Università degli Studi di Ferrara

DOCTORAL COURSE IN

"Translational Neurosciences and Neurotechnologies"

CYCLE XXXIV

COORDINATOR Prof. Luciano Fadiga

Novel Therapeutic Strategies for secondary prevention of Temporal Lobe Epilepsy

Scientific/disciplinary sector (SDS): BIO/14

Candidate
Dott. Guarino Annunziata

_____

Supervisor
Prof. Simonato Michele

_____

Year 2018/2021

Abstract (italiano)

L'epilessia del lobo temporale mesiale è una delle forme più frequenti di epilessia, spesso gravata da comorbidità che hanno un impatto significativo sulla qualità della vita. Ad oggi la terapia farmacologica è solo sintomatica e circa un terzo dei pazienti è farmaco-resistente. Questo tipo di epilessia ha frequentemente, alla base, un evento precipitante seguito da un periodo definito "epilettogenesi", nel quale si verificano molteplici fenomeni fisiopatologici come perdita di neuroni, plasticità neuronale e neurogenesi aberrante, modificazioni delle cellule gliali, alterazione della barriera emato-encefalica, neuroinfiammazione. Tutti questi fenomeni portano, a distanza anche di anni, allo sviluppo della malattia.

Durante il mio dottorato di ricerca ho indagato possibili nuove strategie terapeutiche, che potessero modulare l'epilettogenesi e prevenire lo sviluppo di epilessia:

- Valutazione dell'ipotesi che il 7,8 diidrossiflavone (7,8- DHF), un agente antiossidante e agonista del recettore TrkB per BDNF, possa esercitare un ruolo anti-epilettogeno. Per raggiungere questo obiettivo, è stato utilizzato il modello litio-pilocarpina nel ratto. L'effetto del 7,8- DHF è stato valutato sia sull'esordio della malattia (in termini di frequenza e gravità delle crisi epilettiche) sia sulle co-morbidità (attraverso l'utilizzo di test comportamentali per la locomozione, l'ansia e la memoria spaziale). Inoltre, è stata eseguita un'analisi ex vivo per comprendere i meccanismi alla base degli effetti del 7,8- DHF. Abbiamo scoperto che il 7,8- DHF a basso (5 mg/kg), ma non ad alto (10 mg/kg) dosaggio, può esercitare forti effetti anti-epilettogeni nel modello litio-pilocarpina, e che questi effetti sono correlati a pattern specifici di fosforilazione di TrkB e di attivazione delle vie di segnalazione TrkB-dipendenti.

- Studio dell'effetto del Peptide di rigenerazione neuronale 2945 (NRP2945) nel modello di pilocarpina di mTLE, utilizzando due diversi paradigmi di somministrazione di NRP2945: (i) a seguito di stato epilettico indotto da pilocarpina, per valutare la sua capacità di prevenire lo sviluppo di epilessia (vale a dire un possibile effetto anti-epilettogeno) e (ii) nella fase cronica dell'epilessia, per valutarne l'effetto sulle crisi spontanee. Abbiamo scoperto che NRP2945 esercita un forte effetto anti-epilettogeno, riducendo la frequenza delle convulsioni spontanee, esercitando un significativo effetto neuroprotettivo e attenuando i comportamenti ansiosi e il deterioramento cognitivo. Questi effetti sembrano dipendere dalla modulazione del processo di epilettogenesi e non dalla soppressione delle crisi, perché NRP2945 non ha ridotto la frequenza o la durata delle crisi spontanee quando somministrato ad animali già epilettici.

Abstract (inglese)

Mesial temporal lobe epilepsy is one of the most frequent forms of epilepsy, often burdened by comorbidities that