly, in addition to the reduced number of recurrent seizures, this study also demonstrated preserved levels of Y2R into the AAV-injected hippocampus, with functional transport of the recombinant NPY to nerve terminals and high levels of release upon induction of neuronal depolarization. In a following report, the same authors delivered NPY using AAV1/2, and observed a widespread transgene expression pattern throughout the injected hippocampi and a potent reduction of seizure frequency, with no detectable evidence of immune response or cognitive impairment (Noè *et al*, 2010).

In contrast with the absence of effects on cognitive function reported when naïve rats were injected with either AAV1 or AAV1/2 overexpressing NPY by Noè et al. (Noè *et al*, 2008, 2010), Sørensen et al. (Sørensen *et al*, 2008) claimed that a delay in hippocampal-based learning may arise in naïve rats overexpressing NPY, as shown by an attenuation of long-term potentiation of Schaffer collateral-CA1 synapses. However, the same author showed seizure protection with no impact on working memory performance tasks in kindled rats injected in both hippocampi with the AAV1/2-pNSE-NPY vector in a following study (Sørensen *et al*, 2009).

As extensively described in the previous sections, NPY ability to control brain excitability is mediated by its target receptors, which are GPCRs acting preferentially via hetero-trimeric Gi/o proteins. NPY binding to Y1R, Y2R and Y5R regulates intracellular calcium levels and glutamate release. Whereas the seizures suppressing effect is clearly

mediated by overexpressing Y2Rs (and, to a lesser extent, by Y5Rs), the effects of the engagement of Y1Rs remains debated. As an example, Olesen et al. (2012b) showed that AAV-mediated Y1 over-expression was associated with a modest-anxiolytic effect accompanied by a modest increase in susceptibility to kainate-induced seizures. Therefore, a simple overexpression of NPY, which could ubiquitously act on the whole family of Y receptors, may become a double-edged sword.

Based on these considerations, a new therapeutic strategy was put forward, based on the intervention aimed not only at increasing the levels of the ligand (NPY), but also at the re-shaping of the receptor response. Several attempts have been made overexpressing YRs alone or in combination with NPY. In particular, Gøtzsche et al. (2012) delivered a pool of AAV vectors expressing NPY in combination with Y5Rs as compared with a single vector expressing Y5R alone. The study showed that no positive effect was detectable with Y5R alone, whereas the combination mediated a seizure suppressant effect (Gøtzsche *et al*, 2012; Olesen *et al*, 2012). Conversely, Y2R, when over-expressed alone, was sufficient to suppress seizures and even more robust effects were obtained with the concurrent over-expression of NPY (Woldbye *et al*, 2010). Further studies, performed by using two separate AAV to deliver the therapeutic genes in the intra-hippocampal kainic acid model, confirmed and extended these data (Nikitidou Ledri *et al*, 2016). Taken together, these data are in line with the hypothesis that NPY and Y2Rs are the key elements in the system for the control of hyper-excitability and therefore hold a strong potential as therapeutic targets. However, the use of a viral pool composed by two different AAV vectors driving the expression of the therapeutic genes faces some limitation especially in the prospect of clinical application. To address this issue, NPY and Y2R were engineered in a single AAV1 vector (Melin et al. in 2019). These authors employed a CAG promoter driving the expression of Y2R and NPY separated by an IRES sequence. The vector, delivered both in the dorsal and ventral hippocampus, has been tested at multiple doses to achieve the optimal response and ultimately resulted in a remarkable decrease of EEG seizure frequency and duration in the kainic acid model of TLE (Melin *et al*, 2019; Szczygieł *et al*, 2020).

Chapter IV - AIM OF THE WORK

During the last few decades, interest for gene therapy has exponentially increased. Epilepsies emerge as a plausible candidate disease among CNS disorders, because one third of the patients do not get control of their seizures with currently available ASDs (Tang *et al*, 2017) and less than 10% are eligible for resective brain surgery. Therefore, gene therapy may represent a doable approach for the unmet medical need of these drug-resistant patients not eligible for surgery.

Great efforts have been made to correct single gene defects in several epilepsy syndromes (Jensen *et al*, 2021). While these efforts are extremely important, a gene therapy approach able to target general disease mechanisms underlying seizure development, would hold an even greater potential. As an example, a lentiviral approach based on an engineered potassium channel have been tested in two preclinical models of focal neocortical seizures and temporal lobe epilepsy and is now the first in human trial of viral-mediated gene therapy for epilepsy (Snowball *et al*, 2019).

In this context, multiple pre-clinical studies have been devoted to the development of gene therapy products exploiting Neuropeptide Y (NPY) for the treatments of epilepsy. Almost all the approaches designed to modulate NPY were based on AAV vectors directly infused in the epileptogenic areas. However, in recent years several attempts have been made overexpressing YRs alone or in combination with NPY aiming and intervein not only at increasing the levels of the ligand, but also at re-shaping the receptor response. Ultimately, the combined overexpression of NPY and Y2 receptors has been suggested as the most promising.

To further improve currently available approaches, we decided to develop a viral-mediated gene transfer platform in which the NPY and Y2 receptors are selectively expressed only in a subset of neurons, i.e. excitatory cells. To this aim, by giving cell specificity and regional specificity to the vector we could ameliorate the therapeutic outcome ensuring an auto-inhibitory negative feedback only in excitatory cells, without affecting GABA release by interneurons.

Finally, the designed gene therapy strategy was tested in a genetic model of epilepsy, to demonstrate that this combinatorial approach may produce a beneficial effect in an epilepsy model with underlying genetic etiology.

Chapter V - RESULTS

Electrophysiological characterization of HSV-1 based viral vector

First of all, we focused our attention on testing the safety and efficacy profile HSV-1 replication defective vectors by generating a JΔNI8 recombinant vector expressing the red fluorescent protein tdTomato (tdT) under the Ubiquitin promoter (Ub). We used rat primary hippocampal neuronal cultures to characterize the effects of viral transduction on neuronal physiology. Surprisingly, our results showed variability between different batches of HSV-1 based vectors, with some able to induce severe electrophysiological alterations. We therefore decided to further characterize these defects in an attempt to shed light on the toxic effect caused by these HSV-derived viral vector batches, that are reminiscent of the fusogenic nature of HSV-1 and of its ability to spread from cell to cell.

HSV replication defective vector transduction alters neuronal physiology

We generated a JΔNI8 vector expressing a red fluorescent reporter gene (tdTomato) under the control of the Ubiquitin promoter to assess transduction efficiency in rat primary hippocampal neurons in vitro (Figure 11). Working at a multiplicity of infection (MOI) of 1, we obtained a very high transduction efficiency, with a percentage of transduced neurons ranging between 80 and 90% (Figure 11C). As expected from the natural neurotropism of HSV, no red fluorescent signal was found in astrocytes (Figure 11D).

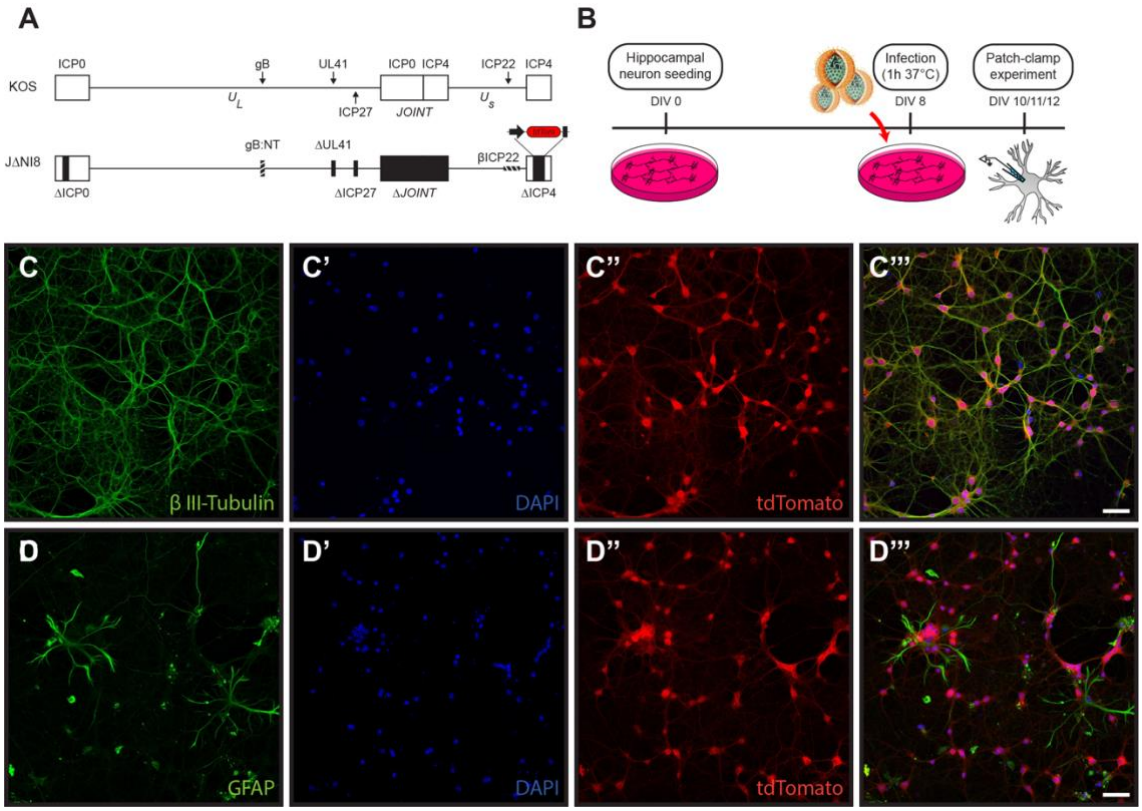

Figure 11 – JΔNI8 transduction of rat primary hippocampal neuron cultures.
A*. Schematic representation of HSV-1 genome (KOS strain) and JΔNI8 recombinant vector in which the Ubiquitin (Ub) promoter driving tdTomato (tdT) expression has been inserted in the ICP4 locus.* ***B****. Schematic representation of the experimental set-up used for primary neurons transduction with HSV-1 based vectors.* ***C, D****. Representative immunofluorescence images showing rat primary hippocampal neurons transduced with JΔNI8-Ub-tdT at a multiplicity of infection of 1. Representative immunofluorescence images for β-III-Tubulin (C, green); DAPI (C', blue), tdTomato (C'', red). Representative immunofluorescence images for GFAP (D, green, DAPI (D', blue), tdTomato (D'', red). Scale bar = 50μm.*

We then asked whether the electrophysiological properties of cells transduced with the JΔNI8 vectors are altered. Primary cultures were infected at 8 days in vitro (DIV) and electrophysiological experiments were performed between 10 and 12 DIV (Figure 12C). Although no gross cytotoxic effect or morphological changes were observed, transduced cells displayed several electrophysiological alterations. In current clamp experiments, tdTomato positive neurons did not displayed alterations of the evoked action potential (AP) shape compared to control neurons (Figure 12A,B). However, spontaneous action potential of transduced neurons display difference in the afterhyperpolarization (AHP) amplitude (i.e., the difference in voltage between RMP and the voltage after the AP peak) when compared to spontaneous AP of control cells (Figure 12I) . In addition, the AP shape at threshold values for the initiation of the AP showed a sharper inflection as

represented by the higher slope value at the peak in transduced neurons compared to controls (Figure 12I).

Moreover, infected neurons were spontaneously firing AP, which were replaced by depolarizing events similar to excitatory post-synaptic potentials (EPSP) when the neurons were too hyperpolarized to reach the AP threshold (Figure 12J). Voltage clamp recordings of these cells confirmed the presence of rhythmic single inward currents or bursts of currents, comparable with the EPSP observed in current clamp (Figure 12D,E). We further analyzed the basic electrophysiological properties of transduced neurons and we measured a strong reduction of both input resistance (Rin) and resting membrane potential (RMP) in JΔNI8 transduced neurons (Figure 12F,G).

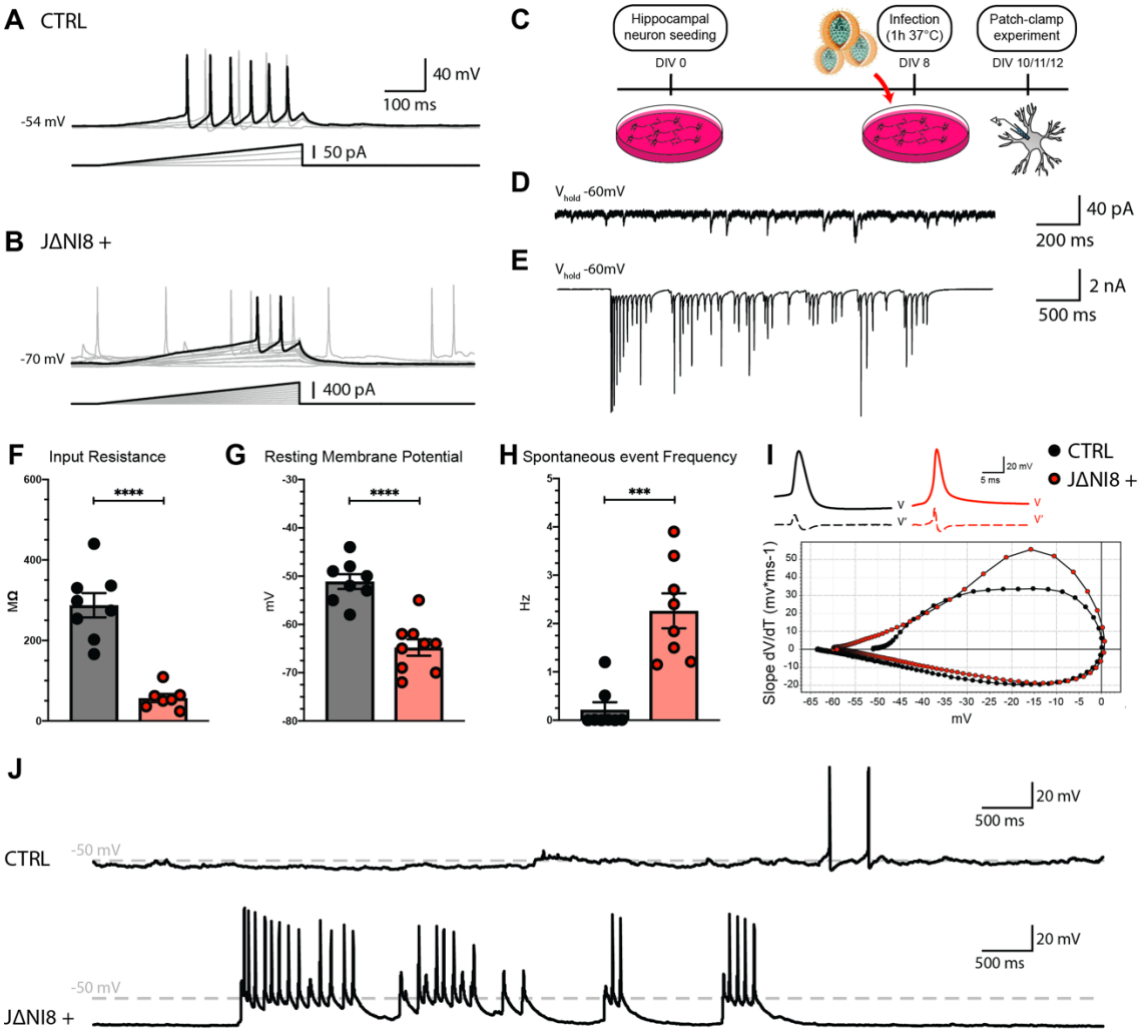

Figure 12 – JΔNI8 transduction induces electrophysiological alterations in rat primary hippocampal neurons.

A, B*. Voltage response of control and JΔNI8+ neurons to suprathreshold depolarizing ramp currents. Note that transduced neurons require injection of greater amplitude currents to elicit action potentials.* ***C.*** *Schematic representation of the experimental set-up used for*

electrophysiological experiments in primary neurons transduced with HSV-1 based vectors. ***D, E****. Voltage-clamp recordings of control and JΔNI8+ neurons (Vhold = -60mV).* ***F-H****. Dot plots summarizing average Input Resistance, Resting Membrane Potential, and spontaneous event Frequency of control and JΔNI8+ neurons. Data are shown as Mean ± SEM. F, CTRL = 287 ± 30 MΩ, JΔNI8+ = 56.8 ± 10 MΩ. G, CTRL = -51.1 ± 1.5 mV, JΔNI8+ = -64.7 ± 1.7 mV. H, CTRL = 0.21 ± 0.15 Hz, JΔNI8+ = 2.26 ± 0.36 Hz. Statistical significance in F-H was calculated using unpaired Student's t test. ***p<0.001, ****p<0.0001.* ***I****. Upper part of the panel shows the average voltage traces (V), and their first derivative (V'), of spontaneous action potential of control (black) and transduced (red) neurons. In the bottom part is represented a phase plot highlighting the slope trajectory during the entire AP cycle. Data are shown as mean of three cells per condition.* ***J****. Examples of spontaneous activity in control (upper trace) and JΔNI8+ neurons (lower trace). Note the bursting activity of transduced cells which arises from a highly hyperpolarized resting membrane potential compared to controls.*

These data should imply a reduction in neuronal excitability, since neurons displaying lower RMPs require bigger currents to get depolarized, with currents that are further dampened by a low membrane resistance. Further experiments are needed in order to characterize a potential transcriptional dysregulation of channel expression, in particular of potassium channels. However, in spite of the reduced excitability of transduced neurons, the net effect on the neuronal culture was an higher frequency of firing spontaneous AP (Figure 12H,J) which may arise from an involvement of the neuronal network, as suggested in the following section.

HSV-induced alterations are associated with fusogenic events between neurons

Alterations similar to the ones we found in hippocampal neurons infected with JΔNI8 were previously described in cells infected with syncytial strains of HSV-1 (Mayer *et al*, 1985). Therefore, we asked whether our vector might be able to form syncytia in our experimental conditions. To this aim, we used a green fluorescent dye (Neurobiotin-488) that was loaded in the patch clamp pipette and allowed to diffuse in the cytoplasm of the patched cell. Interestingly, the fluorescent Neurobiotin was able to diffuse and label also tdTomato positive neighboring cells, that should therefore be fused with the patched one, but not within tdTomato negative neurons (Figure 13A,B and Figure 14A).

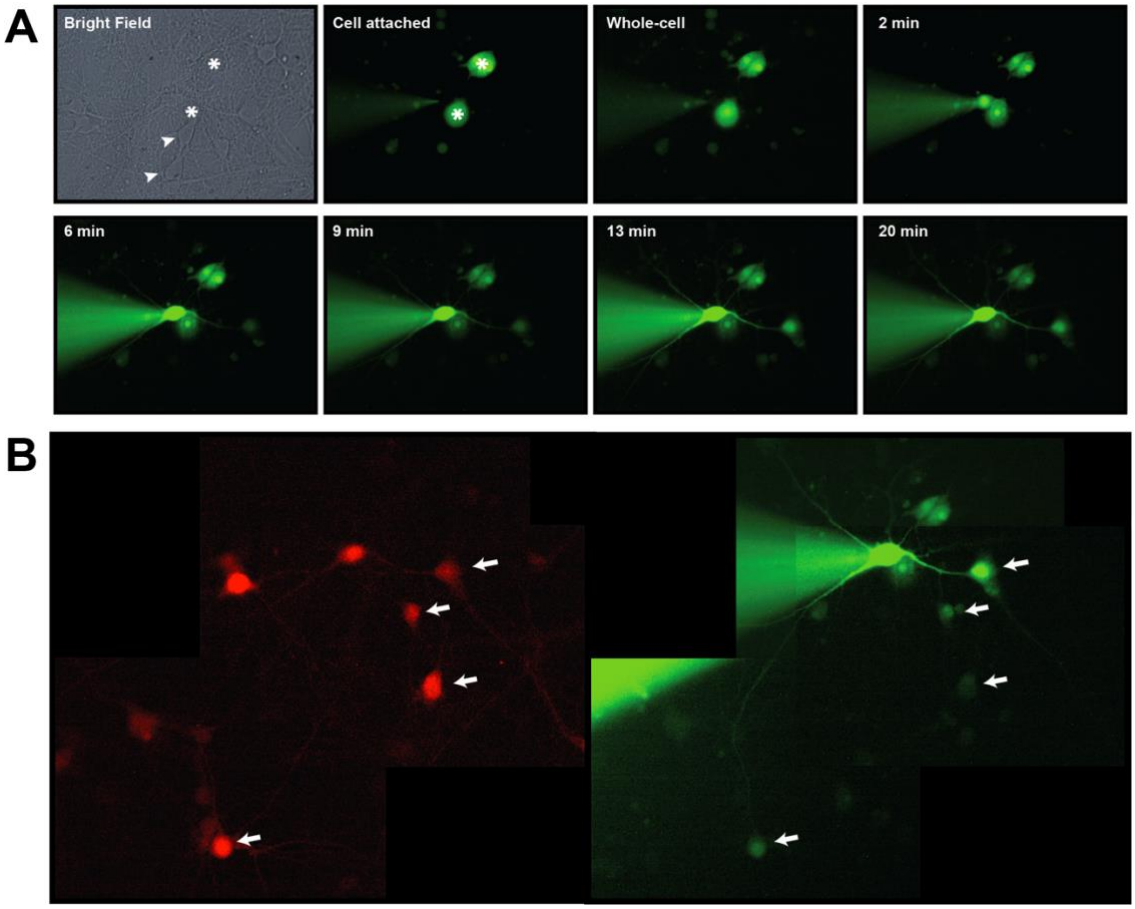

Figure 13 – Dye coupling of neurons transduced with JΔNI8 viral vectors.
A.. *Representative sequential images showing green fluorescence monitoring at different time points during voltage clamp recording of JΔNI8 transduced neuron using a Neurobiotin-488 loaded pipette. Note the dye diffusing through the cytoplasm of the patched cell; asterisks represent aspecific labelling of dead cells prior to patching. As shown in* ***B****, Neurobiotin-488 is able to diffuse in neighboring tdTomato+ cells (arrows), but not within tdTomato- neurons (indicated by arrowheads in the bright field image in A).*

We could also demonstrate the formation of syncytia using paired patch-clamp recordings of tdTomato positive neurons (Figure 14B), corroborating the hypothesis that these JΔNI8 vector batches retained some fusogenic properties of the wild-type virus. Altogether, our results suggest that viral vector-induced cell fusion can produce electrical coupling between hippocampal neurons, resulting in increased spontaneous activity.

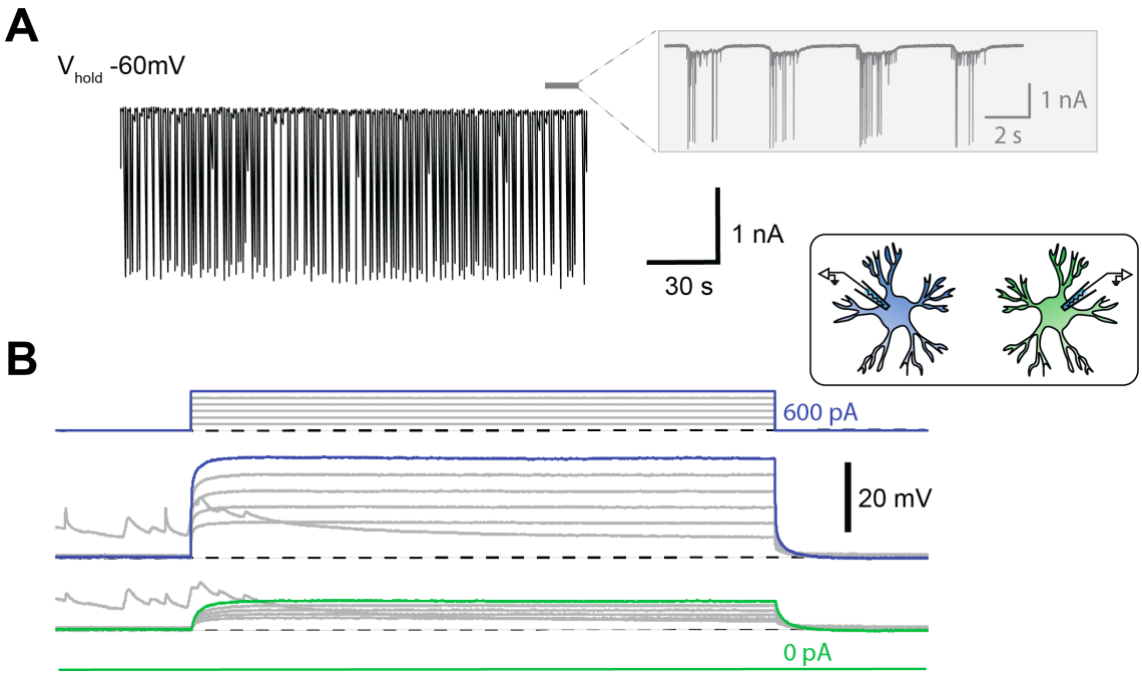

Figure 14 – Electrical coupling of neurons transduced with JΔNI8 viral vectors.
A. *Voltage clamp recording during the dye loading of the patched cell. Inset represents a higher magnification of the bursting activity.* ***B****. Dual patch clamp recording of two JΔNI8+ neurons demonstrating the electrical coupling of putatively fused cells. Note that only one neuron (blue) has been injected with suprathreshold depolarizing current steps, while the second (green) displays a coherent and synchronous response.*

Voltage-gated calcium channels are involved in neurons increased excitability after JΔNI8 transduction

To further characterize the alterations that might occur in ion homeostasis, intracellular calcium levels of control and transduced neurons were measured with Fura-2, a ratiometric calcium indicator. Following the same protocol used for patch-clamp experiments, primary cultures were infected at 8 DIV and calcium was measured at DIV11-12 in resting conditions. As shown in Figure 15, transduced neurons displayed increased Ca^{2+} levels compared to control cells.

Because the fusogenic event occurs between transduced neurons, we wanted to clarify if the uncontrolled spiking was dependent of synaptic transmission. Both treatment with TTX (a blocker of voltage-gated sodium channels) and NNC-55-0396 (a blocker of T-type calcium channels) block the uncontrolled spiking of infected neurons (Figure 15C-E). Conversely, bath application of NBQX (an AMPA receptor blocker) did not stop, but only partially reduced the uncontrolled spiking induced caused by fusogenic events. Although these data are only scratching the surface of a more complex underlying

mechanism, they nonetheless support the idea that the electrical coupling is the predominant driving force of the uncontrolled firing frequency rather than a synaptically mediated mechanism.

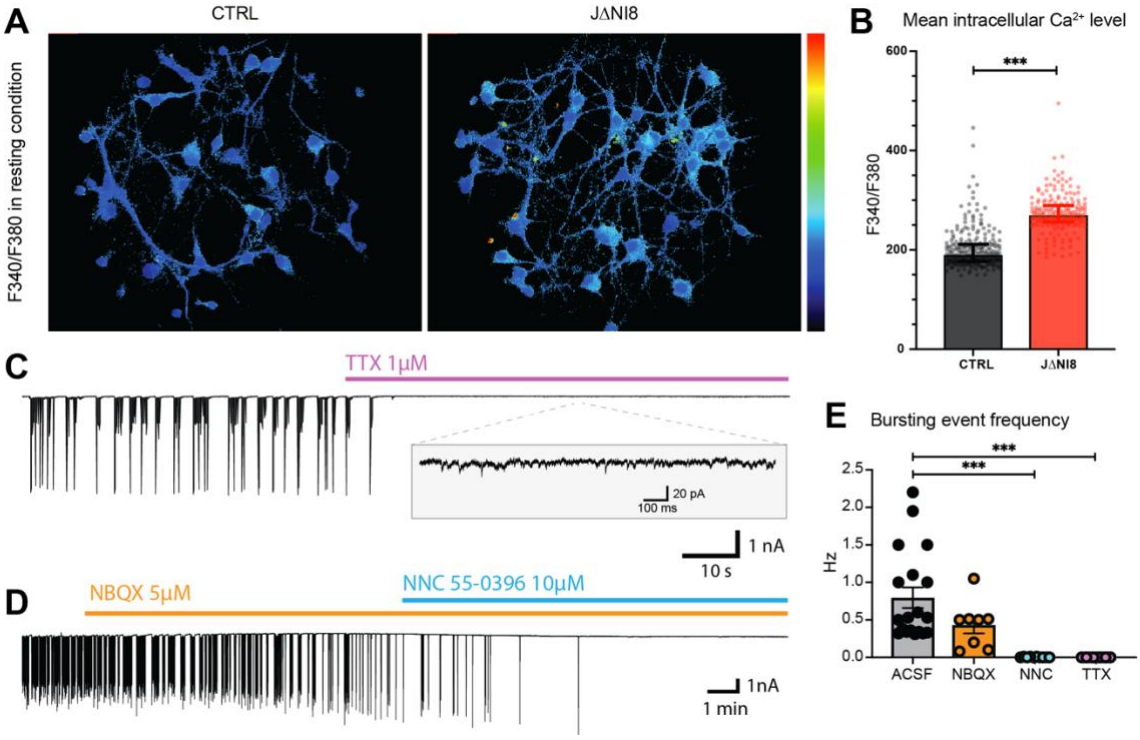

Figure 15 – Electrical coupling of neurons transduced with JΔNI8 viral vectors.
A. *Pseudocolored images representative of calcium level measured in control and JΔNI8+ neurons at resting state with Fura-2 Ca^{2+} dye.* ***B****. Dot plot summarizing mean intracellular calcium levels in resting condition. Data are shown as Median with interquartile range; CTRL = 190, n = 258 cells of 5 independent experiments, JΔNI8+ = 269, n = 202 cells of 5 independent experiments. Statistical significance in B was calculated using Mann-Whitney U test. ***p<0.001.* ***C****. Voltage clamp recording of transduced neurons during bath application of the voltage gated sodium channels blocker TTX (1µM).* ***D****. Voltage clamp recording of transduced neurons during bath application of the AMPA antagonist, NBQX (5µM) and the T-type calcium channel blocker NNC 55-0396 (10µM).* ***E****. Dot plot summarizing average bursting frequency. Data are shown as Mean ± SEM. ACSF = 0.795 ± 0.137 Hz (black), NBQX = 0.431 ± 0.111 Hz (orange), NNC = 0.003 ± 0.001 Hz (light-blue) and TTX = 0 Hz (magenta). Statistical significance in E was calculated using the Kruskal–Wallis one-way analysis of variance followed by the Dunn's post hoc test. *** p<0.001.*

HSV-1 based amplicon vectors do not cause fusogenic events between neurons

We then decided to test other HSV-1 based vectors to determine if the fusogenic ability was an intrinsic feature of the whole family of HSV-1 based vectors or whether it was

restricted to those replication-defective. We therefore tested Amplicon vectors based on the HSV-1 genome provided by the company Bioviron® (Figure 16).

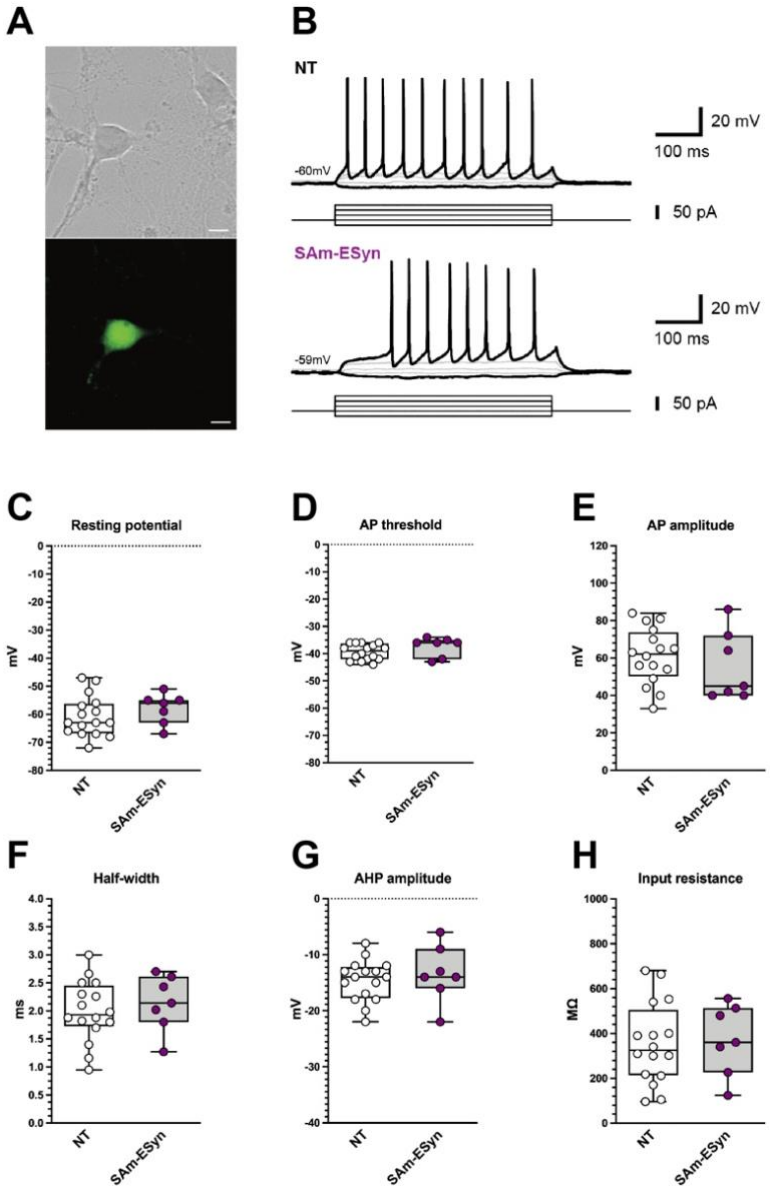

Figure 16 – Amplicon vector transduction of rat primary hippocampal neurons.
A. *Representative image of native EGFP signal in a vSAm-ESyn-LiG2 transduced hippocampal neuron (Scale bar: 10µm).* ***B.*** *Current-clamp recordings of a hippocampal neuron showing voltage response to step current injections at various amplitudes in mock (NT) and vSAm-ESyn-LiG2infected cells.* ***C-H****. Basic electrophysiological parameters of mock (white circles; n = 16) and vSAm-ESyn-LiG2 transduced neurons (purple circles; n = 7). Statistical difference was assessed with unpaired t test. Mean ± SEM are: C, NT = -60.7 ± 1.8 mV, SAm-Esyn = -57.5 ± 1.7 mV. D, NT = -39.4 ± 0.7 mV, SAm-Esyn = -38.2 ± 1.1 mV. E, NT = 61 ± 3.7 mV, SAm-Esyn = -55.8 ± 5.9 mV. F, NT = -1.9 ± 0.1 ms, SAm-Esyn = 2.1 ± 0.1 ms. G, NT = -14.6 ± 0.9 mV, SAm-Esyn = -13 ± 1.5 mV. H, NT = -354.6 ± 45 MΩ, SAm-Esyn = -378.4 ± 46 MΩ. AP, action potential; AHP, afterhyperpolarization. Results published in (Soukupová* et al*, 2021).*

As we recently published in (Soukupová *et al*, 2021), rat primary hippocampal neurons were mock-transduced or transduced with the amplicon vector vSAm-ESyn-LiG2 at a MOI of 1 (Figure 16), and whole-cell patch-clamp experiments were performed like in the previous experiments (i.e. transduction at DIV 8, recording between DIV10-12). Similarly, no gross morphological alteration was detected in transduced neurons; furthermore, current-clamp experiments displayed a similar voltage response in both infected and mock neurons (Figure 16B). No significant differences were detected on the following basic electrophysiological parameters: resting membrane potential, action potential amplitude, after-hyperpolarization amplitude, action potential half-width, action potential threshold, and input resistance (Figure 16C-H). Altogether, this data demonstrate that amplicon vectors do not cause fusogenic events between transduced primary neurons as suggested by the absence of alterations at the basic electrophysiological properties analyzed in these conditions.

Different batches of JΔNI8 vectors cause different degrees of electrophysiological alterations in neurons

We then focused our attention specifically on replication-defective JΔNI8 vectors and tested viral batches generated by our collaborators in Pittsburgh (JΔNI8 P). While several batches of vectors produced in our laboratory (JΔNI8 M) consistently caused electrophysiological alterations in transduced cells, neurons transduced with JΔNI8 P viral batches were more similar to control cell. Indeed, spontaneous spiking frequency and Rin were similar to the control ones (Figure 17A,C). However, the resting membrane potential of cells infected with JΔNI8 P was hyperpolarized compared to controls (Figure 17B) and current clamp experiments showed that a subset of the recorded cells (12%) displayed defects similar to those observed in cells infected with JΔNI8 M vectors produced in Milan (Figure 17D). However, no electrical coupling was found in any of the paired recording trials we performed with JΔNI8 P.

Taken together, we hypothesized that the fusogenic properties of viral batches might vary, despite employment of an identical protocol production and propagation protocol, due to spontaneous mutations in viral genes arising during vector production, and that amplicon vectors did not induce and alteration because they carry almost no viral gene. Therefore, we decided to sequence several batches of vector by extracting viral genome

DNA (Figure 17E). In collaboration with the Center of Omics Science (COSR) of the IRCCS Ospedale San Raffale Institute, we extracted DNA of eight samples (listed in Table 2) and performed whole-genome sequencing. Samples were sequenced with short-reads Illumina NGS technology (read length = 250bp) and the wild-type HSV-1-KOS strain (NCBI Accession N. JQ673480) was used as reference for detecting variations.

Samples	Description
BAC M	Bacterial Artificial Chromosome containing HSV-defective genome (Milan)
BAC P	Bacterial Artificial Chromosome containing HSV-defective genome (Pittsburgh)
JΔNI8 M1	Joint deleted no immediate 8 vector (produced in Milan, batch 1)
JΔNI8 M2	Joint deleted no immediate 8 vector (produced in Milan, batch 2)
JΔNI8 M3	Joint deleted no immediate 8 vector (produced in Milan, batch 3)
JΔNI8 M4	Joint deleted no immediate 8 vector (produced in Milan, batch 4)
JΔNI8 M5	Joint deleted no immediate 8 vector (produced in Milan, batch 5)
JΔNI8 P1	Joint deleted no immediate 8 vector (Pittsburgh, batch 1)

Table 2 – List of samples sequenced using NGS Illumina technology
In collaboration with the Center of Omics Science, eight samples have been sequenced. The two top samples (BAC M and BAC P) consist in the bacterial chromosome used to prepare the respective viral batches. All the JΔNI8 M (M1-M5) samples have been generated in the laboratories of Prof. Simonato in Milan, while JΔNI8 P1 has been prepared in the laboratory of Prof. Glorioso in Pittsburgh.

Before proceeding with the variant calling analysis, we performed a quality control assay to check the correct mapping of the sequenced DNA on the reference DNA and we noted that two samples (JΔNI8_M2 and JΔNI8_M5) showed very low coverages. Therefore, we decided to perform read classification analysis to check where the reads map. We used Kraken2, a taxonomic classification tool used to find the lowest common ancestor of a given sequence and, by performing classification analysis, we noted that many reads do not align with the HSV-1 reference. In the two BAC samples (BAC_M and BAC_P), 60% of the reads consistently align to a viral reference and approximately 22% of the non-aligned reads mostly align to a bacterial reference. In the JΔNI8 samples, the percentage of reads mapping to the host cell genome (human) dramatically increases, reaching over 80% in some samples (*e.g.,* JΔNI8_M2 and JΔNI8_M5), while the percentage of reads mapped to a viral reference span between 3 and 22% (Figure 17F). This data suggests a strong DNA contamination in the viral batches that probably results from the viral production process itself, which includes cell lysis as the last step before harvesting viral particles.

We thus decided to filter the analysis only on the DNA aligned to the HSV viral reference. We excluded the samples JΔNI8_M2 and JΔNI8_M5 for further analysis due to their low coverage on the HSV-1 reference genome. A representation in Integrative Genome Viewer (IGV) of the mapped reads against the reference genome is presented in Figure 17G.

We subsequentially preformed a variant calling analysis on filtered reads consistently mapping on the viral HSV genome. As shown in Figure 17H, most of the detected variants were single nucleotide polymorphisms (SNPs). To check the sensitivity of the variant detection we applied the "minfrac" parameter (minimum proportion of reads which differ from the reference) in a range from 0.85 to 0.95. The number of detected variables slightly decreases by increasing the minfrac parameter. For BAC samples, the number of detected SNPs remains unchanged by varying minfrac, while the insertion (INS) event decreases. A minfrac value of 0.90 was chosen for further evaluation.

Last, we looked at the detected SNPs, and found that most of the mutations either fall in intergenic regions or were causing silent synonymous mutations. Furthermore, some missense mutations were present only in specific viral batches and were spanning across the whole genome of HSV. In particular, three missense mutations in the UL27 gene (encoding for the glycoprotein B; Figure 17I) were consistently present in all the six samples analyzed (D285N, A315T, A549I). Among these, the A315T mutation was new, while the D285N mutation was expected as described in the previous work of (Uchida *et al*, 2010). However, in the same work a second mutation (A549T) was found in the gB gene, resulting in the gB N/T (D285N, A549T) variants which were described to increase transduction efficiency. To our surprise, we noted that the third detected mutation (A549I) was on the same residue previously mutated and described to increase gB transduction efficiency. Hence, in all the viral batches (and BACs) analyzed we found a new gB N/T/I (D285N, A315T, A549I) variant.

Based on these data, we hypothesize that the conversion of the original gB N/T (D285N, A549T) into the new gB N/T/I (D285N, A315T, A549I) might be responsible to a mild syncytial phenotype, as observed with electrophysiological experiments with JΔNI8 P vectors. Furthermore, additional missense mutations present in gB (or other viral genes), but only in specific viral batches, might be responsible for the more severe phenotype observed with JΔNI8 M vectors. These results suggest that a strict quality

control is needed in order to ensure that the viral genome is stable and that mutations in potentially toxic genes (e.g., gB) do not arise during viral production and propagation.

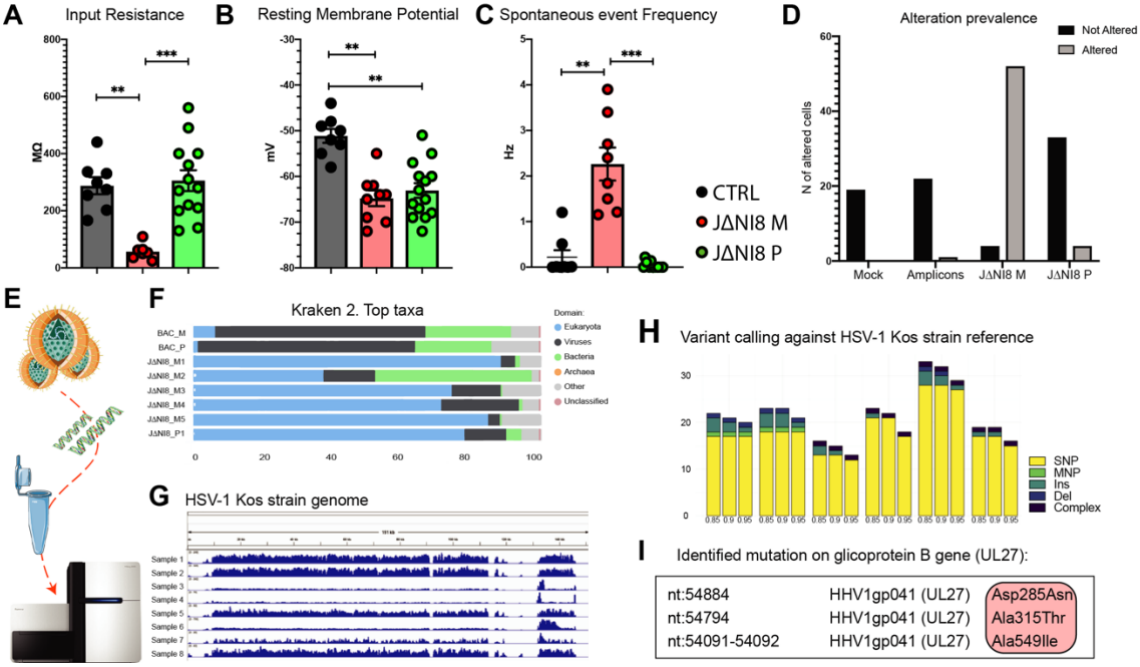

Figure 17 – Different batches of JΔNI8 vectors cause different degrees of electrophysiological alterations in neurons.

A-C. *Dot plots summarizing average Input Resistance, Resting Membrane Potential, and spontaneous event Frequency of control, JΔNI8_M, and JΔNI8_P. Data are shown as Mean ± SEM. A, CTRL = 287 ± 30 MΩ, JΔNI8 M = 56.8 ± 10 MΩ, JΔNI8 P = 305 ± 36 MΩ. B, CTRL = -51.1 ± 1.5 mV, JΔNI8 M = -64.7 ± 1.7 mV, JΔNI8 P = -64.7 ± 1.7 mV. C, CTRL = 0.21 ± 0.15 Hz, JΔNI8 M = 2.26 ± 0.36 Hz, JΔNI8 P = 0.04 ± 0.02 Hz. Statistical significance was calculated using Kruskal–Wallis one-way analysis of variance followed by the Dunn's post hoc test. **p <0.01; ***p<0.001.* ***D****. Histograms summarizing the alteration prevalence in neurons infected with different types of HSV-1 viral vectors. Cells displaying bursting activity were 0 out of 19 (Mock), 1 out of 23 (Amplicons), 4 out of 33 (JΔNI8 P), 52 out of 56 (JΔNI8 M).* ***E****. Schematic representation of the sample preparation for viral genome DNA sequencing.* ***F****. Kraken 2 classifications summary, showing the % of reads falling into the top 5 taxa across different ranks.* ***G****. IGV snapshot of BigWig file showing the coverage across HSV-1 Kos strain. Each sample auto-scaled individually.* ***H.*** *Variant calling summary with different detection sensitivity parameters* ***I****. Identified mutation in the HSV-1 replication defective viral batches sequenced.*

Exploiting a combinatorial gene therapy approach of NPY and Y2R

As shown in the former section, JΔNI8 viral vectors will require more work and quality control to ensure readiness and justify further pre-clinical studies. In collaboration with Professor Joe Glorioso from the University of Pittsburgh Medical Center, we decided to begin working on ameliorating their design and quality (see discussion section). Meanwhile, we also decided to move to a more characterized tool for the delivery of the therapeutic cassette comprising NPY and its receptor Y2. We chose an approach based on lentiviral vectors. In particular, since Y2 receptors act mainly at the presynaptic level and reduce neurotransmitter release by lowering Ca^{2+} uptake, we thought to further improve our strategy by driving Y2R expression specifically in excitatory neurons, through the minimal CamKII(0.4) promoter. The vision is to enhance an autoinhibitory feedback that will control glutamate release upon high frequency discharge of excitatory hippocampal neurons, thereby mitigating epileptic hyperexcitability.

NPY and Y2 receptor overexpression in rat primary hippocampal neurons

The design of the therapeutic bicistronic vectors (LV_mCamK-NPY for NPY alone and LV_mCamK-Y2-NPY for NPY and Y2R) are shown in Figure 18A. As a control, we used a monocistronic vector expressing EGFP under the minimal CamKII(0.4) promoter (LV_mCamK-EGFP).

We first tested our vectors in primary rat hippocampal neurons to measure both NPY and Y2 receptor expression. As shown in Figure 18B, a few NPY positive cells are present in neurons transduced with LV_mCamK-EGFP, while the neuropeptide expression is dramatically increased in neurons transduced with either LV_mCamK-NPY (Figure 18C) or LV_mCamK-Y2-NPY (Figure 18D). We confirmed these data by performing a western blot analysis on protein extracts from neurons transduced with the three different vectors. As shown in Figure 18E,F, the levels of NPY are significantly increased in both LV_mCamK-NPY and LV_mCamK-Y2-NPY. Moreover, Y2 receptor expression was also confirmed by western blot analysis, where Y2R expression was detected with the FLAG antibody and, as expected, is present only in LV_mCamK-Y2-NPY transduced cells (Figure 18E). As already described in litterature (Mizuguchi *et al*, 2000), the lower levels of EGFP expression in therapeutic vectors compared to the control vector (Figure 18E) are due to the presence of the IRES element in the therapeutic vectors.

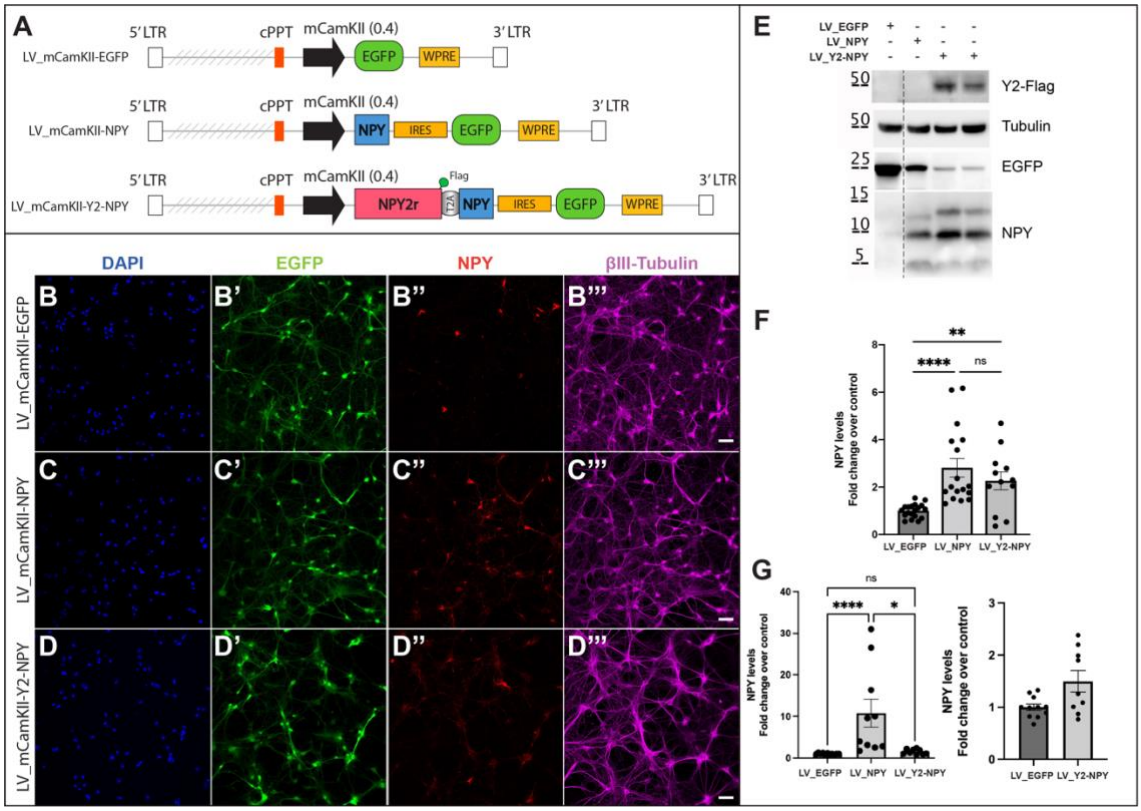

Figure 18 – Characterization of Lentiviral vectors in primary hippocampal neurons.
A. *Schematic representation of the lentiviral viral vector design.* ***B-D.*** *Representative confocal images of primary hippocampal neurons transduced with either LV_mCamK-EGFP (B-B'''), LV_mCamK-NPY (C-C'''), or LV_mCamK-Y2-NPY (D-D'''). Immunofluorescence staining for DAPI (blue), EGFP (green), NPY (red), ßIII-tubulin (magenta). Scale bar = 20µm.* ***E, F*** *Representative western blot and quantification of the indicated proteins from total cell extracts of rat primary hippocampal neurons transduced with the indicated vectors. Protein signals were quantified and then normalized for loading (Tubulin). Results are presented as mean ± SEM of at least three independent experiments, with protein levels shown as fold change over control (LV_mCamK-EGFP = 1 ± 0.06; LV_mCamK-NPY = 2.81 ± 0.3; LV_mCamK-Y2-NPY = 2.26 ± 0.37).* ***G****. ELISA measurement of NPY extracellular levels. Results are presented as mean ± SEM of at least three independent experiments, with protein levels shown as fold change over control (LV_mCamK-EGFP = 1 ± 0.06; LV_mCamK-NPY = 10.75 ± 3.3; LV_mCamK-Y2-NPY = 1.49 ± 0.2). Statistical significance in F and G was calculated using the Kruskal–Wallis one-way analysis of variance followed by the Dunn's post hoc test. * p<0.05; **p<0.01; **** p<0.0001.*

Finally, we performed an ELISA assay to measure neuronal NPY basal release. As expected, cells transduced with LV_mCamK-NPY showed the highest level of NPY release (Figure 18G). Although at a lower level, however, also LV_mCamK-Y2-NPY transduced cells displayed a greater release of NPY compared to cells transduced with the control vector.

Lentiviral vector transduction in the hippocampus of WT mice

We then moved to test our viral vector platform *in vivo*. Wild-type mice were positioned onto a stereotaxic frame and injected with a single dose of vector in the hippocampus. Two uL of viral preparation were diluted to 1x10^9 TU/mL and injected in the brain parenchyma using the following coordinates: -1.8 AP; ±1.8; -2.0 to aim at the mossy fibers projecting from the granular cells of the DG toward the CA3 of the dorsal hippocampus. LV_mCamK-EGFP was used to characterize viral vector spread and promoter specificity. As shown in Figure 19A, lentiviral transduction is achieved for the most part in the granular neurons of the ipsilateral hippocampus. We were also able to detect EGFP-positive commissural fibers labeling the inner molecular layer in the contralateral hippocampus (Figure 19A'). However, few pyramidal neurons of the CA3 were occasionally transduced due to viral diffusion at the injection site (Figure 19B) resulting in the positive signal at the level of the stratum radiatum in the contralateral hippocampus (Figure 19B').

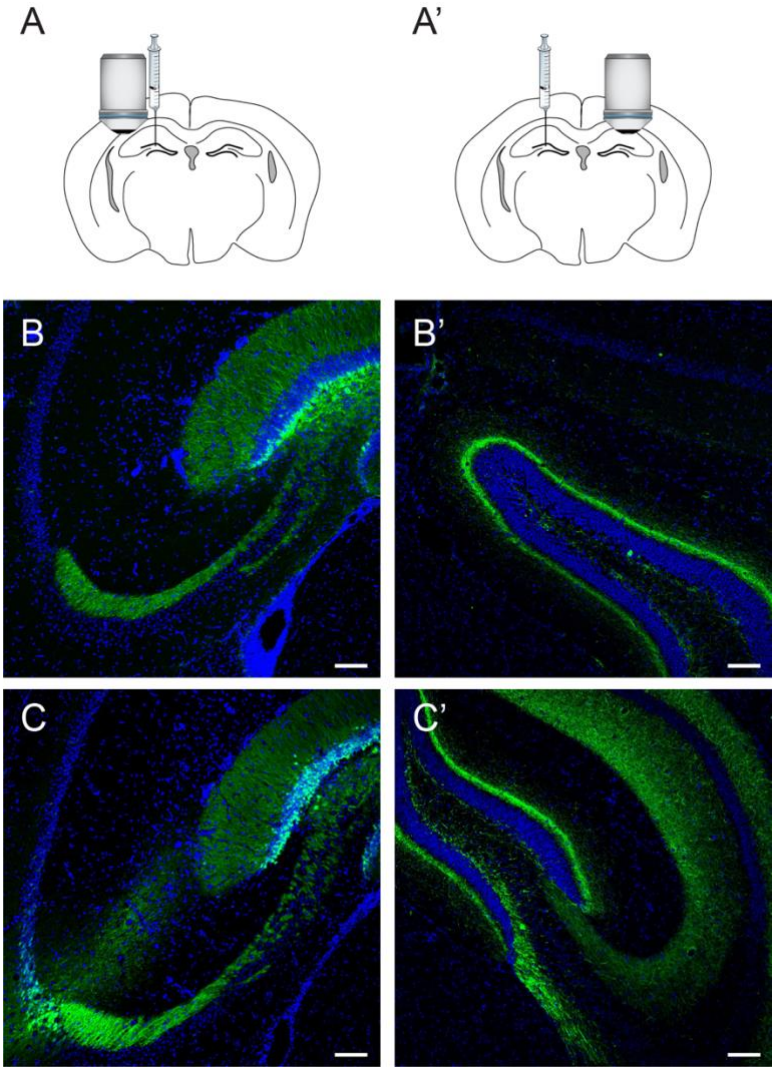

Figure 19 – Viral spread and expression in the mouse hippocampus.
A. Schematic representation of the mouse brain showing the site of viral vector injection and the field imaged in the lower panels. ***B, C****. Representative images of the hippocampus ipsilateral to the injection.* ***B', C'.*** *Representative images of the contralateral hippocampus. (scale bar = 100μm).*

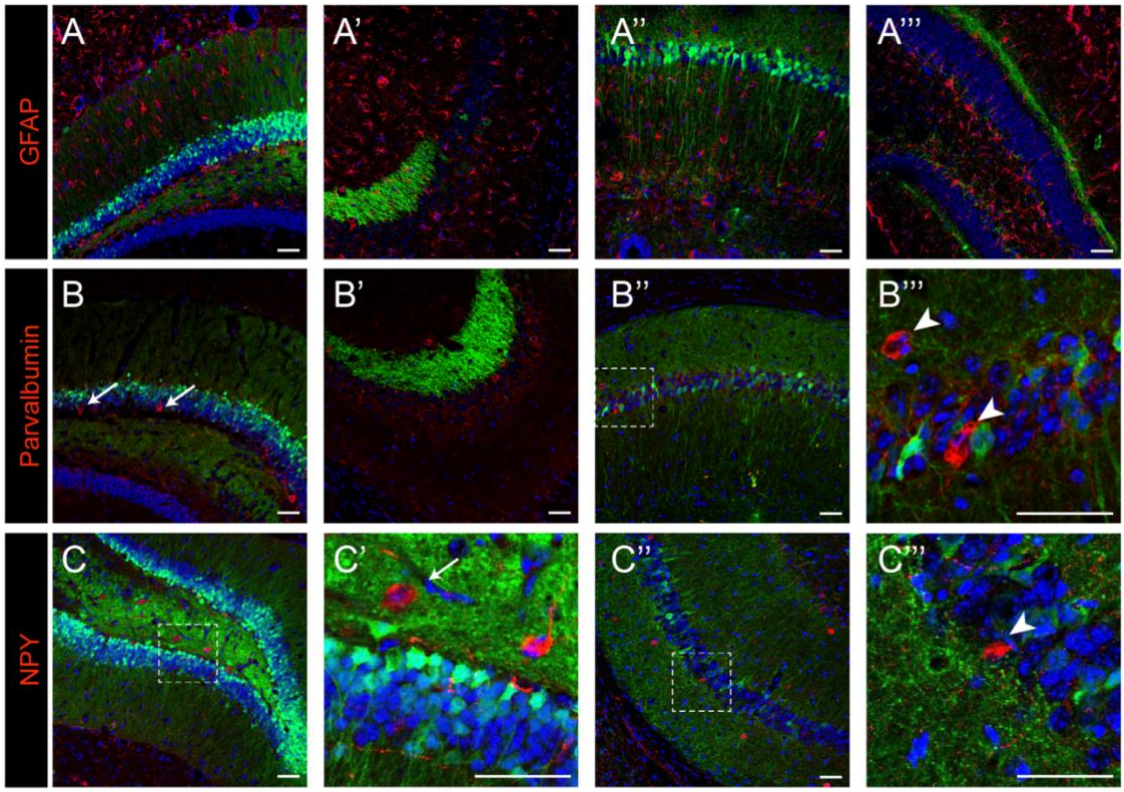

Figure 20 – The mCamKII(0.4) promoter drives EGFP expression specifically in excitatory neurons.

Representative immunofluorescence of WT mice injected with LV_mCamK-EGFP. In all the images transduced neurons expressing EGFP are shown in green; nuclei labeled with DAPI in blue. ***A-A'''*** *show GFAP immunofluorescence of different areas of the mouse hippocampus. A, hilus and dentate gyrus; A', mossy fiber projecting to CA3 pyramidal neurons, A'', CA1 pyramidal neurons, A''' and contralateral hippocampus.* ***B-B'''*** *show Parvalbumin immunofluorescence staining of different areas of the mouse hippocampus. B, hilus and dentate gyrus; B', mossy fiber projecting to CA3 pyramidal neurons; B'', CA1 pyramidal neurons, B''', higher magnification of CA1 Parvalbumin positive (PV+) interneurons. Arrows in B and arrowheads in B''' show PV+ interneurons in the DG and CA1 respectively.* ***C-C'''*** *show NPY immunofluorescence staining of different areas of the mouse hippocampus. C, hilus and dentate gyrus; C', higher magnification of DG NPY positive neurons; C'', CA1 pyramidal neurons, C''', higher magnification of CA1 NPY positive neurons. Arrows in C' and arrowheads in C''' show NPY positive neurons in the DG and CA1 respectively (scale bar = 50µm).*

To assess cell specificity of the mCamKII(0.4) promoter, we checked expression patterns using LV_mCamK-EGFP and co-stained hippocampal slices with different cellular markers. We used antibodies against GFAP and Parvalbumin to rule out EGFP expression in astrocytes and a sub-set of GABAergic interneurons, respectively. Consistent with a previous characterization of the promoter activity in cortical cells (Dittgen *et al*, 2004), no signal was found in astrocytes nor in PV+ interneurons (Figure 20).

NPY and Y2 receptor overexpression in the hippocampus of WT mice

We then focused our attention on the expression of NPY and its receptor Y2 *in vivo*. Wild-type animals were injected with a single dose of viral vector in the dorsal hippocampus and expression of NPY and Y2 was assessed both by immunofluorescence and western blot.

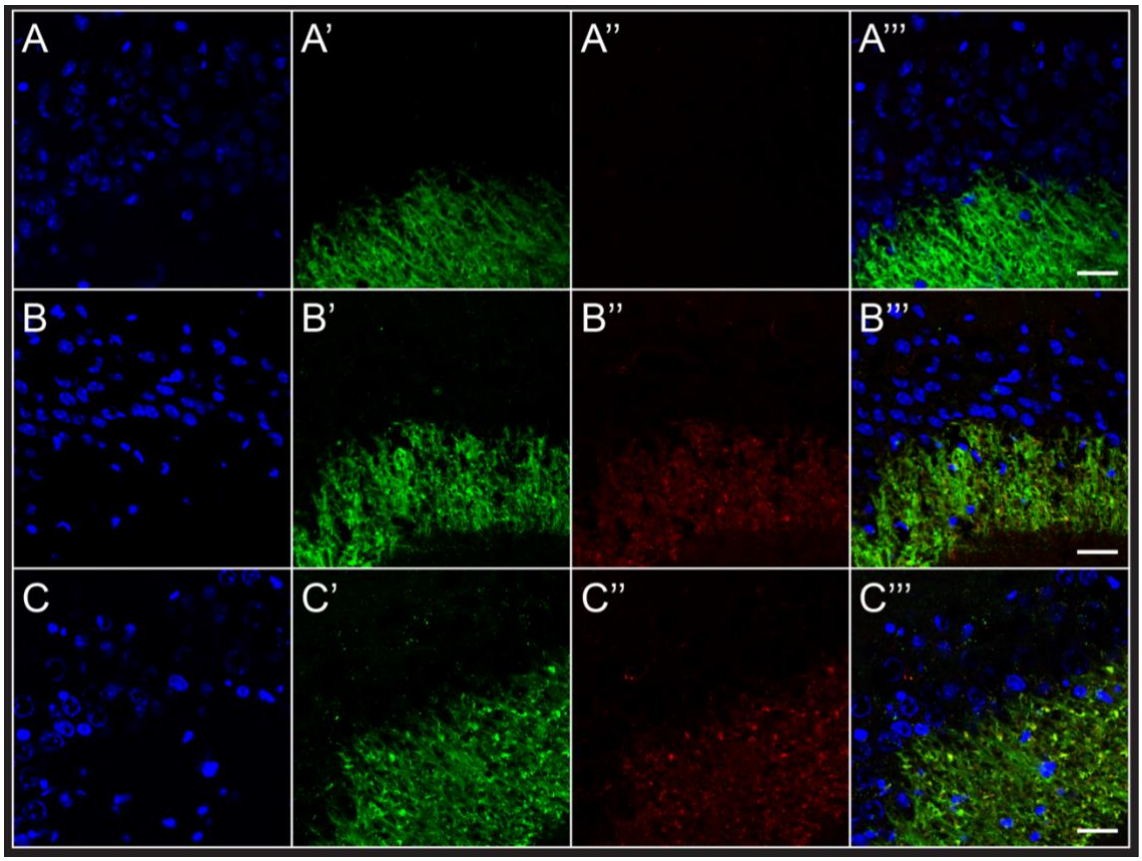

Figure 21 – Lentiviral mediated NPY overexpression in the mossy fiber terminals of WT mice.
A. *Representative confocal images of WT mice injected with either LV_mCamK-EGFP (**A-A'''**), LV_mCamK-NPY (**B-B'''**), or LV_mCamK-Y2-NPY (**C-C'''**). Immunofluorescence staining for DAPI (blue), EGFP (green), NPY (red), are shown and represent the mossy fiber pathway of dentate granule cells projecting their axons onto CA3 pyramidal neurons (scale bar = 20µm).*

Consistent with *in vitro* experiments, a strong overexpression of NPY was detected in transduced neurons. Of note, the mossy fiber pathway, which consist of the unmyelinated axons of granule cells projecting to CA3, was strongly immunoreactive to NPY staining in animals that received either LV_mCamK-NPY or LV_mCamK-Y2-NPY, but not in LV_mCamK-EGFP treated-animals (Figure 21).6

In parallel, we also assessed NPY2R expression in mossy fibers. Although several works in the literature describe NPY2R expression on presynaptic terminals of mossy fiber in rat, mouse and human samples (Stanić *et al*, 2006, 2011; Furtinger *et al*, 2001; Sperk *et al*, 1992), we have not been able to detect a convincing staining in wild type animals or animals treated with LV_mCamK-EGFP. However, Y2R immune-positive fibers were detected in LV_mCamK-Y2-NPY transduced animals, with a pattern resembling the one previously described (Stanić *et al*, 2011) (Figure 22).

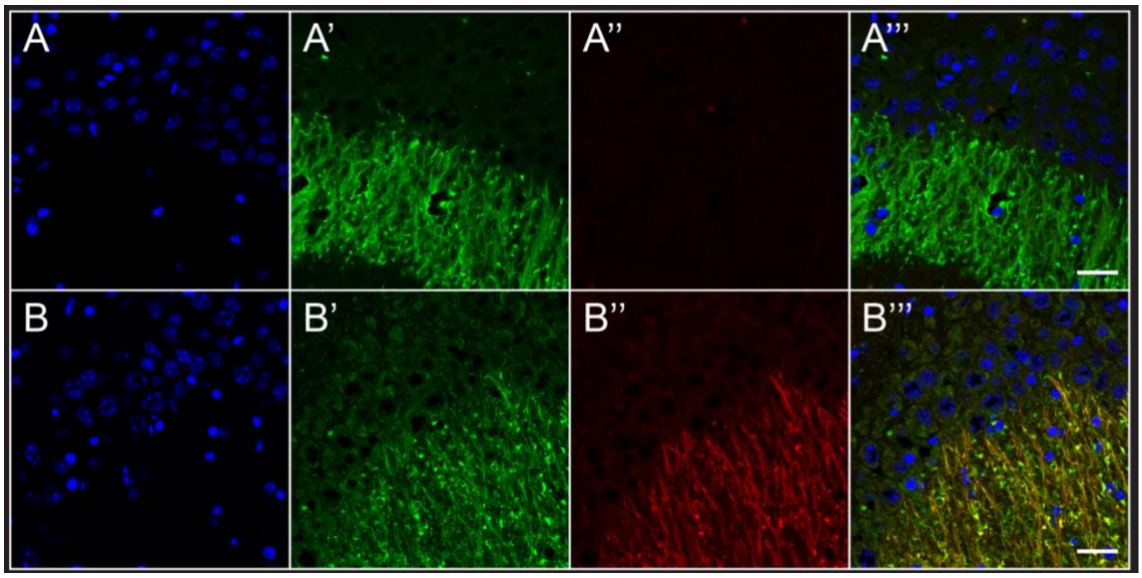

Figure 22 – Lentiviral mediated Y2 receptor overexpression in the mossy fiber terminals of WT mice.
*Representative confocal images of WT mice injected with either LV_mCamK-EGFP (**A-A'''**), or LV_mCamK-Y2-NPY (**B-B'''**). Immunofluorescence staining for DAPI (blue), EGFP (green), Y2 receptor (red), are shown and represent the mossy fiber pathway of dentate granule cells projecting their axons onto CA3 pyramidal neurons. Scale bar = 20µm.*

Consistent with the results obtained so far, western blot analysis of injected animals confirmed a significant overexpression of both NPY and Y2 receptor. As shown in Figure 23, hippocampal extracts from WT animal injected with either LV_mCamK-EGFP, LV_mCamK-NPY or LV_mCamK-Y2-NPY showed a higher level of NPY expression (Figure 23A,B). Y2 receptor expression was also confirmed analyzing extracts of hippocampal microsomal fractions by western blot with a FLAG antibody, showing Y2R expression only in LV_mCamK-Y2-NPY transduced tissue (Figure 23C).

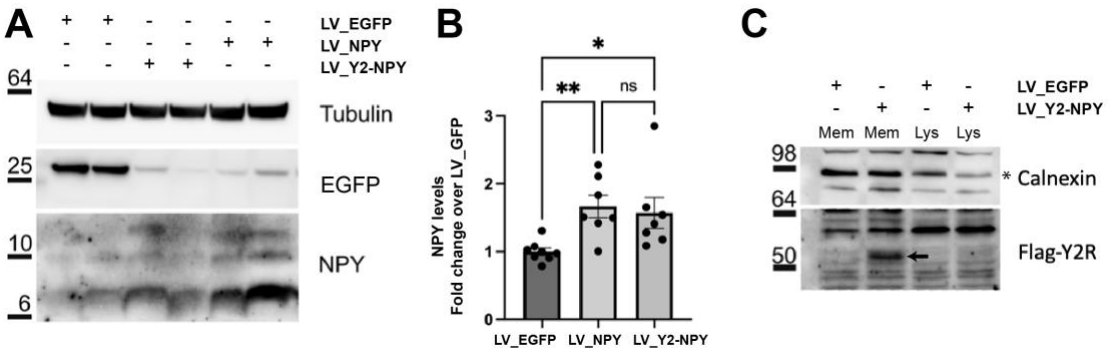

Figure 23 – Western blot analysis of NPY and Y2 expression in WT mice.
*Representative western blot (**A-C**) and quantification (**B**) of the indicated proteins in extracts from hippocampi of wild-type mice injected with the indicated vectors (* highlight Calnexin; Arrow indicates Flag-Y2 Receptor). Protein levels are shown as fold change over control (LV_mCamK-EGFP = 1 ± 0.05, n= 8 mice; LV_mCamK-NPY = 1.66 ± 0.16, n = 7 mice; LV_mCamK-Y2-NPY = 1.57 ± 0.22, n=7 mice). Protein levels are normalized for loading (Tubulin) and shown as fold change over control (LV_mCamK-EGFP). Mem = membrane fraction; Lys = total extract. Statistical significance was calculated using the Kruskal–Wallis one-way analysis of variance followed by the Dunn's post hoc test. * p<0.05; ** p<0.01.*

Upregulation of NPY in the mossy fiber terminals of Synapsin TKO animals during epileptogenesis

As described in the introduction, there is a strong evidence of the antiseizure effect of NPY in classic pharmacology studies, transgenic animals, and gene therapy approaches. In addition, it has been demonstrated that the epileptogenic process and epilepsy itself modify the expression pattern of the genes encoding NPY and its receptors in both rodents and humans. Hence, NPY rises great interest as a potential target for the treatment of epilepsy. Indeed, NPY is up-regulated in epileptic hippocampal samples despite the loss of NPY^+ GABAergic interneurons in the hilus and it has been demonstrated to be ectopically produced in neurons of the dentate gyrus after an epileptic insult, i.e., during the epileptogenic process.

Although the high amount of data in chemically induced animal models of epilepsy and in human specimens from mTLE patients, little is known about NPY regulation in genetic models of epilepsy. We thus evaluated the synapsin triple KO (TKO) mouse model, in which deletion of the three synaptic genes results in spontaneous seizures. As previously described (Cambiaghi *et al*, 2013), these animals do not display any gross defect nor epileptic phenotype during early development. Conversely, TKO display relatively rare spontaneous seizures and increased susceptibility to evoked seizures between 60 and 100 days of life. Hence, we asked whether TKO animals display reactive

up-regulation of NPY in a window ranging from 30 to 120 days of life. First of all, we performed an immunohistochemistry analysis on NPY on wild-type and TKO animals at 100 days. This specific time-point was chosen based on previous data showing a peak of seizure frequency at 90 days of age (Cambiaghi *et al*, 2013).

As shown in Figure 24, TKO animals show an ectopic NPY expression in the mossy fiber terminals which is not present in control animals.

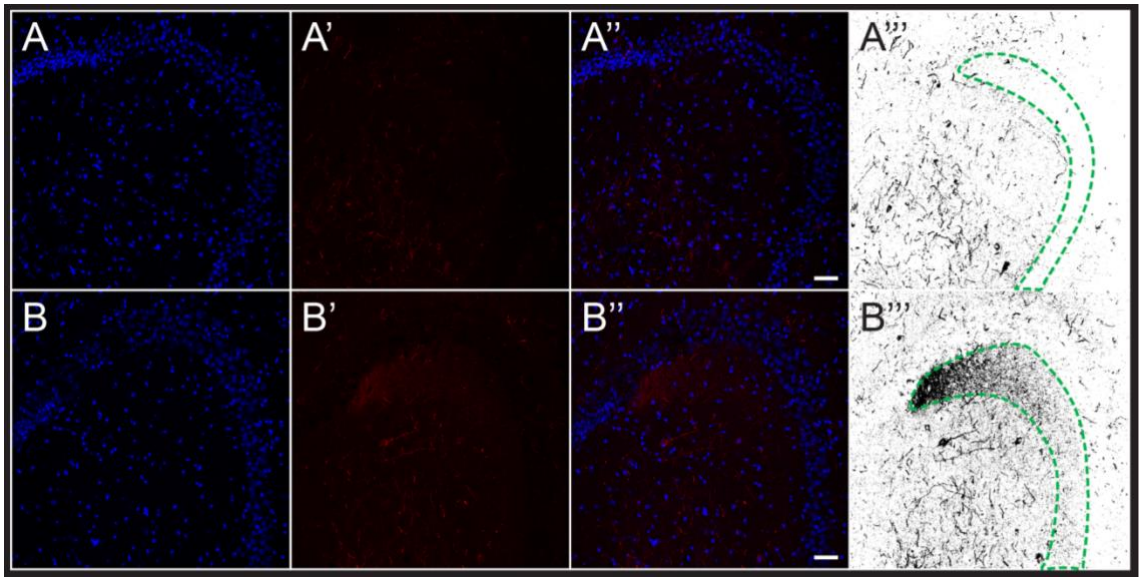

Figure 24 – NPY up-regulation in the mossy fiber terminals of TKO mice.
*Representative confocal images of WT (**A-A'''**) and TKO (**B-B'''**) mice at 100 days of age. Immunofluorescence staining for DAPI (blue) and NPY (red and binary) are shown. Note the mossy fiber pathway containing dentate granule projecting axons onto CA3 pyramidal neurons which is ectopically expressing NPY only in the TKO mice. Scale bar = 50µm.*

We then confirmed and extended these data by performing a western blot analysis on protein extracts from hippocampi of WT and TKO mice at different timepoints. In line with the already mentioned timeline of seizure occurrence, we analyzed animals at 30, 60, 90, and 120 post-natal days (PND; Figure 25). Interestingly, in TKO animals, NPY expression shows an increase at 90 PND. However, the level of NPY in the hippocampus of TKO mice was not significantly higher compared to WT mice at a later timepoint (120 PND).

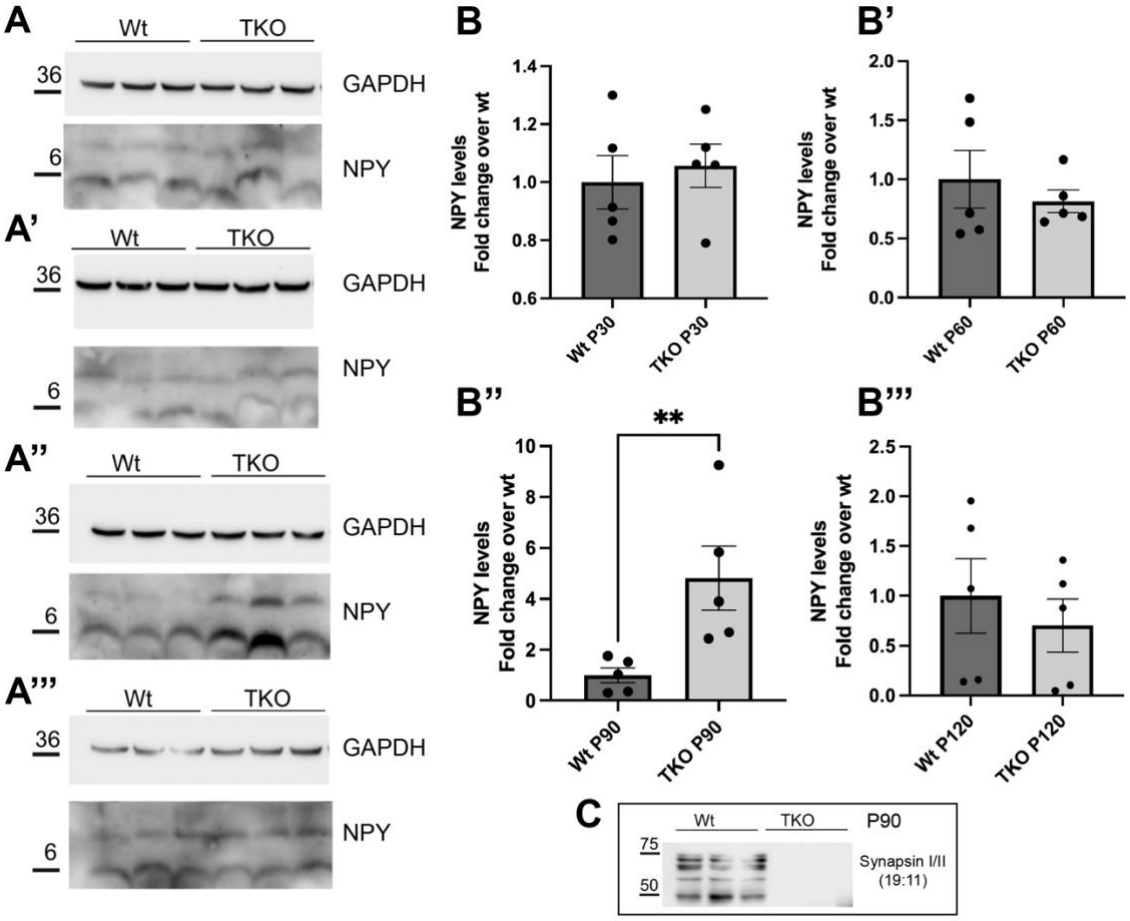

Figure 25 – Temporal NPY up-regulation in the hippocampi of Wt and TKO animals.
*Representative western blot (**A**) and quantification (**B**) of NPY in extracts from hippocampi of TKO mice at the indicated time points. A and B = p30; A' and B' =p60; A'' and B'' = p90; A''' and B''' = p120. Protein levels are shown as fold change over control (Wt). Protein levels are normalized for loading (GAPDH) and shown as fold change over control. Data re shown as Mean ± SEM. (B) WT = 1 ± 0.09; TKO = 1.05 ± 0.07; (B') WT = 1 ± 0.24; TKO = 0.08 ± 0.09; (B'') WT = 1 ± 0.29; TKO = 4.82 ± 1.26; (B''') WT = 1 ± 0.37; TKO = 0.7 ± 0.26. Statistical significance was calculated using the Mann–Whitney U test (n=5 mice for each genotype for each time point). ** p<0.01. (**C**) Representative western blot of Synapsin in extracts from hippocampi of WT and TKO mice (p90).*

Combined overexpression of NPY and Y2 receptor reduces frequency and duration of seizures in TKO mice

The increased levels of NPY at P90 can be interpreted as an adaptive reaction to the development of seizures, that has an onset around P60. In the view of anticipating and reinforcing this response, we decided to treat TKO mice in a time window when seizures are not yet present and the ectopic expression of NPY is still absent, or at least undetectable. The hypothesis is to prevent seizure occurrence by delaying the epileptogenic process.

As shown in the schematic representation of Figure 26A, we chose to inject the viral vector at p45, implant a telemetry probe 1 week later, and start monitoring the animals at p60 for 3 consecutive weeks. TKO littermates were treated with two injections, bilaterally in the hippocampus, with either the LV_mCamK-EGFP or LV_mCamK-Y2-NPY. At P60, animals were transferred in the telemetry system where video-EEG monitoring was performed 24/24h for 7 days a week to detect EEG and behavioral generalized seizures (Figure 26B).

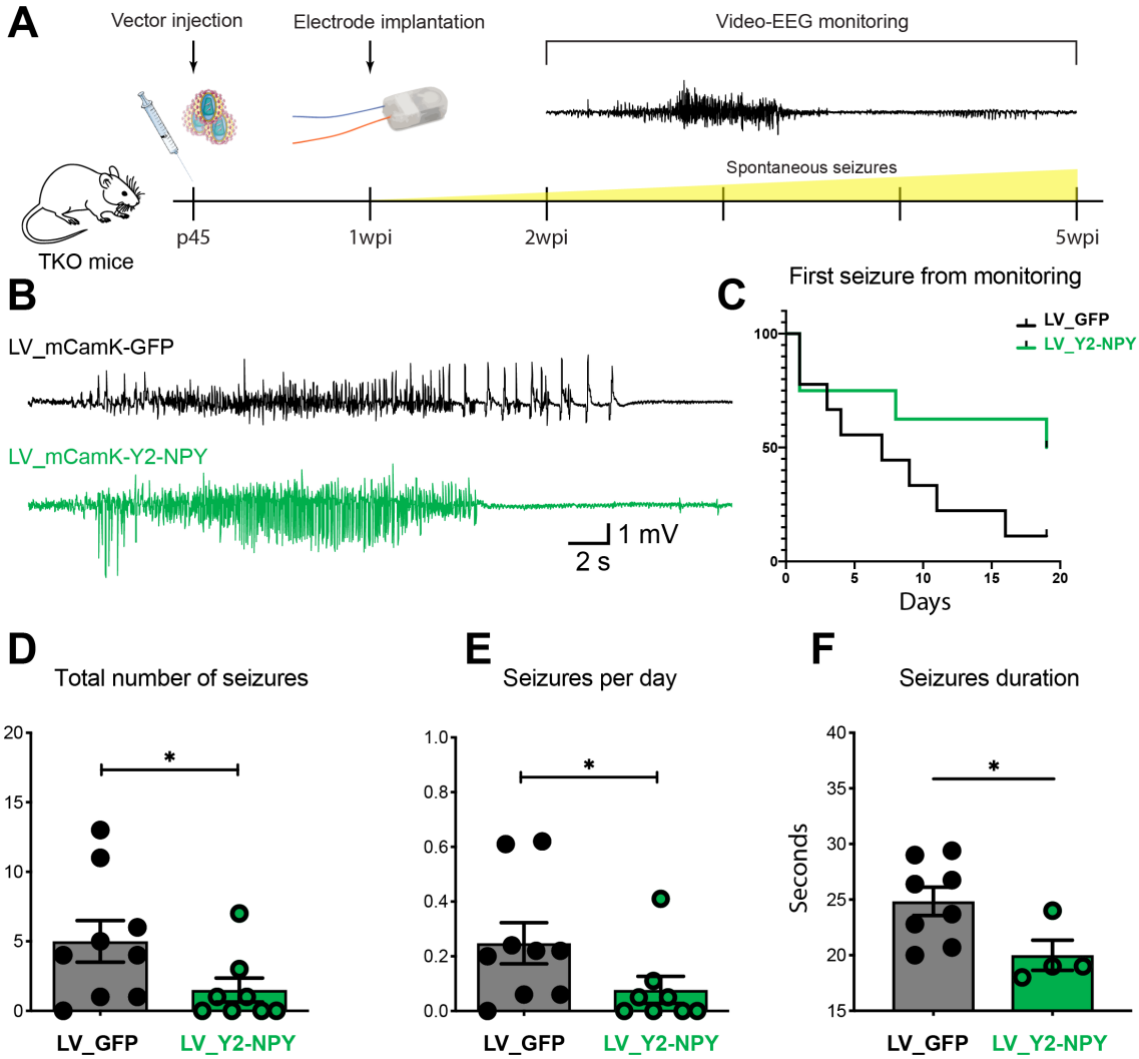

Figure 26 – Lentiviral mediated overexpression of NPY and Y2R reduce seizures in TKO mice.

A. *Schematic illustration of the experimental set-up for in vivo treatment of TKO mice. Viral vector injection of either LV-mCamK-EGFP or LV-mCamK-Y2-NPY at P45. EEG telemetry electrode implantation 1 week post viral injection. Video-EEG monitoring 2 weeks post-electrode implantation (P60).* ***B.*** *Representative EEG traces of seizures in LV_mCamK-EGFP and LV_mCamK-Y2-NPY TKO mice.* ***C.*** *Time to first seizure from the initiation of monitoring (n=9 TKO mice treated with LV_EGFP, n=8 TKO mice treated with LV_Y2-NPY). p=0.06; Log-rank (Mantel-cox) test. Data are shown as mean ± SEM. (**D**) Total number of seizures (LV_mCamK-*

*EGFP = 5 ± 1.4, and LV_mCamK-Y2-NPY = 1.5 ± 0.8), (**E**) seizures per day (LV_mCamK-EGFP = 0.24 ± 0.7, and LV_mCamK-Y2-NPY = 0.07 ± 0.04), and (**F**) mean seizure duration (LV_mCamK-EGFP = 24.8 ± 1.2 s n= 8 mice, and LV_mCamK-Y2-NPY = 20 ± 1.3s n=4 mice) *p<0.05; Mann-Whitney U test.*

The combined expression of NPY and Y2Rs significantly reduced the total number of seizures and seizure per day in treated animals compared to control (Figure 26D,E). Moreover, the duration of individual seizures was significantly reduced (Figure 26F). Last, given that monitoring was performed from day 60, and therefore we do not have evidence of spontaneous seizures occurring before this specific time-point, animals treated with LV-mCamK-Y2-NPY displayed a clear tendency towards a delay in time to first seizures (p = 0.06; Figure 26C).

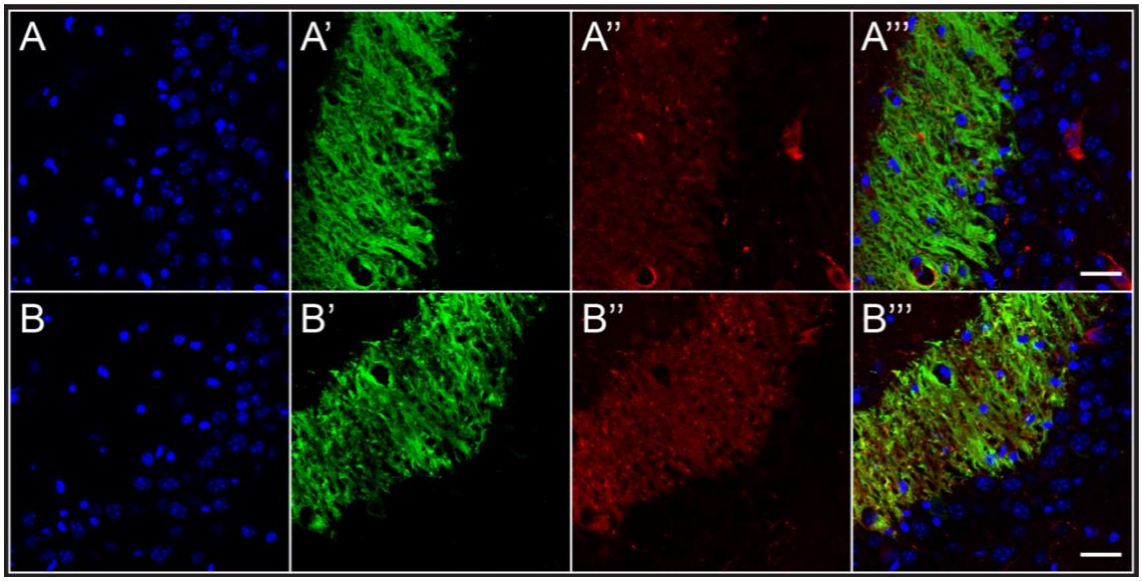

Figure 27 – NPY immunofluorescence in TKO animals after video-EEG monitoring.
*Representative confocal images of TKO mice following video-EEG monitoring treated with either LV-mCamK-EGFP (**A-A'''**) or LV_mCamK-Y2-NPY (**B-B'''**). Immunofluorescence staining for DAPI (blue), EGFP (green), and NPY (red) are shown. Scale bar = 20µm*

A subset of animals (n=3 animals per group) was sacrificed at the end of the monitoring to confirm the correct expression of the transgenes in the TKO background. In line with what was previously shown, TKO mice treated LV-mCamK-EGFP showed an increased expression of NPY in response to seizure occurrence (Figure 27A) that was further increased by injection of the vector LV-mCamK-Y2-NPY (Figure 27B).

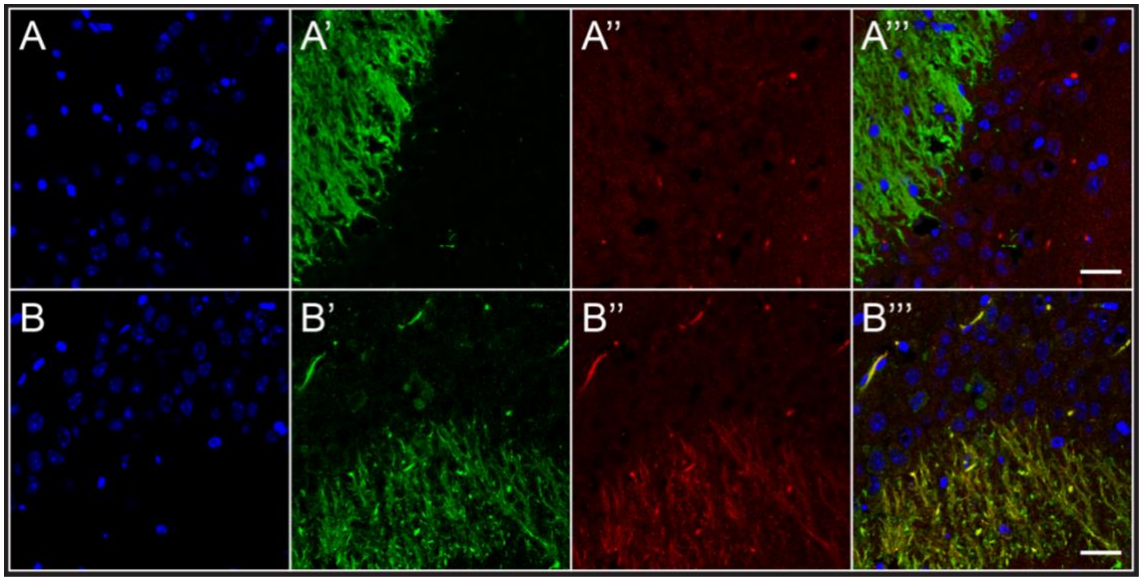

Figure 28 – Y2 receptor immunofluorescence in TKO animals after video-EEG monitoring. *Representative confocal images of TKO mice treated with either LV-mCamK-EGFP (**A-A'''**) or LV_mCamK-Y2-NPY (**B-B'''**). Immunofluorescence staining for DAPI (blue), EGFP (green), and Y2R (red) are shown. Scale bar = 20µm*

Moreover, no Y2 positive fibers were detectable in control treated TKO animals, while many Y2 positive terminals were observed at the level of the mossy fiber in the region of the CA3 in animals treated with LV-mCamK-Y2-NPY (Figure 28B).

Chapter VI – DISCUSSION

Electrophysiological characterization of HSV-1 replication-defective vectors

Gene therapy has enjoyed success in the last few years. Originally devoted to the treatment of rare monogenic diseases, it seems now to reach a maturity level for broader approaches to treat diseases affecting millions of people worldwide. Among the most prominent viral-based gene therapies platform available, lentiviral vectors have been extensively used for gene delivery to bone marrow stem cells. Other applications involving gene transfer to the retina and whole organs, such as the liver, have made effective use of AAV.

Both LV and AAV have been already brought to clinical trials for treating CNS disorders (Snowball *et al*, 2019; Szczygieł *et al*, 2020; Jensen *et al*, 2021; Jablonka *et al*, 2022). However, while these vectors are proving effective for some applications, future gene therapies might require delivery of large genes, complex multigene cassettes, or sophisticated genome editing approaches (Colasante *et al*, 2020; Lubroth *et al*, 2021) that greatly exceed the payload capacity of lentiviral and AAV vectors. Although several strategies either to miniaturize the expression cassette (Xu *et al*, 2021) or to split it in multiple viral vectors (Tornabene *et al*, 2019) are under the lens of many laboratories, it remains highly uncertain if these processes will ultimately result in satisfactory efficacy.

In the past few years, great advancements in the field of viral vectors based on HSV-1 have been made by our and other laboratories. The most attractive feature of these vectors is that they can accommodate very large payloads, amplifying the potential impact of our future gene therapy approaches. However, some degree of concern remains regarding their safety and, therefore, potential for applicability to treat human CNS pathologies.

We studied the effects of HSV-1 replication-defective vectors on neuronal physiology. We used the JΔNI8 vector backbone, which has been previously described to induce persistent transgene expression in the mouse brain for several months without any sign of morphological alteration nor inflammatory response (Verlengia *et al*, 2017; Miyagawa *et al*, 2017). However, the impact of these viral vectors on cell physiology has not been studied yet.

In the present work, we report that a JΔNI8 vector expressing tdTomato under the Ubiquitin promoter maintains its exclusively neuronal tropism in vitro without promoting expression of the reporter gene in astrocytes. Furthermore, we confirm that no morphological alterations are observed in transduced neurons. However, whole-cell patch-clamp experiments revealed several electrophysiological alterations after JΔNI8 transduction comprising an increase in the spontaneous network activity, and reduction of RMP and Rin.

In particular, spontaneous spiking activity was observed as single spike discharges or bursts of action potentials interspersed by a quiescent state of highly hyperpolarized RMP. Occasionally, at hyperpolarized potential, the spontaneous depolarizing events appeared similar to excitatory post-synaptic potentials. Despite the high firing frequency of transduced hippocampal neurons, resting membrane potential was strongly hyperpolarized compared to control cells. Spontaneous action potential shape also displayed differences in the AHP between transduced and mock neurons which may result from difference in K+ conductance. However, further experiments are needed to verify this hypothesis.

In the frame of studies on the pathophysiological basis for certain types of HSV-1 induced neuralgias, similar electrophysiological alterations were recorded in primary cultures of dissociated sensory neurons infected with syncytial HSV-1 strains (Mayer *et al*, 1985). In line with what we observed, and despite the use of a WT HSV-1 replication-competent syncytial strain, these authors claimed that no marked morphological changes were observed in neuronal cultures. In fact, even at the latest time point we employed for patch-clamp experiments (i.e., 96 h after transduction), cells appeared viable without signs of cytotoxicity.

This prompted us to investigate the possibility that JΔNI8 vectors might have conserved a fusogenic/syncytial ability, even if highly deficient and non-replicative. We first addressed this question by performing a dye coupling experiment demonstrating that the fluorescent Neurobiotin dye was able to diffuse and label neighboring cells. This was indeed the case and, importantly, the diffusion of the dye was restricted exclusively to other tdTomato positive cells. Assuming that fusogenic events take place between cells, we cannot disambiguate whether all the tdTomato(+) cells were directly infected by viral

particles, or the pores formed between membranes allowed the movement of proteins like tdTomato.

Although several dyes with increasing molecular weight could be tested to address this question, we decided to further confirm our hypothesis by performing dual cell patch-clamp experiments of tdTomato positive neurons. Our results clearly show that JΔNI8 transduction of hippocampal neurons leads to electrical coupling and, consequently, to increased spontaneous activity. Thus, electrical coupling confirms the hypothesis that membrane fusion is present in transduced neurons.

As a consequence of fusogenic events, Rin reduction could be interpreted as the result of the increased membrane surface as expected following Ohm's law. Conversely, the strong RMP reduction cannot be directly explained by a process involving membrane fusion. In this respect, we speculate that other mechanisms capable of modifying neuronal physiology and ion channels expression, downregulation, or internalization might take place. For example, an alteration of Ca2+ homeostasis has been reported to occur following HSV-1 infection of rat dorsal root ganglion neuron-like hybrid cell line (Zhang *et al*, 2017). Other studies identified calcium-activated chloride channels as responsible for the after-hyperpolarization of primary neuronal cultures (Mayer, 1986). However, differences could arise from testing different neuronal subtypes and more detailed studies are needed to define the potential role of HSV-1 on specific channels regulating the RMP.

Since changes in intracellular Ca2+ concentration regulate a variety of intracellular pathways, including neuron excitability and synaptic transmission (Brini *et al*, 2014), we decided to test whether JΔNI8 transduction could influence basal calcium levels. Under normal conditions, calcium homeostasis is a tightly regulated process that keeps intracellular concentration in a range from nanomolar to micromolar through sequestration of calcium in organelles and transport outside the cell. Voltage changes, such as action potentials, cause big increases in intracellular calcium concentrations. In particular, we hypothesized that the hyperactivity of neurons resulting from fusogenic events between neurites could directly cause a massive increase in intracellular calcium. As showed by Fura-2 experiments, hippocampal neurons transduced with JΔNI8 viral vectors display significantly higher intracellular calcium levels compared to controls. However, high intracellular calcium is cytotoxic and adaptation mechanisms should take place. In our conditions, electrophysiological alterations were present from the earliest

(48h) to the latest (96h) time point analyzed. It is surprising that these overstimulated neurons do not die by apoptosis or necrosis. Notably, one study suggests that the HSV-1 antiapoptotic protein pUs3 could promote cell survival despite cellular insults (Jerome *et al*, 1999) which might explain why JΔNI8 transduced cells could remain alive under cytotoxic conditions for days.

Finally, we explored mechanisms that may stop the uncontrolled firing of transduced neurons. Expectedly, TTX treatment was able to completely block the uncontrolled firing, which indicates sodium-dependence of this event. Conversely, NBQX, a potent AMPA receptor antagonist, was only partially able to block spontaneous spiking of transduced cells, supporting the idea that excessive firing is not dependent on excitatory synaptic activity and, therefore, that electrical coupling resulting from fusogenic events is the main driving force for the alterations detected. Interestingly, blocking T-type calcium channel mirrored the effect of TTX, suggesting a key role also for T-type calcium channel.

Altogether, these results demonstrate that fusogenic events might arise after transduction of neurons with JΔNI8 viral vectors, leading to increased firing frequency, alterations of basic membrane parameters such as RMP and Rin, and an overall increase in basal intracellular calcium levels. Moreover, our data suggest that T-type calcium channels are involved in this process. Further experiments are mandatory to completely characterize the mechanism underlying these fusogenic events and electrophysiological alterations, but these are outside the scope of this thesis. However, the natural ability of HSV-1 to spread from cell-to-cell through the fusion of cell membranes (Ambrosini & Enquist, 2015) may be a feature that is maintained even in highly defective, replication incompetent viral vectors like JΔNI8. Hence, we asked whether the phenotype that we observed could be an unsurmountable limitation for the application of HSV-1 based vectors to treat CNS disorders.

We hypothesized that mutations in key genes may be at the bases of the fusogenic phenotype. To test this hypothesis, we decided to use a category of HSV-1 vectors in which almost all viral genes are absent, i.e., amplicons. Patch-clamp experiments have been therefore conducted in rat primary hippocampal neurons transduced with amplicon vectors provided by the company Bioviron®. Electrophysiological properties of transduced neurons have been evaluated at the same timepoints used for JΔNI8

experiments. Overall, no amplicon vectors did not alter any electrophysiological property of transduced primary neurons (Soukupová *et al*, 2021). Hence, we asked whether some batches of JΔNI8 could have acquired fusogenic properties due to genome recombination during the viral vector production process.

In our study, we found that different JΔNI8 vector preparations cause different levels of electrophysiological alterations. Therefore, we analyzed a batch of JΔNI8 vectors produced and purified by our collaborators at the University of Pittsburgh Medical Center (JΔNI8 P), that show a milder fusogenic phenotype compared to some of the batches produced in Milan (JΔNI8 M), namely spontaneous firing activity and Rin similar to controls but RMP similar to JΔNI8 M. Moreover, by the identification of cells based on the presence of random single spike discharges or bursts of action potentials, we counted a much small number of neurons displaying such alterations with JΔNI8 P than with JΔNI8 M.

We thus hypothesized that the variability between batches of viral vectors might result from DNA recombination during vector production. We analyzed the DNA sequence of 8 samples, 2 containing the BAC sequence used in Milan and Pittsburgh, and 6 consisting of 5 batches of JΔNI8 M and 1 of JΔNI8 P. Unfortunately, the DNA quality of two JΔNI8 M samples (JΔNI8 M2 and M5) showed a low coverage on HSV-1 viral genomes and, for that reason, were excluded from further analysis. Regarding the BAC sequences, the two samples were found to be completely overlapping, suggesting that no mutations were introduced during the routine handling of BAC DNA between the two laboratories. However, three missense mutations (D285N, A315T, A549I) in the UL27 gene (encoding the glycoprotein B) were consistently present in both the BAC DNA sequences and in all the JΔNI8 viral batches analyzed. Among these mutations, A315T was a de novo mutation that was not present in the former generations of the JΔNI vector family (Uchida *et al*, 2010), whereas the mutation D285N was expected as described in the work of Uchida et al (2010) in which the authors described a higher transduction efficiency by vectors generated with a variant of the gB gene called gB N/T (D285N, A549T). The third mutation we detected (A549I) also differs from the expected and results in a new variant of gB N/T/I (D285N, A315T, A549I) containing three mutated residues.

Interestingly, three different strains of replication-competent PRV (a member of the *Herpesviridae* family) strains, namely a virulent Becker strain (PRV-151), an avirulent

Becker strain not expressing the glycoprotein B (PRV-223 gB null), and an attenuated Bartha strain (PRV-152) have been previously compared (McCarthy *et al*, 2009). These viruses were used to characterize the effect of PRV on rat superior cervical ganglia through electrophysiological experiments. When the virus was added to the cultured neurons the authors noted that the PRV-151 displayed several electrophysiological alterations comprising high firing frequency in infected cells, a spikelet-like event during the hyperpolarized state, and formation of pore between neurons (demonstrated by both dye and electrical coupling). Conversely, such alterations never occurred in the gB null mutant (i.e. PRV-233), whereas alterations with PRV-152 were delayed over time compared to the PRV-151.

Altogether, these data are strongly in line with our results, despite the different viruses (PRV vs. HSV-1) and neuronal types (superior cervical ganglion vs. hippocampal neurons) that were investigated. While further modulatory mechanisms that might cause the time-dependent development of fusogenic events remain to be explored, the involvement of gB in the fusogenic mechanism seems a key element in both studies.

We thus speculate that conversion of the original gB N/T (D285N, A549T) into the new gBN/T/I (D285N, A315T, A549I) might be responsible for a mild syncytial phenotype, as observed with electrophysiological experiments. Further mutations that we found exclusively in JΔNI8 M vectors may account for their strong phenotype. Additionally, the occurrence of mutations in other genes involved in the fusogenic process such as gK, gH, gI, (Dogrammatzis *et al*, 2020; Granstedt *et al*, 2013) might be present in the samples analyzed. However, we could not explore this possibility in detail because a limitation in our sequencing approach is that the depth might not be sufficient to detect rare mutations that occur in a limited number of genome copies of a single viral batch.

Finally, it is interesting to note that, although in cultures exposed to pseudorabies virus (PRV) it was possible to observe fused cells in vitro, the same phenotype was not equally evident in vivo (McCarthy *et al*, 2009; Granstedt *et al*, 2013; Ambrosini & Enquist, 2015). These data may explain why our previous results showed that the stereotaxic injection of JΔNI8 vectors in various brain regions did not result in overt cell toxicity or induction of inflammatory cell infiltrates while persisting in the rat brain up to 6 months.

The variability of different viral batches asks for further engineering

In collaboration with Prof. Joseph Glorioso of the University of Pittsburg Medical Center we have now planned to further characterize and improve the JΔNI viral platform. To this aim, our collaborators are working on a new version of the JΔNI vector family which maintains all the modifications of the viral backbone except for gB, which will be reverted to its wt form. Subsequently, electrophysiological experiments will be performed on hippocampal neurons transduced with this new vector to confirm our hypothesis.

However, our results shed light on a potential limitation for HSV-1 based replication-defective vectors. In particular, we speculate that, during the manufacturing process, random mutations in the viral genome might arise, leading to potentially toxic viral batches. Indeed, although rare, DNA recombination might take place during repetitive cycles of cell infection during viral production.

In fact, we did not identify any alteration when using the amplicon-based vector. This is not surprising because this class of viral vectors is almost completely devoid of HSV-1 genes, containing only the *ori* and *pac* sequences, which are responsible for genome replication and packaging.

In any case, further engineering and routine quality control on HSV-1 replication-defective vectors is mandatory to further develop this class of vectors. Hence, our future perspectives aim at designing JΔNI variants deleted of gB or other viral genes such as Us9, that has been suggested to be involved in the formation of fusions in the asso-assonic conjunctions between neurons by mediating the transport of gB along the axon (Granstedt *et al*, 2013). However, this aim will imply the generation of new complementing cells and specific tests to assess the safety and efficiency of these new viral vector generations. Therefore, we decided to switch to LV vectors as an alternative platform for testing a combinatorial gene therapy for epilepsy.

Exploiting a combinatorial gene therapy approach in a genetic model of epilepsy

In the present thesis, we demonstrate the antiseizure effect of the hippocampal overexpression of NPY and its receptor Y2 in the Synapsin triple-KO model of epilepsy. Overexpression was achieved through the use of a single lentiviral vector expressing both Y2R and NPY under a minimal CamKII(0.4) promoter, which drives gene expression exclusively in excitatory neurons. In general terms, we demonstrate that this combinatorial gene therapy strategy exerts a significant seizure-suppressant effect in a genetic model of epilepsy.

We first characterized our viral platform in vitro by assessing NPY and Y2 receptor over-expression in rat primary hippocampal cultures. We demonstrate that both LV_mCamK-NPY (expressing only NPY) and LV_mCamK-Y2R-NPY (expressing NPY and Y2) are able to increase the intracellular level of NPY. However, we were able to measure a significant increase in the level of extracellular NPY release only in the media of neurons transduced with the Lentiviral vector expressing NPY alone. Nevertheless, it should be considered the possibility that NPY release in neurons transduced with the vector LV_mCamK-Y2R-NPY, which constitutively overexpresses NPY and its receptor Y2, might result in a reduction of NPY release itself. To address this question, we plan to measure extracellular NPY in neurons under depolarizing conditions and to compare the three experimental groups (EGFP, NPY, and Y2-NPY).

Furthermore, we characterize the minimal version of the CamKII promoter (mCamkII(0.4)) by showing that it is sufficient to drive transgene expression in hippocampal excitatory neurons. Indeed, by using the control LV_mCamK-EGFP vector, in which EGFP is driven by mCamkII(0.4), we found that no EGFP expression can be detected either in astrocytes (GFAP+), Parvalbumin positive interneurons, or NPY expressing interneurons in both the dentate gyrus and at the level of the CA1 of the hippocampus. However, since one of the novel aspects of the present approach is to specifically direct the transgene expression in the excitatory neuronal population of the hippocampus, further experiments have been planned to increase the panel of markers to determine promoter specificity. In particular, we will analyze EGFP expression in hippocampal slices co-stained with a general GABAergic marker such as GAD65 and

other interneuron markers such as Somatostatin (SST), Cholecystokinin (CCK), Vasoactive Intestinal Peptide (VIP).

We then demonstrate that both LV_mCamK-NPY (expressing only NPY) and LV_mCamK-Y2R-NPY (expressing NPY and Y2 together) efficiently overexpress NPY in the mossy fiber terminals of the dentate gyrus excitatory neurons, even if this cell population do not normally express it under basal conditions. To strengthen our hypothesis, it would be of great interest to study the effect of our approach on neuronal excitability by performing local field potential experiments stimulating the granule cell layer and recording in the stratum lucidum of CA3, in order to assess whether a reduction of the evoked excitatory post synaptic potential is achieved in slices of mice co-expressing NPY and Y2 compared to control.

Although several gene therapy approaches based on NPY and Y2R have been investigated in pre-clinical models of acquired epilepsy (Noè et al, 2008, 2010; Nikitidou Ledri et al, 2016; Szczygieł et al, 2020), none employed a promoter capable of inducing selective expression in excitatory neurons. Furthermore, only one study so far has been conducted in a genetic model of epilepsy, namely the rat model of Genetic Generalized Epilepsy with absence seizures (Powell et al, 2018). In the latter, NPY was delivered in the thalamus and somatosensory cortex via AAV-mediated transduction and proved effective in suppressing absence-like seizures. Here, we report for the first time a combinatorial approach based on the delivery of both NPY and its receptor Y2 in hippocampal excitatory neurons and in a genetic model of epilepsy.

We show that the TKO model that we exploited reproduces features of the NPY pathophysiological regulation that have been extensively described in both rodents and human epileptic samples (Sperk *et al*, 1992; Furtinger *et al*, 2001). Indeed, during the epileptogenic process NPY is ectopically expressed by dentate gyrus granule cells, while NPY+ GABAergic interneurons are lost. In line with this evidence, we hypothesized that analogous adaptive mechanisms might arise in animal models with a defined onset and progression of disease. In the TKO model, although the epileptogenic focus has not been mapped so far, we found that NPY up-regulation follows the epileptogenic process reaching its peak at approximately 90 days of age, coherent with the peak of seizure frequency (Cambiaghi *et al*, 2013). In particular, we performed a spatiotemporal analysis of NPY upregulation with both quantitative measurements at several time points (P30-

P60-P90-P120) and a qualitative localization of NPY upregulation specifically in the mossy fiber terminals of TKO mice. However, the level of NPY in the hippocampus of TKO mice was not significantly higher compared to WT mice at a later timepoint (120 PND). To better clarify this aspect, we have now planned a longitudinal analysis of WT and TKO mice to measure NPY level at different timepoints (P30-P60-P90-P120) to see if there is a physiological regulation in the level of the neuropeptide that might be shifted in the TKO genetic model. Moreover, in our opinion the ectopic upregulation of NPY should not be considered as a requirement for the therapeutic effect, but rather as a pathophysiological adaptive system that strengthens the hypothesis of the anti-seizure effect of NPY and Y2 combined over-expression.

We then decided to treat TKO animals with LV_mCamK-Y2-NPY before the initiation of the adaptive NPY up-regulation, in order to anticipate it and thereby prevent or attenuate spontaneous seizures. Indeed, the combination of NPY and Y2R reduced both the total number and the mean duration of seizures.

These data demonstrate that overexpression of NPY and Y2R mainly in granular cells is sufficient to exert a robust anti-seizure effect in a genetic model. This approach was designed to induce the NPY inhibition through pre-synaptic Y2Rs specifically in excitatory cells, thereby establishing an auto-inhibitory loop to decrease glutamate release. Previous attempts used ubiquitous (Szczygieł *et al*, 2020) or neuron-specific (Nikitidou Ledri *et al*, 2016), but not excitatory-specific promoters, thus driving NPY and Y2R expression also on inhibitory interneurons. Although interneurons only count for about 10-15% of the total neuronal hippocampal population (Pelkey *et al*, 2017), their distribution and morphology allow them to deeply integrate within the hippocampal formation and tightly regulate hippocampal circuitry. Consequently, avoiding the overexpression of these therapeutic genes in this cellular population is desirable to avoid a reduced GABA release. These considerations lead to the suggestion that gene therapy-mediated overexpression of NPY or NPY receptors can be more efficient if excitatory neurons are specifically targeted. Of note, it would be of interest to assess whether there is a correlation between NPY expression levels and anti-seizures effects. Unfortunately, brains of TKO mice that underwent video-EEG recordings were collected and cryopreserved to perform immunofluorescence staining to verify transgene expression at the end of the monitoring period. For this reason, we did not have the opportunity to

perform a quantitative correlation between NPY over-expression and seizure reduction in our conditions.

However, it has been recently proposed that mossy cell of the hilus might have a protective role in the hippocampal network by preventing seizures progression (Bui *et al*, 2018). Although other studies argue against this hypothesis (Botterill *et al*, 2019), it would be of interest to study the mossy cell population survival in the TKO treated mice compared to controls. Furthermore, hypoexcitability of mossy cell has been reported in the Synpasin II-/- genetic model prior to the symptomatic phase, which may render the hippocampal circuitry more prone to hyperxcitability in adult animals (Toader *et al*, 2013).

Finally, to further support our data, it would be interesting to test the proposed strategy in other epilepsy models. In particular, to measure the therapeutic effects of the combinatorial expression of NPY and Y2 specifically in excitatory neurons in either a chronic model of epilepsy or a second genetic model. Moreover, another interesting aspect would be to test if our strategy is effective in other regions of the brain, such as the cortex, where the ectopic up-regulation of NPY seen in the hippocampus has not been observed yet. To this aim, a focal neocortical seizures model (Mainardi *et al*, 2012) could be used.

Concluding remarks

A growing number of studies demonstrate that most classically defined idiopathic forms of epilepsy have a complex genetic component. In the last decades, several genes have been identified as directly responsible for epilepsy syndromes. Among these, many voltage-dependent and ligand-gated ion channels including $GABA_A$ receptors (Harkin *et al*, 2002), nicotinic receptors (Steinlein *et al*, 1995), and sodium channels (Lopez-Santiago & Isom, 2019). In addition, among the gene families added to the list of predisposing factors, were identified genes involved in synaptic transmission (Giovedí *et al*, 2014), genes related to metabolic pathways (Brockmann *et al*, 2001, 1), and genes related to protein synthesis (Vatsa & Jana, 2018).

Animal models KO for a specific gene or gene families have been generated to study the molecular mechanisms underlying epilepsy in which these genes are involved. The synapsin TKO mice are an example of this approach. Even if not all these models may be

ideal, as they do not reflect the human pathology or show no or very mild epileptic phenotype, the increasing number of studies on these genetic animal models and the stratification of patients based on their genetic predisposing mutations will allow to better align preclinical and clinical studies, hopefully leading to personalized therapies. As an example, in this work we provide the first evidence of the possibility of using an approach that aims to target a general mechanism (rebalancing excitation/inhibition by promoting self-inhibition of excitatory cells) to treat a genetic model of epilepsy in which three genes involved in synaptic transmission are knockout.

Outlook for the use of viral vector-based strategies in humans

Despite the complexity of the NPY-system, antiseizure gene therapy strategies have been pursued in several pre-clinical animal models of acquired epilepsy. In all these studies, the targeting of NPY alone, or in combination with Y2 or Y5 receptors, proved successful in controlling seizures. Concern may arise on the potential impact of NPY and Y2 receptor ectopic expression on normal neuronal activity. However, recent studies showed that no effect was observed on body weight nor on short- or long-term memory in rats treated with the combination of NPY and Y2 and tested in the Y-maze or Morris water maze tests (Szczygieł *et al*, 2020).

Here, we refined this approach in terms of vector design and extended it to a genetic model. Notably, the first-in-human gene therapy trial for drug-resistant epileptic patients has been recently approved (NCT04601974) and will soon start recruiting. This trial will employ LV vectors to deliver an engineered potassium channel. Our results, that lay on a previous, robust literature and employ last generation LV vectors, contribute to form the basis for future gene therapy clinical trials based on the use of NPY and its family of genes.

Chapter VII - MATERIALS AND METHODS

Materials

Cell culture media and reagents, if not otherwise stated, were from Lonza (Basel, Switzerland). Culture flasks and multiwell plates were from Nalgene (Thermofisher). Petri dishes were from Falcon BD (Franklin Lakes, NJ, USA).

DL-2-amino-5-phosphonopentanoic acid (APV), tetrodotoxin (TTX), NNC55-0396, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBX) disodium salt were from Tocris (Bristol, UK). Other chemicals for general use were from Sigma-Aldrich (St Louis, MO, USA).

Antibodies

Primary antibodies used in the present thesis were:

- α-Tubulin
    - WB – (Sigma; mouse mAb, T9026); 1:6000
- β-III-Tubulin
    - ICC – (Covance); 1:1000
- Neuropeptide Y (NPY)
    - WB – (Cell signaling; Rabbit, mAb #11976); 1:1000
    - ICC – (Cell signaling; Rabbit, mAb #11976); 1:150
    - IHC – (Cell signaling; Rabbit, mAb #11976); 1:250
- Glial fibrillary acidic protein (GFAP)
    - ICC – (Sigma; mouse mAB, G3893); 1:250
    - IHC – (Sigma; mouse mAB, G3893); 1:250
- Green fluorescent protein (EGFP)
    - WB – (Abcam; Chicken, ab13970); 1:250
    - ICC – (Abcam; Chicken, ab13970); 1:250
    - IHC – (Abcam; Chicken, ab13970); 1:250
- Flag
    - WB – (Sigma; mouse mAb 1804); 1:1000
- Parvalbumin (PV)
    - IHC – (Sigma; mouse mAb; P3088); 1:500
- Y2 Receptor (Y2R)

    - IHC – (Neuromics; Rabbit, RA14112); 1:200
- Calnexin
    - WB – (Enzo life science); 1:2000
- Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
    - WB – (Sigma, rabbit G9545); 1:6000
- Synapsin
    - WB – 1:1000

Secondary antibodies used in the present thesis were:

- Alexa Fluor 647 Goat anti-mouse
    - ICC – (Invitrogen; A-21235); 1:250
    - IHC – (Invitrogen; A-21235); 1:300
- Alexa Fluor 594 Goat anti-rabbit
    - ICC – (Invitrogen; A-11012); 1:250
    - IHC – (Invitrogen; A-11012); 1:300
- Alexa Fluor 594 Goat anti-mouse
    - IHC – (Invitrogen; A-11005); 1:300
- Alexa Fluor 488 Goat anti-chicken
    - ICC – (Invitrogen; A-11039); 1:250
    - IHC – (Invitrogen; A-11039); 1:300

Molecular biology techniques

Plasmids

Construct used in the present thesis were:

- pEnter Ub-tdTomato (already present in the lab)
    - It is composed by a Ubiquitin promoter (pUbc) driving the expression of the tdTomato reporter gene followed by SV40 polyA. The expression cassette is flanked by two attL sites for Gateway Recombination mediated by LR Clonase.
- pLenti mCamKII(0.4)-EGFP
    - Plasmid containing the minimal CamKII(0.4) promoter sequence was kindly donated by Dr. Marco Ledri (Lund University). PCR-amplified

sequence of the promoter was extracted with Primer #1 and #2 (see Table 3) and subcloned into a lentiviral transfer plasmid using EcoRV and AgeI.

- pLenti mCamKII(0.4) -NPY-IRES-EGFP
    - Adapter sequences (#3 and #4) were used to insert a NheI site through BamHI digestion ahead IRES sequence in a plasmid containing the IRES-EGFP sequence (present in the lab).
    - Adapters (#5 and #6) were used to insert a SalI downstream EGFP coding sequence.
    - PCR-amplified sequence of NPY sequence (Primer #7 and #8) have been extracted from a pcDNA3.1-NPY plasmid (present in the lab) and subcloned upstream to the IRES-EGFP through XbaI/NheI digestion. Since, cleavage with XbaI and NheI produces compatible ends, correct insertion of the sequence was checked before proceeding.
    - Subsequently, NPY-IRE-EGFP were cloned into the pLenti mCamKII(0.4)-EGFP by AgeI/SalI digestion removing the EGFP sequence.
- pLenti mCamKII(0.4)-Y2R(flag)-T2A-NPY-IRES-EGFP
    - Adapters (#9 and #10) were used to insert MluI and XbaI through AgeI/NheI digestion upstream the IRES-EGFP sequence.
    - Adapters (#11 and #12) were used to insert a Flag-T2A sequence through MluI/XbaI digestion.
    - PCR-amplified sequence of Y2R sequence (Primer #13 and #14) was extracted from a pcDNA3.1-Y2R plasmid (present in the lab) and subcloned upstream to the Flag-T2A-IRES-EGFP through AgeI/MluI digestion in frame with the Flag tag.
    - Y2R(Flag)-T2A-IRES-EGFP sequence were cloned into the pLenti mCamKII(0.4)-EGFP by AgeI/SalI digestion removing the EGFP sequence.
    - PCR-amplified sequence of NPY (Primer #7 and #8) was finally inserted downstream the T2A sequence in frame with Y2R by

XbaI/NheI digestion to create pLenti mCamKII(0.4)-Y2R(flag)-T2A-NPY-IRES-EGFP

#N	Strand	Primer ID	Sequence
#1	FW	EcoRV_miniCamK	AAACGATATCTGACTTGTGGACTAAG
#2	RW	EGFP	GAACTTGTGGCCGTTTAC
#3	FW	NheI with BamHI	GATCCACGTGCTAGCACTGGC
#4	RW	NheI with BamHI	GATCGCCAGTGCTAGCACGTG
#5	FW	SalI with NotI	GGCCGTATTCAGGTCGACTAGGC
#6	RW	SalI with NotI	GGCCGCCTAGTCGACCTGAATAC
#7	FW	XbaI-AgeI-NPY	TTAATCTAGATACCGGTCCACCATGCTAGGTAACAAGC
#8	RW	NheI-NPY	TAAGCTAGCTCATCACCAC
#9	FW	AgeI-MluI-XbaI-NheI	CCGGTTACGCGACGCGTCGTAGGCTCTAGAGCTAAAG
#10	RW	AgeI-MluI-XbaI-NheI	CTAGCTTTAGCTCTAGAGCCTACGACGCGTCGCGTAA
#11	FW	(MluI) Flag-T2A (XbaI)	CGCGTGATTACAAGGATGACGACGATAAGGGCTCCGGCGAGGGCAGGGGAAGTCTTCTAACATGCGGGGACGTGGAGGAAAATCCCGGCCCAT
#12	RW	(MluI) Flag-T2A (XbaI)	CTAGATGGGCCGGGATTTTCCTCCACGTCCCCGCATGTTAGAAGACTTCCCCTGCCCTCGCCGGAGCCCTTATCGTCGTCATCCTTGTAATCA
#13	FW	AgeI-Y2R	GCAGCACCGGTCGCCACCATGGTTC
#14	RW	MluI-Y2R	ATTTACGCGTCACATTGGTAGCC

Table 3 – List of primer used for generating the plasmids employed in the present thesis.

Oligonucleotide phosphorylation and annealing

Dehydrated oligonucleotides were synthetized and delivered by Sigma-Aldrich. After reconstituted with sterile mqH2O separate phosphorylation reactions were set up for each oligo using T4 Polynucleotide Kinase (NEB) according to manufacturer's instruction.

Specific pairs of phosphorylated oligos were then annealed by incubation at 95°C for 3 minutes with a 1:1 ratio. Reaction mix was then cooled down at room RT for 30 minutes and quantified and stored at -20°C.

DNA digestion and purification

Plasmids, BAC DNAs and PCR products were digested with restriction enzymes (NEB/Roche), according to the manufacturer's instructions. rSAP (Shrimp Alkaline Phosphatase, NEB) was added to the digestion mix of the receiving plasmid vector.

The DNA fragments were run on a 0.5-2% agarose gel supplemented 1x SYBR-Safe DNA stain (Invitrogen). DNA purification extracted from gel/PCR was performed using

NuceloSpin Gel and PCR Clean-up (Machery-Nagel) according to the manufacturer's instructions.

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. In the present work it has been used to transfer the Ub-tdTomato expression cassette from the donor plasmid (pEnter-Ub-tdTomato) into the HSV-containing BAC. This reaction exploits 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 clone. LR 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) according to manufacturer's instruction.

Electroporation

The transformation of the BAC requires electroporation. Electrocompetent bacteria (ElectroMAXTM DH10BTM 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 overnight.

DNA ligation and transformation of competent bacteria

Linearized DNA fragments were ligated together (generally at a 1:3 vector/insert ratio) with T4 DNA ligase (NEB) at RT for 2 hours. Subsequently, approximately 5uL were transferred in 50uL of chemically competent Subcloning Efficiency™ DH5α Competent

Cells (Thermo Fisher scientific) for bacteria transformation according to the manufacturer's instructions.

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. For colony-PCR screening, bacteria were gently picked from agar plate and resuspended in 10uL of sterile mqH_2O. 2uL of bacteria containing solution were directly used as template for PCR screening.

DNA fragments for subcloning were amplified by Polymerase Chain Reaction (PCR) using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher scientific).

Sanger sequencing

To verify that cloning was properly executed, plasmid DNA extracted from bacteria was sequenced by the external company Mycrosynth AG accordingly to the fragment to be verified.

Herpes Simplex Virus-1-based viral vector procedures

JΔNI8- HSV-1 BAC

JΔNI8 BAC-DNA construct was donated by Prof. Joseph Gloriso from the University of Pittsburgh. It 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. Important modifications of this backbone have been made by Dr Yoshitaka Miyagawa.

Transfection of BAC-DNA into U2OS-ICP4

BAC HSV-1 DNAs were transfected into U2OS-ICP4/ICP27 cells 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. After 24 h cells (80% confluent) 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% CO2 and mCherry expression was monitored until viral plaques formation and spreading was observed.

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

Titration of viral supernatant by plaque assay was performed on 48-well plates of U2OS-ICP4/ICP27 cells at a density of 120,000 cells per well to achieve 80% confluency at the time of infection. Cells were infected with serial 10-fold dilutions of viral supernatant in 120µl of DMEM (1% P/S, serum-free) media and incubated at 37°C in 5% CO2 for approximately 3 hours. Subsequently, 100 µl of DMEM (1% P/S, serum-free) per well was added and the cells were incubated at 33° C with 5% CO2 until lysis plaques form (typically 3-4 days). Plaque counts were performed by light or fluorescence microscopy and the result expressed as p.f.u./ml (plaque forming units per ml of viral preparation).

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% CO2.

Four to five 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.

JΔNI8 transduction of primary neurons

To prevent osmotic shock at the time of infection, 1/3 (approximately 400uL) of the culture media of primary neurons seeded on glass slides in a 24-weell plate (see below) were collected every three days and replace with fresh media. The collected media was stored till the 8th day and used to resuspend the viral preparation. On the 8th day after seeding, the entire medium was collected and approximately 300uL/well of stored medium were supplemented with the proper amount of viral volume (MOI 1; 200.000 neurons per slide) and added to the cells. Neurons were incubated for 1h at 37°C in 5% CO2. Subsequently, the viral vector containing medium was discarded, washed with 300uL of stored medium, and replaced with 2/3 of stored conditioned media supplemented with 1/3 of fresh one.

DNA extraction from JΔNI8 viral preparation

20uL of pure viral preparation were used to extracted DNA sequencing. DNA was extracted with DNeasy Blood & Tissue Kits (Qiagen) according to manufacturer's instruction. Once purified DNA was delivered to the Center for Omics Science at San Raffaele Scientific Institute.

Lentiviral vector procedures

Lentiviral vectors production

To produce the viral vectors HEK293T cells were used. The day before transfection cells were plated at a density of 9.000.000 cells per dish in 5 15 cm2 culture dishes for each lentivirus and kept in 20 mL of IMDM medium (10% FBS (Euroclone), 2% L-glutamine (Sigma), 1% Pen/Strep (Sigma) in IMDM (Sigma)). The day after, medium was changed with 22,5 mL of fresh IMDM, 2 hours before transfection and the transfection mixes were prepared with the plasmidic DNA for the transfer vectors (sgRNA 1-5 and sgLacZ) and packaging plasmids, including VSV-G, Gag/Pol, Advantage and REV in different 15 mL Falcon tubes for each lentivirus. The mix was then brought to a final volume of 1125 µL using 0,1X TE (made by diluting 1:10 a stock of 1X TE - 10 mM Tris at pH 8, 1 mM EDTA pH 8). The mixes were briefly vortexed and 125 µL of 2.5 M CaCl2 were added. Right after, 1250 µL of 2X HBSS (for 50 mL, made of 0,82 g of NaCl, 1,2 g of HEPES and 0,0106 g of Na2HPO4) were added dropwise

while vortexing to avoid precipitation of the DNA in the tubes and the mixes were immediately used to transfect cells. Hek293 cells were cultured at 37°C with 5% CO2 for 12 to 14 hours and then their medium was changed with fresh IMDM. After 30 hours from transfection, supernatants from every dish were collected, filtered once in 0,22 μm stericups and ultracentrifuged at 45000g for 2 hours at 20°C in a SW32.Ti rotor (Beckman Coulter). Supernatants were then discarded and the pellets were dissolved in 60 μL of 1X PBS and collected in 1,5 mL Eppendorf tubes. The mixes were then resuspended and left rotating on a wheel for 30 minutes at room temperature and stored at -80°C until further use.

Cell Cultures

Cell lines

U2OS Human Osteosarcoma and HEK 293T Human Epithelial Kidney cell lines were purchased from ATCC (Manassas, VA). 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 and grown in Dulbecco's Modified Minimum Essential Medium (DMEM, Lonza) supplemented with 10% Fetal Bovine Serum (Gibco, Thermofisher), 2 mM L-glutamine (Gibco, Thermofisher), 100 U/mL Penicillin (Merck, Millipore), and 100 μg/ml Streptomycin (Merck, Millipore).

Primary culture of rat hippocampal neurons

The Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute approved the animal use procedures. Primary cultures of hippocampal neurons were prepared according to (Bettegazzi *et al*, 2021) from 2 to 3 day-old Sprague–Dawley rats. Briefly, after brain removal from the skull, and quick subdivision of hippocampi into small pieces, the tissue was incubated into Hank's solution containing 3.5 mg/mL trypsin type IX (Sigma-Aldrich) and 0.5 mg/mL DNase type IV (Calbiochem, La Jolla, CA, USA) for 5 min. The pieces were then mechanically dissociated in a Hank's solution supplemented with 12 mM MgSO4 and 0.5 mg/mL DNase IV. After centrifugation, cells were plated onto poly-ornithine coated coverslips and maintained in MEM supplemented with 20 mM glucose, B27 (Life Technologies, Carlsbad, CA, USA), 2 mM glutamax, 5%

serum (fetal clone III - FCIII; Hyclone, South Logan, UT, USA) and 5 μM 1-β-D-cytosine-arabinofuranoside (Ara-C; Sigma). Cultures were maintained at 37°C in a 5% CO2 humidified incubator.

Calcium measurement with Fura-2 calcium indicator

Ca2+ measurements were performed in Krebs-Ringer-Hepes buffer (KRH - 5 mM KCl, 125 mM NaCl, 2 mM CaCl2, 1.2mM MgSO4, 1.2 mM KH2PO4, 6 mM glucose and 20 mM Hepes, pH 7.4).

Cells were loaded with 4 μM fura-2 acetoxymethyl ester (Calbiochem)for 40 min at 37°C. After dye loading, cells were washed twice with KRH and kept in the same buffer for the entire duration of the experiments.

The single cell experiments were performed with a videoimaging setup consisting of an Axioskope 2 microscope (Zeiss, Oberkochen, Germany) and a Polychrome IV (Till Photonics, GmbH, Martinsried, Germany) light source. Fluorescence images were collected by a cooled CCD videocamera (PCO Computer Optics GmbH, Kelheim, Germany). The 'Vision' software (Till Photonics) was used to control the acquisition protocol and to perform data analysis (Codazzi, 2006).

Immunofluorescence on glass slide

Cells, plated on glass coverslips, were fixed with 4% paraformaldehyde + 4% sucrose/PBS for 15 min, then permeabilized with 0.1 % Triton X-100/PBS and blocked 15 min in 1% normal goat serum/PBS. Primary antibodies were incubated 1 h at RT, diluted in blocking solution (1% normal goat serum/PBS). After incubation cells were washed 3 times (5 minutes each) with PBS, then secondary antibodies were incubated diluted in the same solution. After one washing step in PBS, coverslips were incubated with DAPI for 5' at RT, deepen in water and mounted on microscope slides with Fluorescence Mounting Medium (DAKO, Agilent).

Biochemical procedures

Cell lysis

For NPY detection primary neurons were lysed by direct addition of 2x sample buffer (100 mM Tris-HCl, pH 6.8, 5 mM EDTA/Na, 4% SDS, 10% glycerol, 0.4 M DTT, 0.02% bromophenol blue).

Mice hippocampi were homogenized in homogenization buffer (250 mM Sucrose, 2 mM EDTA/Na, 20 mM Hepes/Na pH 7.5, protease and phosphatase inhibitors) with 25 strokes of a glass-Teflon homogenizer and centrifuged at 500 g, 4°C for 5 min. The supernatant S1 was then centrifuged at 100000g, 4°C for 45 min. The supernatant was discarded, while the pellet, containing membranes, was resuspended in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl (pH 8), 1% Tx-100, 0.5% Na-deoxycholate and 0.1% SDS, protease and phosphatase inhibitors), incubated on ice for 15 min and then centrifuged at 10000g, 4°C for 10 min. The protein content of the membrane extract was analyzed by BCA (ThermoFisher Scientific, Waltham, MA, USA).

Tissue homogenization

Mice hippocampi were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl (pH 8), 1% Tx-100, 0.5% Na-deoxycholate and 0.1% SDS, protease and phosphatase inhibitors) with 25 strokes of a glass-Teflon homogenizer and centrifuged at 15,000 g, 4°C for 15 min. The protein content was analyzed by BCA (ThermoFisher Scientific, Waltham, MA, USA).

Western blot

About 50 ug of proteins were separated by standard SDS-PAGE and transferred onto nitrocellulose membrane. The nitrocellulose filter was stained with Ponceau S (0.2% in 3% trichloroacetic acid) and de-stained with double distilled water for protein visualization. After 1 h of blocking with TBST (10 mM Tris/HCl, 150 mM NaCl, 0.1% Tween-20) containing 5% bovine serum albumin (Roche diagnostics, Basel, Switzerland) or skimmed powdered milk, the membranes were incubated overnight with the primary antibodies and, after extensive washing, with horseradish peroxidase-conjugated anti-rabbit or mouse secondary antibody (Bio-Rad, Hercules, CA, USA). For loading controls membranes were stripped in acidic buffer (0.2 M glycine, 0.1% SDS, 1% Tween-20, pH 2.2) and re-probed with the appropriate antibody. Proteins were revealed by direct acquisition using the Biorad Chemidoc Imaging system by Super Signal West

Chemiluminescent Substrate (ThermoFisher Scientific). Bands were quantified using ImageJ and protein levels normalized against the loading control. Details on the antibodies employed in Western Blot analysis are reported in Antibody section.

ELISA assay

Total secreted NPY, in the culture media of primary rat hippocampal neurons, was evaluated by the rat specific ELISA assay (Merk; Millipore) according to the manufacturer's instructions.

Electrophysiology

In vitro electrophysiological experiment on primary neurons

Primary culture slides were submerged in a recording chamber mounted on the stage of an upright BX51WI microscope (Olympus, Japan) equipped with differential interference contrast optics (DIC). Slides were continuously perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 25 $NaHCO_3$, 1 $MgCl_2$ and 11 D- glucose saturated with 95% O_2, 5% CO_2 (pH 7.3) flowing at a rate of 2-3ml/min at room temperature. Whole-cell patch-clamp recordings were performed using glass pipettes filled with a solution containing the following (in mM): 30 KH_2PO_4, 100 KCl, 2 $MgCl_2$, 10 NaCl, 10 HEPES, 0.5 EGTA, 2 Na_2-ATP, 0.02 Na-GTP, (pH 7.2, adjusted with KOH; tip resistance: 6-8 MΩ). For the dye-coupling experiments internal solution was modified by additional loading of 0.1% of Neuriobiotin-488 (LabVector).

All recordings were performed using a MultiClamp 700B amplifier interfaced with a PC through a Digidata 1440A (Molecular Devices, Sunnyvale, CA, USA). Data were acquired and analyzed using pClamp10 software (Molecular Devices). Voltage- and current-clamp traces were sampled at a frequency of 30 kHz and low-pass filtered at 2 kHz.

Animals

Mice were housed under controlled temperature (22 ± 1 °C) and humidity (50%) conditions following a 12 h light/dark cycle. Food and water were provided ad libitum. TKO mice were kindly provided by Dr.ssa Elena Monzani in accordance with Prof. Flavia

Valtorta. Mice were maintained and bred at the animal house of Ospedale San Raffaele in compliance with institutional guidelines and international laws (EU Directive 2010/63/EU EEC Council Directive 86/609, OJL 358, 1, December 12, 1987, NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All efforts were made to minimize animal suffering.

Intracardial perfusion, brain collection and fixation

Mice intended to immunohistochemistry examination were anesthetized with an intraperitoneal injection of a mixture of ketamine/xylazine (100mg/kg and 10mg/kg, respectively). Transcardial perfusion was performed with 25ml of ice-cold 1x phosphate buffered saline (PBS) and subsequent 25ml of 4% paraformaldehyde in PBS (pH 7.4). After decapitation, brain was rapidly removed from the skull and kept in 4% paraformaldehyde in PBS for 16h at 4°C. Brains were then washed three times with 1x PBS for 10 minutes and cryoprotected in 30% sucrose in 1x PBS for 2 days. Brains were rapidly frozen and 15µm-thick coronal sections were cut with a CM3050s cryostat (Leica Microsystems) and placed onto SuperFrost slides, allow to dry at room temperature, and kept at -20°C for further procedures.

Immunofluorescence on brain slices

Slices were incubated with a blocking solution containing 1% normal goat serum (NGS) and 0.3% Triton in 1x PBS for 1 hour at 4°C. Subsequently incubation with primary antibodies diluted in the same solution were carried overnight at 4°C.

Slices were rinsed 2 times with 15 minutes of 1x PBS and 15 minutes of blocking solution kept at 4°C. Subsequently they were incubated with a secondary antibody diluted in the blocking solution for 90 minutes at room temperature in a dark room. Appropriate fluorophore-conjugated (Alexa Fluor® 488, 594, 647; Molecular probes) secondary antibodies were used according to manufacturer's instructions. Slices were then rinsed 3 times with 15 minutes of 1x M PBS and 15 minutes of blocking solution, incubated for 5 minutes with 5mg/ml DAPI (Sigma check) and mounted with DAKO mounting medium (Agilent).

Immunofluorescence with Y2R antibody required heat-induced antigen retrieval. Thus, prior to the described immunofluorescence protocol, brain slices positioned onto

super frost glass slides were placed in a tray containing sodium citrate buffer (10mM Sodium Citrate; pH 6.0). The buffer tray was heated in a microwave, heating was stopped before reaching boiling temperature, and the buffer was let chill at RT for 30' before proceeding.

Imaging and analysis

All the confocal images have been acquired in the Advanced Light and Electron Microscopy BioImaging Center (Alembic®) with a Sp8 Leica confocal system.

Image analysis was performed with Fiji ImageJ software. For binary processed image only: after the subtraction of the same arbitrary threshold, images were transformed into binary to easily visualize the difference.

Image composition

Image composition and drawing were done with the use of Adobe Illustrator cc 2017 (Adobe System, San Jose, CA, USA).

Surgical procedures

All the surgical procedures were performed on anesthetized mice place in a stereotaxic frame (Stoelting co.). Anesthesia was indued in a plastic chamber saturated with a mixture of 4% isoflurane/ 0.5% O_2 (Harvard apparatus). Subsequently, mice were positioned in the stereotaxic frame with the nose inserted into a mask for anesthesia where a mixture of 2% isoflurane/ 1% O_2 was continuously flowing for the entire time of the procedure.

Viral vector stereotaxic injection in the mouse hippocampus

Mice used for biochemical and histological examination were injected with viral vectors at 60-days of age. TKO mice used for video-EEG experiment were injected with viral vectors at 45-days of age.

After quick shaving of the head of the mouse, the skull of mice steadily positioned in the stereotaxic frame was exposed trough a cut along the anteroposterior axis. A drill was used to prepare a burr hole at coordinates: AP: -1.8; ML: ± 1.8; DV: -2.0. The coordinates were measured in relation to bregma after alignment of the bregma-lambda axis. Mice

intended for biochemical and histological examination were injected homolaterally. Mice that underwent video-EEG monitoring were injected bilaterally.

Infusion was performed trough a 34G stainless steel needle (Hamilton company) connected to a 25 µL Hamilton syringe. The syringe was mounted onto a peristaltic pump (Legato® 130 syringe pump; KD scientific) positioned directly on the arm of the stereotaxic frame. 2µl of viral vector were infused at a rate of 200nl/min (10 min total). After the injection, the needle was left in place for an additional minute to allow the diffusion of vector particles before being slowly withdrawn from the brain.

Telemetry transmitter implant for video-EEG monitoring

TKO mice intended for video-EEG experiment underwent surgery for transmitter implant between 52-55 days of age. The ETA-F10 transmitter (Data Sciences International, St. Paul, MN) was positioned in a sub cutaneous pocket on the back of the mouse with the wires guided to the skull. The recording electrode was placed on *dura mater* above the hippocampus, and the reference electrodes were placed contralaterally on *dura mater*, anterior to bregma. Once the electrodes were in position, dental cement (Harvard apparatus) was added to cover and attach the implant to the skull.

Video-EEG monitoring

Neuroscore (Data Sciences International, St. Paul, MN) was used for EEG analysis. All traces were visually inspected for the detection of seizures and duration was measured as the period of paroxysmal activity of high frequency (> 5 Hz) characterized by a 3-fold higher amplitude over baseline with a minimum duration of at least 5 sec.

Statistical Analysis

Datasets were compared using GraphPad Prism 8.0 (La Jolla, USA). Results are given as dot plots or histogram with mean ± SEM or median as specified in the figure legends. All the experiments have been conducted at least in triplicate. In general, a value of $p<0.05$ was considered to be statistically significant if not otherwise specified in the figure legend.

Chapter VIII – REFERENCES

Abid K, Rochat B, Lassahn PG, Stöcklin R, Michalet S, Brakch N, Aubert JF, Vatansever B, Tella P, De Meester I, *et al* (2009) Kinetic study of neuropeptide Y (NPY) proteolysis in blood and identification of NPY3-35. A new peptide generated by plasma kallikrein. *Journal of Biological Chemistry*

Aicher SA, Springston M, Berger SB, Reis DJ & Wahlestedt C (1991) Receptor-selective analogs demonstrate NPY/PYY receptor heterogeneity in rat brain. *Neuroscience Letters* 130: 32–36

Allen J, Novotny J, Martin J & Heinrich G (1987) Molecular structure of mammalian neuropeptide Y: Analysis by molecular cloning and computer-aided comparison with crystal structure of avian homologue. *Proceedings of the National Academy of Sciences of the United States of America*

Ambrosini AE & Enquist LW (2015) Cell-fusion events induced by α-herpesviruses. *Future Virology* 10: 185–200

Argnani R, Lufino M, Manservigi M & Manservigi R (2005) Replication-competent herpes simplex vectors: design and applications. *Gene Ther* 12: S170–S177

Artusi S, Miyagawa Y, Goins W, Cohen J & Glorioso J (2018) Herpes Simplex Virus Vectors for Gene Transfer to the Central Nervous System. *Diseases* 6: 74

Asadi-Pooya AA (2018) Lennox-Gastaut syndrome: a comprehensive review. *Neurol Sci* 39: 403–414

Atanasova KR & Reznikov LR (2018) Neuropeptides in asthma, chronic obstructive pulmonary disease and cystic fibrosis. *Respiratory Research* 19

Atasoy D, Aponte Y, Su HH & Sternson SM (2008) A FLEX Switch Targets Channelrhodopsin-2 to Multiple Cell Types for Imaging and Long-Range Circuit Mapping. *J Neurosci* 28: 7025–7030

Axelsen TM & Woldbye DPD Gene Therapy for Parkinson's Disease, An Update. 21

El Bahh B, Balosso S, Hamilton T, Herzog H, Beck-Sickinger AG, Sperk G, Gehlert DR, Vezzani A & Colmers WF (2005) The anti-epileptic actions of neuropeptide Y in the hippocampus are mediated by Y2 and not Y5 receptors.

European Journal of Neuroscience

El Bahh B, Cao JQ, Beck-Sickinger AG & Colmers WF (2002) Blockade of neuropeptide Y2 receptors and suppression of NPY's anti-epileptic actions in the rat hippocampal slice by BIIE0246. *British Journal of Pharmacology*

Baldock PA, Allison SJ, Lundberg P, Lee NJ, Slack K, Lin EJD, Enriquez RF, McDonald MM, Zhang L, During MJ, *et al* (2007) Novel role of Y1 receptors in the coordinated regulation of bone and energy homeostasis. *Journal of Biological Chemistry*

Baltimore D (1971) Expression of animal virus genomes. *Bacteriological Reviews* 35: 235–241

Banasik MB & McCray PB (2010) Integrase-defective lentiviral vectors: progress and applications. *Gene Ther* 17: 150–157

Baraban SC (2004) Neuropeptide Y and epilepsy: Recent progress, prospects and controversies. *Neuropeptides* doi:10.1016/j.npep.2004.04.006 [PREPRINT]

Beghi E & Giussani G (2018) Aging and the Epidemiology of Epilepsy. *Neuroepidemiology* 51: 216–223

Benarroch EE (2009) Neuropeptide Y: Its multiple effects in the CNS and potential clinical significance. *Neurology* 72: 1016–1020

Bettegazzi B, Sebastian Monasor L, Bellani S, Codazzi F, Restelli LM, Colombo AV, Deigendesch N, Frank S, Saito T, Saido TC, *et al* (2021) Casein Kinase 2 dependent phosphorylation of eIF4B regulates BACE1 expression in Alzheimer's disease. *Cell Death Dis* 12: 1–14

Blömer U, Naldini L, Verma IM, Trono D & Gage FH (1996) Applications of gene therapy to the CNS. *Human Molecular Genetics* 5: 1397–1404

Bloom DC, Giordani NV & Kwiatkowski DL (2010) Epigenetic regulation of latent HSV-1 gene expression. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1799: 246–256

Boehmer PE & Lehman IR (1997) Herpes Simplex Virus Dna Replication. *Annual Review of Biochemistry* 66: 347–384

Bonanni P, Malcarne M, Moro F, Veggiotti P, Buti D, Ferrari AR, Parrini E, Mei D, Volzone A, Zara F, *et al* (2004) Generalized epilepsy with febrile seizures plus

(GEFS+): clinical spectrum in seven Italian families unrelated to SCN1A, SCN1B, and GABRG2 gene mutations. *Epilepsia* 45: 149–158

Botterill JJ, Lu Y-L, LaFrancois JJ, Bernstein HL, Alcantara-Gonzalez D, Jain S, Leary P & Scharfman HE (2019) An Excitatory and Epileptogenic Effect of Dentate Gyrus Mossy Cells in a Mouse Model of Epilepsy. *Cell Reports* 29: 2875-2889.e6

Boutell C & Everett RD (2013) Regulation of alphaherpesvirus infections by the ICP0 family of proteins. *Journal of General Virology* 94: 465–481

Bregola G, Dumont Y, Fournier A, Zucchini S, Quirion R & Simonato M (2000) Decreased levels of neuropeptide Y5 receptor binding sites in two experimental models of epilepsy. *Neuroscience*

Brini M, Calì T, Ottolini D & Carafoli E (2014) Neuronal calcium signaling: function and dysfunction. *Cell Mol Life Sci* 71: 2787–2814

Brockmann K, Wang D, Korenke CG, Von Moers A, Ho Y-Y, Pascual JM, Kuang K, Yang H, Ma L, Kranz-Eble P, *et al* (2001) Autosomal dominant Glut-1 deficiency syndrome and familial epilepsy. *Ann Neurol* 50: 476–485

Brumovsky PR, Shi TJ, Matsuda H, Kopp J, Villar MJ & Hökfelt T (2002) NPY Y1 receptors are present in axonal processes of DRG neurons. *Experimental Neurology* 174: 1–10

Bui AD, Nguyen TM, Limouse C, Kim HK, Szabo GG, Felong S, Maroso M & Soltesz I (2018) Dentate gyrus mossy cells control spontaneous convulsive seizures and spatial memory. 5

Buiting K, Williams C & Horsthemke B (2016) Angelman syndrome — insights into a rare neurogenetic disorder. *Nat Rev Neurol* 12: 584–593

Caberlotto L (1997) Localization of neuropeptide Y Y1 mRNA in the human brain: Abundant expression in cerebral cortex and striatum. *European Journal of Neuroscience* 9: 1212–1225

Cabrele C & Beck-Sickinger AG (2000) Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *Journal of Peptide Science* 6: 97–122 doi:10.1002/(SICI)1099-1387(200003)6:3<97::AID-PSC236>3.0.CO;2-E [PREPRINT]

Cambiaghi M, Cursi M, Monzani E, Benfenati F, Comi G, Minicucci F, Valtorta

F & Leocani L (2013) Temporal evolution of neurophysiological and behavioral features of synapsin I/II/III triple knock-out mice. *Epilepsy Research* 103: 153–160

Cattaneo S, Verlengia G, Marino P, Simonato M & Bettegazzi B (2021) NPY and Gene Therapy for Epilepsy: How, When,... and Y. *Frontiers in Molecular Neuroscience* 13

Cavarsan CF, Malheiros J, Hamani C, Najm I & Covolan L (2018) Is mossy fiber sprouting a potential therapeutic target for epilepsy? *Frontiers in Neurology* 9: 1–13

Cerdá-Reverter JM & Larhammar D (2000) cNeuropeptide Y family of peptides: Structure, anatomical expression, function, and molecular evolution. *Biochemistry and Cell Biology* 78: 371–392

Chan KY, Jang MJ, Yoo BB, Greenbaum A, Ravi N, Wu W-L, Sánchez-Guardado L, Lois C, Mazmanian SK, Deverman BE, *et al* (2017) Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nat Neurosci* 20: 1172–1179

Chronwall BM, DiMaggio DA, Massari VJ, Pickel VM, Ruggiero DA & O'donohue TL (1985) The anatomy of neuropeptide-y-containing neurons in rat brain. *Neuroscience*

Codazzi F (2006) Synergistic Control of Protein Kinase C Activity by Ionotropic and Metabotropic Glutamate Receptor Inputs in Hippocampal Neurons. *Journal of Neuroscience* 26: 3404–3411

Colasante G, Lignani G, Brusco S, Berardino CD, Carpenter J, Giannelli S, Valassina N, Bido S, Ricci R, Castoldi V, *et al* (2020) dCas9-Based Scn1a Gene Activation Restores Inhibitory Interneuron Excitability and Attenuates Seizures in Dravet Syndrome Mice. *Molecular Therapy* 28: 235–253

Colmers WF, Klapstein GJ, Fournier A, St-Pierre S & Treherne KA (1991) Presynaptic inhibition by neuropeptide Y in rat hippocampal slice in vitro is mediated by a Y2 receptor. *British Journal of Pharmacology* 102: 41–44

Colmers WF, Lukowiak K & Pittman QJ (1985) Neuropeptide Y reduces orthodromically evoked population spike in rat hippocampal CA1 by a possibly presynaptic mechanism. *Brain Research* 346: 404–408

Colmers WF, Lukowiak K & Pittman QJ (1987) Presynaptic action of neuropeptide Y in area CA1 of the rat hippocampal slice. *The Journal of Physiology* 383: 285–299

Colmers WF, Lukowiak K & Pittman QJ (1988) Neuropeptide Y action in the rat hippocampal slice: Site and mechanism of presynaptic inhibition. *Journal of Neuroscience* 8: 3827–3837

Conway JE, Rhys C ap, Zolotukhin I, Zolotukhin S, Muzyczka N, Hayward GS & Byrne BJ (1999) High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap. *Gene Ther* 6: 986–993

Corey LA, Pellock JM, Kjeldsen MJ & Nakken KO (2011) Importance of genetic factors in the occurrence of epilepsy syndrome type: A twin study. *Epilepsy Research* 97: 103–111

Criscione L, Rigollier P, Batzl-Hartmann C, Rüeger H, Stricker-Krongrad A, Wyss P, Brunner L, Whitebread S, Yamaguchi Y, Gerald C, *et al* (1998) Food intake in free-feeding and energy-deprived lean rats is mediated by the neuropeptide Y5 receptor. *Journal of Clinical Investigation*

Cuchet D, Potel C, Thomas J & Epstein AL (2007) HSV-1 amplicon vectors. *null* 7: 975–995

Curia G, Longo D, Biagini G, Jones RSG & Avoli M (2008) The pilocarpine model of temporal lobe epilepsy. *Journal of Neuroscience Methods* 172: 143–157

Dalwadi DA, Calabria A, Tiyaboonchai A, Posey J, Naugler WE, Montini E & Grompe M (2021) AAV integration in human hepatocytes. *Molecular Therapy* 29: 2898–2909

D'Andrea Meira I, Romão TT, Pires do Prado HJ, Krüger LT, Pires MEP & da Conceição PO (2019) Ketogenic Diet and Epilepsy: What We Know So Far. *Front Neurosci* 13: 5

Dhindsa RS, Bradrick SS, Yao X, Heinzen EL, Petrovski S, Krueger BJ, Johnson MR, Frankel WN, Petrou S, Boumil RM, *et al* (2015) Epileptic encephalopathy-causing mutations in DNM1 impair synaptic vesicle endocytosis. *Neurology Genetics* 1

Dittgen T, Nimmerjahn A, Komai S, Licznerski P, Waters J, Margrie TW, Helmchen F, Denk W, Brecht M & Osten P (2004) Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring in vivo. *Proceedings of the National Academy of Sciences* 101: 18206–18211

Dogrammatzis C, Waisner H & Kalamvoki M (2020) "Non-Essential" Proteins of HSV-1 with Essential Roles In Vivo: A Comprehensive Review. *Viruses* 13: 17

Domenger C & Grimm D (2019) Next-generation AAV vectors—do not judge a virus (only) by its cover. *Human Molecular Genetics* 28: R3–R14

Dum E, Fürtinger S, Gasser E, Bukovac A, Drexel M, Tasan R & Sperk G (2017) Effective G-protein coupling of Y2 receptors along axonal fiber tracts and its relevance for epilepsy. *Neuropeptides*

Dumont Y, Fournier A, St-Pierre S & Quirion R (1993) Comparative characterization and autoradiographic distribution of neuropeptide Y receptor subtypes in the rat brain. *Journal of Neuroscience* 13: 73–86

Dumont Y, Fournier A, St-Pierre S, Schwartz TW & Quirion R (1990) Differential distribution of neuropeptide Y1 and Y2 receptors in the rat brain. *European Journal of Pharmacology* 191: 501–503

Dumont Y, Jacques D, Bouchard P & Quirion R (1998) Species differences in the expression and distribution of the neuropeptide Y Y1, Y2, Y4, and Y5 receptors in rodents, guinea pig, and primates brains. *Journal of Comparative Neurology*

Dumont Y, Martel JC, Fournier A, St-Pierre S & Quirion R (1992) Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. *Progress in Neurobiology* doi:10.1016/0301-0082(92)90038-G [PREPRINT]

Eddy CM, Rickards HE & Cavanna AE (2011) The cognitive impact of antiepileptic drugs. *Ther Adv Neurol Disord* 4: 385–407

Elfvin LG, Holmberg K, Emson P, Schemann M & Hökfelt T (1997) Nitric oxide synthase, choline acetyltransferase, catecholamine enzymes and neuropeptides and their colocalization in the anterior pelvic ganglion, the inferior mesenteric ganglion and the hypogastric nerve of the male guinea pig. *Journal of Chemical Neuroanatomy*

Engel Jr. J, Pitkänen A, Loeb JA, Edward Dudek F, Bertram III EH, Cole AJ, Moshé SL, Wiebe S, Jensen FE, Mody I, *et al* (2013) Epilepsy biomarkers. *Epilepsia*

54: 61–69

Epstein AL (2009) Progress and prospects: Biological properties and technological advances of herpes simplex virus type 1-based amplicon vectors. *Gene Ther* 16: 709–715

Eriksson H, Wirdefeldt K, Åsberg S & Zelano J (2019) Family history increases the risk of late seizures after stroke. *Neurology* 93: e1964–e1970

Falcicchia C, Trempat P, Binaschi A, Perrier-Biollay C, Roncon P, Soukupova M, Berthommé H & Simonato M (2016) Silencing Status Epilepticus-Induced BDNF Expression with Herpes Simplex Virus Type-1 Based Amplicon Vectors. *PLOS ONE* 11: e0150995

FDA Commissioner O of the (2021) FDA, NIH, and 15 private organizations join forces to increase effective gene therapies for rare diseases. *FDA*

Feigin VL, Vos T, Nichols E, Owolabi MO, Carroll WM, Dichgans M, Deuschl G, Parmar P, Brainin M & Murray C (2020) The global burden of neurological disorders: translating evidence into policy. *The Lancet Neurology* 19: 255–265

Fenno LE, Mattis J, Ramakrishnan C, Hyun M, Lee SY, He M, Tucciarone J, Selimbeyoglu A, Berndt A, Grosenick L, *et al* (2014) Targeting cells with single vectors using multiple-feature Boolean logic. *Nat Methods* 11: 763–772

Ferenczy MW & DeLuca NA (2011) Reversal of Heterochromatic Silencing of Quiescent Herpes Simplex Virus Type 1 by ICP0. *J Virol* 85: 3424–3435

Fiest KM, Sauro KM, Wiebe S, Patten SB, Kwon C-S, Dykeman J, Pringsheim T, Lorenzetti DL & Jetté N (2017) Prevalence and incidence of epilepsy: A systematic review and meta-analysis of international studies. *Neurology* 88: 296–303

Fisher RS, Acevedo C, Arzimanoglou A, Bogacz A, Cross JH, Elger CE, Engel Jr J, Forsgren L, French JA, Glynn M, *et al* (2014) ILAE Official Report: A practical clinical definition of epilepsy. *Epilepsia* 55: 475–482

Fisher RS, Boas W van E, Blume W, Elger C, Genton P, Lee P & Engel Jr. J (2005) Epileptic Seizures and Epilepsy: Definitions Proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 46: 470–472

Fisher RS, Cross JH, French JA, Higurashi N, Hirsch E, Jansen FE, Lagae L,

Moshé SL, Peltola J, Roulet Perez E, *et al* (2017) Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology. *Epilepsia* 58: 522–530

Flood JF & Morley JE (1989) Dissociation of the effects of neuropeptide Y on feeding and memory: Evidence for pre- and postsynaptic mediation. *Peptides* 10: 963–966

French JA, White HS, Klitgaard H, Holmes GL, Privitera MD, Cole AJ, Quay E, Wiebe S, Schmidt D, Porter RJ, *et al* (2013) Development of new treatment approaches for epilepsy: Unmet needs and opportunities. *Epilepsia* 54: 3–12

Fujii T, Ito Y, Takahashi S, Shimono K, Natsume J, Yanagihara K & Oguni H (2016) Outcome of ketogenic diets in GLUT1 deficiency syndrome in Japan: A nationwide survey. *Brain and Development* 38: 628–637

Furtinger S, Pirker S, Czech T, Baumgartner C, Ransmayr G & Sperk G (2001) Plasticity of Y1 and Y2 receptors and neuropeptide Y fibers in patients with temporal lobe epilepsy. *Journal of Neuroscience* 21: 5804–5812

Gariboldi M, Conti M, Cavaleri D, Samanin R & Vezzani A (1998) Anticonvulsant properties of BIBP3226, a non-peptide selective antagonist at neuropeptide Y Y1 receptors. *European Journal of Neuroscience* 10: 757–759

Gehlert DR, Gackenheimer SL & Schober DA (1992) [Leu31-Pro34] neuropeptide Y identifies a subtype of 125I-labeled peptide YY binding sites in the rat brain. *Neurochemistry International* 21: 45–67

Gerald C, Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, Smith KE, Vaysse P, Durkin MM, Laz TM, Linemeyer DL, *et al* (1996) A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* 382: 168–171

Gershkovich MM, Groß VE, Kaiser A PS (2019) Pharmacological and functional similari...n the FLP_NPR system _ Enhanced Reader.pdf. *Cell Commun Signal* 17: 123

Gicquiaux H, Lecat S, Gaire M, Dieterlen A, Mély Y, Takeda K, Bucher B & Galzi JL (2002) Rapid internalization and recycling of the human neuropeptide Y Y1 receptor. *Journal of Biological Chemistry*

Giordano F, Zicca A, Barba C, Guerrini R & Genitori L (2017) Vagus nerve

stimulation: Surgical technique of implantation and revision and related morbidity. *Epilepsia* 58: 85–90

Giovedí S, Corradi A, Fassio A & Benfenati F (2014) Involvement of Synaptic Genes in the Pathogenesis of Autism Spectrum Disorders: The Case of Synapsins. *Frontiers in Pediatrics* 2

Glass MJ, Chan J & Pickel VM (2002) Ultrastructural localization of neuropeptide Y Y1 receptors in the rat medial nucleus tractus solitarius: Relationships with neuropeptide Y or catecholamine neurons. *Journal of Neuroscience Research* 67: 753–765

Glorioso JC (2014) Herpes Simplex Viral Vectors: Late Bloomers with Big Potential. *Hum Gene Ther* 25: 83–91

Gobbi M, Gariboldi M, Piwko C, Hoyer D, Sperk G & Vezzani A (1998) Distinct changes in peptide YY binding to, and mRNA levels of, Y1 and Y2 receptors in the rat hippocampus associated with kindling epileptogenesis. *Journal of Neurochemistry* 70: 1615–1622

Gøtzsche CR, Nikitidou L, Sørensen AT, Olesen M V., Sørensen G, Christiansen SHO, Ängehagen M, Woldbye DPD & Kokaia M (2012) Combined gene overexpression of neuropeptide Y and its receptor Y5 in the hippocampus suppresses seizures. *Neurobiology of Disease*

Grandt D, Schimiczek M, Rascher W, Feth F, Shively J, Lee TD, Davis MT, Reeve JR & Michel MC (1996) Neuropeptide Y 3-36 is an endogenous ligand selective for Y2 receptors. *Regulatory Peptides*

Granstedt AE, Bosse JB, Thiberge SY & Enquist LW (2013) In vivo imaging of alphaherpesvirus infection reveals synchronized activity dependent on axonal sorting of viral proteins. *Proceedings of the National Academy of Sciences* 110: E3516–E3525

Greber S, Schwarzer C & Sperk G (1994) Neuropeptide Y inhibits potassium-stimulated glutamate release through Y2 receptors in rat hippocampal slices in vitro. *British Journal of Pharmacology*

Greco B, Managò F, Tucci V, Kao H-T, Valtorta F & Benfenati F (2013) Autism-related behavioral abnormalities in synapsin knockout mice. *Behav Brain Res* 251: 65–74

Guo HUI, Castro PA, Palmiter RD & Baraban SC (2002) Y5 receptors mediate neuropeptide Y actions at excitatory synapses in area CA3 of the mouse hippocampus. *Journal of Neurophysiology*

Hacein-Bey-Abina S, Kalle CV, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, *et al* (2003) LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science*

Han F, Miyagawa Y, Verlengia G, Ingusci S, Soukupova M, Simonato M, Glorioso JC & Cohen JB (2018) Cellular Antisilencing Elements Support Transgene Expression from Herpes Simplex Virus Vectors in the Absence of Immediate Early Gene Expression. *J Virol* 92

Hanaya R & Arita K (2016) The New Antiepileptic Drugs: Their Neuropharmacology and Clinical Indications. *Neurol Med Chir (Tokyo)* 56: 205–220

Harkin LA, Bowser DN, Dibbens LM, Singh R, Phillips F, Wallace RH, Richards MC, Williams DA, Mulley JC, Berkovic SF, *et al* (2002) Truncation of the GABAA-Receptor g2 Subunit in a Family with Generalized Epilepsy with Febrile Seizures Plus. *Am J Hum Genet*: 7

Harkness JM, Kader M & DeLuca NA (2014) Transcription of the Herpes Simplex Virus 1 Genome during Productive and Quiescent Infection of Neuronal and Nonneuronal Cells. *J Virol* 88: 6847–6861

Hauser WA & Kurland LT (1975) The Epidemiology of Epilepsy in Rochester, Minnesota, 1935 Through 1967. *Epilepsia* 16: 1–66

He T, Itano MS, Earley LF, Hall NE, Riddick N, Samulski RJ & Li C (2019) The Influence of Murine Genetic Background in Adeno-Associated Virus Transduction of the Mouse Brain. *Human Gene Therapy Clinical Development* 30: 169–181

Helbig I & Ellis CA (2020) Personalized medicine in genetic epilepsies – possibilities, challenges, and new frontiers. *Neuropharmacology* 172: 107970

Henshall DC (2017) Chapter 41 - Poststatus Epilepticus Models: Focal Kainic Acid. In *Models of Seizures and Epilepsy (Second Edition)*, Pitkänen A Buckmaster PS Galanopoulou AS & Moshé SL (eds) pp 611–624. Academic Press

Hill A (1995) Herpes simplex virus turns off the TAP to evade host immunity.

375: 5

Howell OW, Doyle K, Goodman JH, Scharfman HE, Herzog H, Pringle A, Beck-Sickinger AG & Gray WP (2005) Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. *Journal of Neurochemistry* 93: 560–570

Hubers SA, Wilson JR, Yu C, Nian H, Grouzmann E, Eugster P, Shibao CA, Billings FT, Kerman SJ & Brown NJ (2018) DPP (dipeptidyl peptidase)-4 inhibition potentiates the vasoconstrictor response to NPY (neuropeptide Y) in humans during renin-angiotensin-aldosterone system inhibition. *Hypertension* 72: 712–719

Ingusci S, Cattaneo S, Verlengia G, Zucchini S & Simonato M (2019a) A Matter of Genes: The Hurdles of Gene Therapy for Epilepsy. *Epilepsy Curr* 19: 38–43

Ingusci S, Verlengia G, Soukupova M, Zucchini S & Simonato M (2019b) Gene Therapy Tools for Brain Diseases. *Front Pharmacol* 10: 724

Jablonka S, Hennlein L & Sendtner M (2022) Therapy development for spinal muscular atrophy: perspectives for muscular dystrophies and neurodegenerative disorders. *Neurol Res Pract* 4: 2

Jacques D, Dumont Y, Fournier A & Quirion R (1997) Characterization of neuropeptide Y receptor subtypes in the normal human brain, including the hypothalamus. *Neuroscience* 79: 129–148

Jallon P & Latour P (2005) Epidemiology of Idiopathic Generalized Epilepsies. *Epilepsia* 46: 10–14

Jensen TL, Gøtzsche CR & Woldbye DPD (2021) Current and Future Prospects for Gene Therapy for Rare Genetic Diseases Affecting the Brain and Spinal Cord. *Frontiers in Molecular Neuroscience* 14

Jerome KR, Fox R, Chen Z, Sears AE, Lee H & Corey L (1999) Herpes Simplex Virus Inhibits Apoptosis through the Action of Two Genes, Us5 and Us3. *J Virol* 73: 8950–8957

De Jong-Brink M, Ter Maat A & Tensen CP (2001) NPY in invertebrates: Molecular answers to altered functions during evolution. *Peptides*

Károly N, Dobó E & and Mihály A (2015) Comparative immunohistochemical study of the effects of pilocarpine on the mossy cells, mossy fibres and inhibitory

neurones in murine dentate gyrus. - PubMed - NCBI. *Acta Neurobiologiae Experimentalis* 75: 220–237

Kato S, Kobayashi K & Kobayashi K (2014) Improved transduction efficiency of a lentiviral vector for neuron-specific retrograde gene transfer by optimizing the junction of fusion envelope glycoprotein. *Journal of Neuroscience Methods* 227: 151–158

Keast JR (1991) Patterns of co-existence of peptides and differences of nerve fibre types associated with noradrenergic and non-noradrenergic (putative cholinergic) neurons in the major pelvic ganglion of the male rat. *Cell and Tissue Research*

Klapstein GJ & Colmers WF (1992) 4-Aminopyridine and low Ca2+ differentiate presynaptic inhibition mediated by neuropeptide Y, baclofen and 2-chloroadenosine in rat hippocampal CA1 in vitro. *British Journal of Pharmacology* 105: 470–474

Klapstein GJ & Colmers WF (1993) On the sites of presynaptic inhibition by neuropeptide y in rat hippocampus in vitro. *Hippocampus* 3: 103–111

Klapstein GJ & Colmers WF (1997) Neuropeptide Y suppresses epileptiform activity in rat hippocampus in vitro. *Journal of Neurophysiology*

Knipe DM (2015) Nuclear sensing of viral DNA, epigenetic regulation of herpes simplex virus infection, and innate immunity. *Virology* 479–480: 153–159

Koblan LW, Erdos MR, Wilson C, Cabral WA, Levy JM, Xiong Z-M, Tavarez UL, Davison LM, Gete YG, Mao X, *et al* (2021) In vivo base editing rescues Hutchinson–Gilford progeria syndrome in mice. *Nature* 589: 608–614

Kofler N, Kirchmair E, Schwarzer C & Sperk G (1997) Altered expression of NPY-Y1 receptors in kainic acid induced epilepsy in rats. *Neuroscience Letters* 230: 129–132

Kooijman SALM & Troost TA (2007) Quantitative steps in the evolution of metabolic organisation as specified by the Dynamic Energy Budget theory. *Biological Reviews* doi:10.1111/j.1469-185X.2006.00006.x [PREPRINT]

Kopp J, Xu ZQ, Zhang X, Pedrazzini T, Herzog H, Kresse A, Wong H, Walsh JH & Hökfelt T (2002) Expression of the neuropeptide Y Y1 receptor in the CNS of rat and of wild-type and Y1 receptor knock-out mice. Focus on immunohistochemical

localization. *Neuroscience* 111: 443–532

Krisky D, Wolfe D, Goins W, Marconi P, Ramakrishnan R, Mata M, Rouse R, Fink D & Glorioso J (1998) Deletion of multiple immediate–early genes from herpes simplex virus reduces cytotoxicity and permits long-term gene expression in neurons. *Gene Ther* 5: 1593–1603

Kullmann DM, Schorge S, Walker MC & Wykes RC (2014) Gene therapy in epilepsy-is it time for clinical trials? *Nat Rev Neurol* 10: 300–304

Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, Johnson MD, Lee EW, Burnett MS, Fricke ST, Kvetnansky R, *et al* (2007) Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. *Nature Medicine*

Kwan P (2011) Drug-Resistant Epilepsy. *The New England Journal of Medicine*: 8

Kwan P, Arzimanoglou A, Berg AT, Brodie MJ, Allen Hauser W, Mathern G, Moshé SL, Perucca E, Wiebe S & French J (2010) Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. *Epilepsia* 51: 1069–1077

Kwan P & Brodie MJ (2009) Early Identification of Refractory Epilepsy. *http://dx.doi.org/101056/NEJM200002033420503*

Kwong AD & Frenkel N (1984) Herpes simplex virus amplicon: effect of size on replication of constructed defective genomes containing eucaryotic DNA sequences. *J Virol* 51: 595–603

Laimbacher AS & Fraefel C (2012) Gene delivery using helper virus-free HSV-1 amplicon vectors. *Curr Protoc Neurosci* Chapter 4: Unit 4.14

Lam WWK, Millichap JJ, Soares DC, Chin R, McLellan A, FitzPatrick DR, Elmslie F, Lees MM, Schaefer GB, Study DDD, *et al* (2016) Novel de novo EEF1A2 missense mutations causing epilepsy and intellectual disability. *Molecular Genetics & Genomic Medicine* 4: 465–474

Larhammar D & Salaneck E (2004) Molecular evolution of NPY receptor subtypes. *Neuropeptides* 38: 141–151

Leblanc GG, Trimmer BA & Landis SC (1987) Neuropeptide Y-like

immunoreactivity in rat cranial parasympathetic neurons: Coexistence with vasoactive intestinal peptide and choline acetyltransferase. *Proceedings of the National Academy of Sciences of the United States of America* 84: 3511–3515

Ledri M, Sørensen AT, Madsen MG, Christiansen SH, Ledri LN, Cifra A, Bengzon J, Lindberg XE, Pinborg LH, Jespersen B, *et al* (2015) Differential Effect of Neuropeptides on Excitatory Synaptic Transmission in Human Epileptic Hippocampus. *Journal of Neuroscience* 35: 9622–31

Lévesque M, Avoli M & Bernard C (2016) Animal models of temporal lobe epilepsy following systemic chemoconvulsant administration. *Journal of Neuroscience Methods* 260: 45–52

Li Q, Bartley AF & Dobrunz LE (2017) Endogenously Released Neuropeptide Y Suppresses Hippocampal Short-Term Facilitation and Is Impaired by Stress-Induced Anxiety. *The Journal of Neuroscience*

Lin EJD, Young D, Baer K, Herzog H & During MJ (2006) Differential actions of NPY on seizure modulation via Y1 and Y2 receptors: Evidence from receptor knockout mice. *Epilepsia* 47: 773–780

Lindner D, Stichel J & Beck-Sickinger AG (2008) Molecular recognition of the NPY hormone family by their receptors. *Nutrition* 24: 907–917 doi:10.1016/j.nut.2008.06.025 [PREPRINT]

Lopez-Santiago L & Isom LL (2019) Dravet Syndrome: A Developmental and Epileptic Encephalopathy. *Epilepsy Curr* 19: 51–53

Löscher W & Rogawski MA (2012) How theories evolved concerning the mechanism of action of barbiturates. *Epilepsia* 53: 12–25

Löscher W & Schmidt D (2011) Modern antiepileptic drug development has failed to deliver: ways out of the current dilemma. *Epilepsia* 52: 657–678

Löscher W & Schmidt D (2012) Perampanel—new promise for refractory epilepsy? *Nat Rev Neurol* 8: 661–662

Lu C, Everhart L, Tilan J, Kuo L, Sun CCJ, Munivenkatappa RB, Jönsson-Rylander AC, Sun J, Kuan-Celarier A, Li L, *et al* (2010) Neuropeptide y and its Y2 receptor: Potential targets in neuroblastoma therapy. *Oncogene*

Lubroth P, Colasante G & Lignani G (2021) In vivo Genome Editing

Therapeutic Approaches for Neurological Disorders: Where Are We in the Translational Pipeline? *Front Neurosci* 15: 632522

Lundberg JM, Rudehill A, Sollevi A, Theodorsson-Norheim E & Hamberger B (1986) Frequency- and reserpine-dependent chemical coding of sympathetic transmission: Differential release of noradrenaline and neuropeptide Y from pig spleen. *Neuroscience Letters*

Lundell I, Rabe Bernhardt N, Johnsson AK & Larhammar D (2011) Internalization studies of chimeric neuropeptide Y receptors Y1 and Y2 suggest complex interactions between cytoplasmic domains. *Regulatory Peptides*

Macdonald SJ, Mostafa HH, Morrison LA & Davido DJ (2012) Genome Sequence of Herpes Simplex Virus 1 Strain KOS. *Journal of Virology*

Maddalena A, Tornabene P, Tiberi P, Minopoli R, Manfredi A, Mutarelli M, Rossi S, Simonelli F, Naggert JK, Cacchiarelli D, *et al* (2018) Triple Vectors Expand AAV Transfer Capacity in the Retina. *Molecular Therapy* 26: 524–541

Magiorkinis E, Diamantis A, Sidiropoulou K & Panteliadis C (2014) Highights in the History of Epilepsy: The Last 200 Years. *Epilepsy Research and Treatment* 2014: 1–13

Mainardi M, Pietrasanta M, Vannini E, Rossetto O & Caleo M (2012) Tetanus neurotoxin-induced epilepsy in mouse visual cortex: TeNT-Induced Epilepsy in Mouse V1. *Epilepsia* 53: e132–e136

Manservigi R, Argnani R & Marconi P (2010) HSV Recombinant Vectors for Gene Therapy. *Open Virol J* 4: 123–156

Marsh DJ, Baraban SC, Hollopeter G & Palmiter RD (1999) Role of the Y5 neuropeptide Y receptor in limbic seizures. *Proceedings of the National Academy of Sciences of the United States of America*

Martire M, Pistritto G, Mores N, Agnati LF & Fuxe K (1993) Region-specific inhibition of potassium-evoked [3H]noradrenaline release from rat brain synaptosomes by neuropeptide Y-(13-36). Involvement of NPY receptors of the Y2 type. *European Journal of Pharmacology* 230: 231–234

Mathern GW, Babb TL, Pretorius JK & Leite JP (1995) Reactive synaptogenesis and neuron densities for neuropeptide Y, somatostatin, and glutamate decarboxylase

immunoreactivity in the epileptogenic human fascia dentata. *Journal of Neuroscience* 15: 3990–4004

Mayer ML (1986) Selective block of inward but not outward rectification in rat sensory neurones infected with herpes simplex virus. *The Journal of Physiology* 375: 327–338

Mayer ML, James MH, Russell RJ, Kelly JS, Wise JCM & Pasternak CA (1985) Spontaneous electrical activity induced by herpes virus infection in rat sensory neuron cultures. *Brain Research* 341: 360–364

McCarthy JB, Walker M, Pierce J, Camp P & White JD (1998) Biosynthesis and Metabolism of Native and Oxidized Neuropeptide Y in the Hippocampal Mossy Fiber System. *Journal of Neurochemistry* 70: 1950–1963

McCarthy KM, Tank DW & Enquist LW (2009) Pseudorabies Virus Infection Alters Neuronal Activity and Connectivity In Vitro. *PLoS Pathog* 5: e1000640

Mcquiston AR & Colmers WF (1996) Neuropeptide Y2 receptors inhibit the frequency of spontaneous but not miniature EPSCS in CA3 pyramidal cells of rat hippocampus. *Journal of Neurophysiology* 76: 3159–3168

Melin E, Nanobashvili A, Avdic U, Gøtzsche CR, Andersson M, Woldbye DPD & Kokaia M (2019) Disease Modification by Combinatorial Single Vector Gene Therapy: A Preclinical Translational Study in Epilepsy. *Molecular Therapy - Methods and Clinical Development* 15: 179–193

Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, Quirion R, Schwartz T & Westfall T (1998) XVI. International union of pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacological Reviews* 50: 143–50

Minth CD, Bloom SR, Polak JM & Dixon JE (1984) Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine. *Proceedings of the National Academy of Sciences of the United States of America*

Miyagawa Y, Marino P, Verlengia G, Uchida H, Goins WF, Yokota S, Geller DA, Yoshida O, Mester J, Cohen JB, *et al* (2015) Herpes simplex viral-vector design for efficient transduction of nonneuronal cells without cytotoxicity. *Proc Natl Acad Sci USA* 112: E1632–E1641

Miyagawa Y, Verlengia G, Reinhart B, Han F, Uchida H, Zucchini S, Goins WF, Simonato M, Cohen JB & Glorioso JC (2017) Deletion of the Virion Host Shut-off Gene Enhances Neuronal-Selective Transgene Expression from an HSV Vector Lacking Functional IE Genes. *Molecular Therapy - Methods & Clinical Development* 6: 79–90

Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E & Hayakawa T (2000) IRES-Dependent Second Gene Expression Is Significantly Lower Than Cap-Dependent First Gene Expression in a Bicistronic Vector. *Molecular Therapy* 1: 376–382

Monnet FP, Fournier A, Debonnel G & De Montigny C (1992) Neuropeptide Y potentiates selectively the N-methyl-D-aspartate response in the rat CA3 dorsal hippocampus. I. Involvement of an atypical neuropeptide Y receptor. *Journal of Pharmacology and Experimental Therapeutics* 263: 1212–1218

Nadler JV, Tu B, Timofeeva O, Jiao Y & Herzog H (2007) Neuropeptide Y in the recurrent mossy fiber pathway. *Peptides* 28: 357–364 doi:10.1016/j.peptides.2006.07.026 [PREPRINT]

Naldini L (2015) Gene therapy returns to centre stage. *Nature* 526: 351–360

Nanobashvili A, Woldbye DPD, Husum H, Bolwig TG & Kokaia M (2004) Neuropeptide Y Y5 receptors suppress in vitro spontaneous epileptiform bursting in the rat hippocampus. *NeuroReport* 15: 339–343

Nectow AR & Nestler EJ (2020) Viral tools for neuroscience. *Nat Rev Neurosci* 21: 669–681

Nguyen AD, Mitchell NF, Lin S, Macia L, Yulyaningsih E, Baldock PA, Enriquez RF, Zhang L, Shi YC, Zolotukhin S, *et al* (2012) Y1 and Y5 receptors are both required for the regulation of food intake and energy homeostasis in mice. *PLoS ONE* 7

Nikitidou Ledri L, Melin E, Christiansen SH, Gøtzsche CR, Cifra A, Woldbye DPD & Kokaia M (2016) Translational approach for gene therapy in epilepsy: Model system and unilateral overexpression of neuropeptide Y and Y2 receptors. *Neurobiology of Disease*

Noè F, Pool AH, Nissinen J, Gobbi M, Bland R, Rizzi M, Balducci C, Ferraguti F, Sperk G, During MJ, *et al* (2008) Neuropeptide Y gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy. *Brain* 131: 1506–1515

Noè FM, Sørensen AT, Kokaia M & Vezzani A (2010) Gene therapy of focal-onset epilepsy by adeno-associated virus vector-mediated overexpression of neuropeptide y. *Epilepsia* 51: 96–96

Olesen MV, Christiansen SH, Gøtzsche CR, Holst B, Kokaia M & Woldbye DPD (2012) Y5 neuropeptide Y receptor overexpression in mice neither affects anxiety- and depression-like behaviours nor seizures but confers moderate hyperactivity. *Neuropeptides* 46: 71–79

O'Loughlin EK, Pakan JMP, McDermott KW & Yilmazer-Hanke D (2014) Expression of neuropeptide Y1 receptors in the amygdala and hippocampus and anxiety-like behavior associated with Ammon's horn sclerosis following intrahippocampal kainate injection in C57BL/6J mice. *Epilepsy and Behavior* 37: 175–183

Paredes MF, Greenwood J & Baraban SC (2003) Neuropeptide Y modulates a G protein-coupled inwardly rectifying potassium current in the mouse hippocampus. *Neuroscience Letters* 340: 9–12

Parker SL, Kane JK, Parker MS, Berglund MM, Lundell IA & Li MD (2001) Cloned neuropeptide Y (NPY) Y1 and pancreatic polypeptide Y4 receptors expressed in Chinese hamster ovary cells show considerable agonist-driven internalization, in contrast to the NPY Y2 receptor. *European Journal of Biochemistry*

Patrylo PR, Van Den Pol AN, Spencer DD & Williamson A (1999) NPY Inhibits Glutamatergic Excitation in the Epileptic Human Dentate Gyrus. *Journal of Neurophysiology* 82: 478–83

Pavone P, Polizzi A, Marino SD, Corsello G, Falsaperla R, Marino S & Ruggieri M (2020) West syndrome: a comprehensive review. *Neurol Sci* 41: 3547–3562

Pechan PA, Fotaki M, Thompson RL, Dunn R, Chase M, Chiocca EA & Breakefield XO (1996) A Novel 'Piggyback' Packaging System for Herpes Simplex Virus Amplicon Vectors. *Human Gene Therapy* 7: 2003–2013

Pedrazzini T, Pralong F & Grouzmann E (2003) Neuropeptide Y: The universal soldier. *Cellular and Molecular Life Sciences* doi:10.1007/s000180300029 [PREPRINT]

Pelkey KA, Chittajallu R, Craig MT, Tricoire L, Wester JC & McBain CJ

(2017) Hippocampal GABAergic Inhibitory Interneurons. *Physiol Rev* 97: 1619–1747

Perucca P, Bahlo M & Berkovic SF (2020) The Genetics of Epilepsy. *Annual Review of Genomics and Human Genetics* 21: 205–230

Pickel VM, Beck-Sickinger AG, Chan J & Wieland HA (1998) Y1 receptors in the nucleus accumbens: Ultrastructural localization and association with neuropeptide Y. *Journal of Neuroscience Research* 52: 54–68

Piguet F, de Saint Denis T, Audouard E, Beccaria K, André A, Wurtz G, Schatz R, Alves S, Sevin C, Zerah M, *et al* (2021) The Challenge of Gene Therapy for Neurological Diseases: Strategies and Tools to Achieve Efficient Delivery to the Central Nervous System. *Human Gene Therapy* 32: 349–374

Pitkänen A, Nehlig A, Brooks-Kayal AR, Dudek FE, Friedman D, Galanopoulou AS, Jensen FE, Kaminski RM, Kapur J, Klitgaard H, *et al* (2013) Issues related to development of antiepileptogenic therapies. *Epilepsia* 54: 35–43

Pohlmann-Eden B, Beghi E, Camfield C & Camfield P (2006) The first seizure and its management in adults and children. *BMJ* 332: 339–342

van den Pol AN (2012) Neuropeptide Transmission in Brain Circuits. *Neuron* doi:10.1016/j.neuron.2012.09.014 [PREPRINT]

Powell KL, Fitzgerald X, Shallue C, Jovanovska V, Klugmann M, Von Jonquieres G, O'Brien TJ & Morris MJ (2018) Gene therapy mediated seizure suppression in Genetic Generalised Epilepsy: Neuropeptide Y overexpression in a rat model. *Neurobiology of Disease* 113: 23–32

Quek AML, Britton JW, McKeon A, So E, Lennon VA, Shin C, Klein CJ, Watson RE Jr, Kotsenas AL, Lagerlund TD, *et al* (2012) Autoimmune Epilepsy: Clinical Characteristics and Response to Immunotherapy. *Archives of Neurology* 69: 582–593

de Quidt ME & Emson PC (1986) Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system-II. Immunohistochemical analysis. *Neuroscience*

Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao G, Wilson JM & Batshaw ML (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Molecular*

Genetics and Metabolism 80: 148–158

Rettenbacher M & Reubi JC (2001) Localization and characterization of neuropeptide receptors in human colon. *Naunyn-Schmiedeberg's Archives of Pharmacology* 364: 291–304

Richichi C, Lin EJD, Stefanin D, Colella D, Ravizza T, Grignaschi G, Veglianese P, Sperk G, During MJ & Vezzani A (2004) Anticonvulsant and Antiepileptogenic Effects Mediated by Adeno-Associated Virus Vector Neuropeptide Y Expression in the Rat Hippocampus. *Journal of Neuroscience*

Ryvlin P, Rheims S, Hirsch LJ, Sokolov A & Jehi L (2021) Neuromodulation in epilepsy: state-of-the-art approved therapies. *The Lancet Neurology* 20: 1038–1047

Saeki Y, Fraefel C, Ichikawa T, Breakefield XO & Chiocca EA (2001) Improved Helper Virus-Free Packaging System for HSV Amplicon Vectors Using an ICP27-Deleted, Oversized HSV-1 DNA in a Bacterial Artificial Chromosome. *Molecular Therapy* 3: 591–601

Samaniego LA, Wu N & DeLuca NA (1997) The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. *J Virol* 71: 4614–4625

Samanta D (2020) Changing Landscape of Dravet Syndrome Management: An Overview. *Neuropediatrics* 51: 135–145

Satoh C, Satoh F, Takahashi K, Murakami O, Sone M, Totsune K, Yabe T, Ohneda M, Fukuda M, Sugimura K, *et al* (1999) Elevated plasma immunoreactive neuropeptide Y concentrations and its increased urinary excretion in patients with advanced diabetic nephropathy. *Endocrine Journal*

Shepard AA & DeLuca NA (1991) A second-site revertant of a defective herpes simplex virus ICP4 protein with restored regulatory activities and impaired DNA-binding properties. *J Virol* 65: 787–795

Shimada K, Ohno Y, Okamatsu-Ogura Y, Suzuki M, Kamikawa A, Terao A & Kimura K (2012) Neuropeptide y activates phosphorylation of ERK and STAT3 in stromal vascular cells from brown adipose tissue, but fails to affect thermogenic function of brown adipocytes. *Peptides*

Silva AP, Xapelli S, Grouzmann E & Cavadas C (2005a) The putative

neuroprotective role of neuropeptide Y in the central nervous system. *Current Drug Targets: CNS and Neurological Disorders* doi:10.2174/1568007054546153 [PREPRINT]

Silva AP, Xapelli S, Pinheiro PS, Ferreira R, Lourenço J, Cristóvão A, Grouzmann E, Cavadas C, Oliveira CR & Malva JO (2005b) Up-regulation of neuropeptide Y levels and modulation of glutamate release through neuropeptide Y receptors in the hippocampus of kainate-induced epileptic rats. *Journal of Neurochemistry* 93: 163–170

Simonato M, Bennett J, Boulis NM, Castro MG, Fink DJ, Goins WF, Gray SJ, Lowenstein PR, Vandenberghe LH, Wilson TJ, *et al* (2013) Progress in gene therapy for neurological disorders. *Nat Rev Neurol* 9: 277–291

Simonato M, Brooks-Kayal AR, Engel J, Galanopoulou AS, Jensen FE, Moshé SL, O'Brien TJ, Pitkanen A, Wilcox KS & French JA (2014) The challenge and promise of anti-epileptic therapy development in animal models. *The Lancet Neurology* 13: 949–960

Simonato M, Manservigi R, Marconi P & Glorioso J (2000) Gene transfer into neurones for the molecular analysis of behaviour: focus on herpes simplex vectors. *Trends in Neurosciences* 23: 183–190

Singh NA, Otto JF, Jill Dahle E, Pappas C, Leslie JD, Vilaythong A, Noebels JL, Steve White H, Wilcox KS & Leppert MF (2008) Mouse models of human KCNQ2 and KCNQ3 mutations for benign familial neonatal convulsions show seizures and neuronal plasticity without synaptic reorganization. *The Journal of Physiology* 586: 3405–3423

Snowball A, Chabrol E, Wykes RC, Shekh-Ahmad T, Cornford JH, Lieb A, Hughes MP, Massaro G, Rahim AA, Hashemi KS, *et al* (2019) Epilepsy Gene Therapy Using an Engineered Potassium Channel. *J Neurosci* 39: 3159–3169

Sørensen AT, Kanter-Schlifke I, Carli M, Balducci C, Noe F, During MJ, Vezzani A & Kokaia M (2008) NPY gene transfer in the hippocampus attenuates synaptic plasticity and learning. *Hippocampus* 18: 564–574

Sørensen AT, Nikitidou L, Ledri M, Lin EJD, During MJ, Kanter-Schlifke I & Kokaia M (2009) Hippocampal NPY gene transfer attenuates seizures without affecting

epilepsy-induced impairment of LTP. *Experimental Neurology* 215: 328–333

Soud K, Jørgensen SH, Woldbye DPD & Sørensen AT (2019) The C-terminal flanking peptide of neuropeptide Y (NPY) is not essential for seizure-suppressant actions of prepro-NPY overexpression in male rats. *Journal of Neuroscience Research* 97: 362–372

Soukupová M, Zucchini S, Trempat P, Ingusci S, Perrier-Biollay C, Barbieri M, Cattaneo S, Bettegazzi B, Falzoni S, Berthommé H, *et al* (2021) Improvement of HSV-1 based amplicon vectors for a safe and long-lasting gene therapy in non-replicating cells. *Molecular Therapy - Methods & Clinical Development* 21: 399–412

Sperk G, Marksteiner J, Gruber B, Bellmann R, Mahata M & Ortler M (1992) Functional changes in neuropeptide Y- and somatostatin-containing neurons induced by limbic seizures in the rat. *Neuroscience* 50: 831–846

Stanić D, Brumovsky P, Fetissov S, Shuster S, Herzog H & Hökfelt T (2006) Characterization of neuropeptide Y2 receptor protein expression in the mouse brain. I. Distribution in cell bodies and nerve terminals. *Journal of Comparative Neurology*

Stanić D, Mulder J, Watanabe M & Hökfelt T (2011) Characterization of NPY Y2 receptor protein expression in the mouse brain. II. Coexistence with NPY, the Y1 receptor, and other neurotransmitter-related molecules. *Journal of Comparative Neurology* 519: 1219–1257

Steinlein OK, Mulley JC, Propping P, Wallace RH, Phillips HA, Sutherland GR, Scheffer IE & Berkovic SF (1995) A missense mutation in the neuronal nicotinic acetylcholine receptor α4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. *Nature Genetics* 11: 201–203

Stjernquist M & Owman C (1990) Further evidence for a prejunctional action of neuropeptide Y on cholinergic motor neurons in the rat uterine cervix. *Acta Physiologica Scandinavica* 138: 95–96

St-Pierre JA, Nouel D, Dumont Y, Beaudet A & Quirion R (2000) Association of neuropeptide Y Y1 receptors with glutamate-positive and NPY-positive neurons in rat hippocampal cultures. *European Journal of Neuroscience* 12: 1319–1330

Szczygieł JA, Danielsen KI, Melin E, Rosenkranz SH, Pankratova S, Ericsson A, Agerman K, Kokaia M & Woldbye DPD (2020) Gene Therapy Vector Encoding

Neuropeptide Y and Its Receptor Y2 for Future Treatment of Epilepsy: Preclinical Data in Rats. *Front Mol Neurosci* 13: 232

Tang F, Hartz AMS & Bauer B (2017) Drug-Resistant Epilepsy: Multiple Hypotheses, Few Answers. *Frontiers in Neurology* 8

Tasan RO, Nguyen NK, Weger S, Sartori SB, Singewald N, Heilbronn R, Herzog H & Sperk G (2010) The central and basolateral amygdala are critical sites of neuropeptide Y/Y2 receptor-mediated regulation of anxiety and depression. *Journal of Neuroscience* 30: 6282–6290

Thijs RD, Surges R, O'Brien TJ & Sander JW (2019) Epilepsy in adults. *The Lancet* 393: 689–701

Thiriet N, Agasse F, Nicoleau C, Guégan C, Vallette F, Cadet JL, Jaber M, Malva JO & Coronas V (2011) NPY promotes chemokinesis and neurogenesis in the rat subventricular zone. *Journal of Neurochemistry*

Thomas RH & Berkovic SF (2014) The hidden genetics of epilepsy—a clinically important new paradigm. *Nat Rev Neurol* 10: 283–292

Thompson HJ, Lifshitz J, Marklund N, Grady MS, Graham DI, Hovda DA & McIntosh TK (2005) Lateral fluid percussion brain injury: a 15-year review and evaluation. *J Neurotrauma* 22: 42–75

Tilan J & Kitlinska J (2016) Neuropeptide Y (NPY) in tumor growth and progression: Lessons learned from pediatric oncology. *Neuropeptides* doi:10.1016/j.npep.2015.10.005 [PREPRINT]

Toader O, Forte N, Orlando M, Ferrea E, Raimondi A, Baldelli P, Benfenati F & Medrihan L (2013) Dentate gyrus network dysfunctions precede the symptomatic phase in a genetic mouse model of seizures. *Front Cell Neurosci* 7

Tomson T, Walczak T, Sillanpaa M & Sander JWAS (2005) Sudden Unexpected Death in Epilepsy: A Review of Incidence and Risk Factors. *Epilepsia* 46: 54–61

Tornabene P, Trapani I, Minopoli R, Centrulo M, Lupo M, Simone S de, Tiberi P, Dell'Aquila F, Marrocco E, Iodice C, *et al* (2019) Intein-mediated protein trans-splicing expands adeno-associated virus transfer capacity in the retina. *Science Translational Medicine*

Tu B, Timofeeva O, Jiao Y & Nadler JV (2005) Spontaneous Release of Neuropeptide Y Tonically Inhibits Recurrent Mossy Fiber Synaptic Transmission in Epileptic Brain. *Journal of Neuroscience* 25: 1718–29

Tucci F, Scaramuzza S, Aiuti A & Mortellaro A (2021) Update on Clinical Ex Vivo Hematopoietic Stem Cell Gene Therapy for Inherited Monogenic Diseases. *Molecular Therapy* 29: 489–504

Uchida H, Chan J, Goins WF, Grandi P, Kumagai I, Cohen JB & Glorioso JC (2010) A Double Mutation in Glycoprotein gB Compensates for Ineffective gD-Dependent Initiation of Herpes Simplex Virus Type 1 Infection. *J Virol* 84: 12200–12209

Vatsa N & Jana NR (2018) UBE3A and Its Link With Autism. *Frontiers in Molecular Neuroscience* 11

Verlengia G, Miyagawa Y, Ingusci S, Cohen JB, Simonato M & Glorioso JC (2017) Engineered HSV vector achieves safe long-term transgene expression in the central nervous system. *Sci Rep* 7: 1507

Verma D, Tasan RO, Herzog H & Sperk G (2012) NPY controls fear conditioning and fear extinction by combined action on Y 1 and Y 2 receptors. *British Journal of Pharmacology*

Vezzani A, Moneta D, Mulé F, Ravizza T, Gobbi M & French-Mullen J (2000) Plastic changes in neuropeptide Y receptor subtypes in experimental models of limbic seizures. *Epilepsia*

Vezzani A & Sperk G (2004) Overexpression of NPY and Y2 receptors in epileptic brain tissue: An endogenous neuroprotective mechanism in temporal lobe epilepsy? *Neuropeptides* doi:10.1016/j.npep.2004.05.004 [PREPRINT]

Vrinda M, Sasidharan A, Aparna S, Srikumar BN, Kutty BM & Shankaranarayana Rao BS (2017) Enriched environment attenuates behavioral seizures and depression in chronic temporal lobe epilepsy. *Epilepsia* 58: 1148–1158

Wagner L, Wolf R, Zeitschel U, Rossner S, Petersén Å, Leavitt BR, Kästner F, Rothermundt M, Gärtner UT, Gündel D, *et al* (2015) Proteolytic degradation of neuropeptide y (NPY) from head to toe: Identification of novel NPY-cleaving peptidases and potential drug interactions in CNS and Periphery. *Journal of*

Neurochemistry

Wahlestedt C, Pich EM, Koob GF, Yee F & Heilig M (1993) Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. *Science*

Wahlestedt C, Yanaihara N & Håkanson R (1986) Evidence for different pre- and post-junctional receptors for neuropeptide Y and related peptides. *Regulatory Peptides* 13: 307–318

Wai SM, Kindler PM, Lam ETK, Zhang A & Yew DT (2004) Distribution of neuropeptide Y-immunoreactive neurons in the human brainstem, cerebellum, and cortex during development. In *Cellular and Molecular Neurobiology*

Walther C, Mörl K & Beck-Sickinger AG (2011) Neuropeptide Y receptors: Ligand binding and trafficking suggest novel approaches in drug development. *Journal of Peptide Science* doi:10.1002/psc.1357 [PREPRINT]

West S, Nevitt SJ, Cotton J, Gandhi S, Weston J, Sudan A, Ramirez R & Newton R (2019) Surgery for epilepsy. *Cochrane Database of Systematic Reviews*

Wickham J, Ledri M, Bengzon J, Jespersen B, Pinborg LH, Woldbye DPD, Andersson M & Kokaia M (2019) Inhibition of epileptiform activity by neuropeptide Y in brain tissue from drug-resistant temporal lobe epilepsy patients. *Scientific Reports* 9: 19393

Wirrell E, Nabbout R, Scheffer IE, Alsaadi T, Bogacz A, French JA, Hirsch E, Jain S, Kaneko S, Riney K, *et al* (2021) Methodology for Classification and Definition of Epilepsy Syndromes: Report of the ILAE Task Force on Nosology and Definitions.

Woldbye DPD, Ängehagen M, Gøtzsche CR, Elbrønd-Bek H, Sørensen AT, Christiansen SH, Olesen MV, Nikitidou L, Hansen TVO, Kanter-Schlifke I, *et al* (2010) Adeno-associated viral vector-induced overexpression of neuropeptide y Y2 receptors in the hippocampus suppresses seizures. *Brain* 133: 2778–2788

Woldbye DPD, Larsen PJ, Mikkelsen JD, Klemp K, Madsen TM & Bolwig TG (1997) Powerful inhibition of kainic acid seizures by neuropeptide Y via Y5- like receptors. *Nature Medicine*

Woldbye DPD, Nanobashvili A, Sørensen AT, Husum H, Bolwig TG, Sørensen G, Ernfors P & Kokaia M (2005) Differential suppression of seizures via Y2 and Y5 neuropeptide Y receptors. *Neurobiology of Disease*

Wood J, Verma D, Lach G, Bonaventure P, Herzog H, Sperk G & Tasan RO (2016) Structure and function of the amygdaloid NPY system: NPY Y2 receptors regulate excitatory and inhibitory synaptic transmission in the centromedial amygdala. *Brain Structure and Function* 221: 3373–3391

Wu N, Watkins SC, Schaffer PA & DeLuca NA (1996) Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding ICP4, ICP27, and ICP22. *J Virol* 70: 6358–6369

Xu X, Chemparathy A, Zeng L, Kempton HR, Shang S, Nakamura M & Qi LS (2021) Engineered miniature CRISPR-Cas system for mammalian genome regulation and editing. *Molecular Cell* 81: 4333-4345.e4

Yang J, Zhou W, Zhang Y, Zidon T, Ritchie T & Engelhardt JF (1999) Concatamerization of Adeno-Associated Virus Circular Genomes Occurs through Intermolecular Recombination. *Journal of Virology*

Yang Z, Han S, Keller M, Kaiser A, Bender BJ, Bosse M, Burkert K, Kögler LM, Wifling D, Bernhardt G, *et al* (2018) Structural basis of ligand binding modes at the neuropeptide y Y1 receptor. *Nature*

Yilmazer-Hanke D, O'Loughlin E & Mcdermott K (2016) Contribution of amygdala pathology to comorbid emotional disturbances in temporal lobe epilepsy. *Journal of Neuroscience Research* 94: 486–503 doi:10.1002/jnr.23689 [PREPRINT]

Zanirati G, Azevedo PN, Venturin GT, Greggio S, Alcará AM, Zimmer ER, Feltes PK & DaCosta JC (2018) Depression comorbidity in epileptic rats is related to brain glucose hypometabolism and hypersynchronicity in the metabolic network architecture. *Epilepsia* 59: 923–934

Zaupa C, Revol-Guyot V & Epstein AL (2003) Improved Packaging System for Generation of High-Level Noncytotoxic HSV-1 Amplicon Vectors Using Cre-loxP Site-Specific Recombination to Delete the Packaging Signals of Defective Helper Genomes. *Human Gene Therapy* 14: 1049–1063

Zeng L-H, Rensing NR, Zhang B, Gutmann DH, Gambello MJ & Wong M (2011) Tsc2 gene inactivation causes a more severe epilepsy phenotype than Tsc1 inactivation in a mouse model of Tuberous Sclerosis Complex. *Human Molecular Genetics* 20: 445–454

Zhang Q, Hsia S-C & Martin-Caraballo M (2017) Regulation of T-type Ca2+ channel expression by herpes simplex virus-1 infection in sensory-like ND7 cells. *J Neurovirol* 23: 657–670

Ziffert I, Kaiser A, Babilon S, Mörl K & Beck-Sickinger AG (2020a) Unusually persistent Gαi-signaling of the neuropeptide Y2 receptor depletes cellular Gi/o pools and leads to a Gi-refractory state. *Cell Communication and Signaling*

Ziffert I, Kaiser A, Hoppenz P, Mörl K & Beck-Sickinger AG (2020b) Shuttling of Peptide-Drug Conjugates by G Protein-Coupled Receptors Is Significantly Improved by Pulsed Application. *ChemMedChem*

Zinn E & Vandenberghe LH (2014) Adeno-associated virus: fit to serve. *Current Opinion in Virology* 8: 90–97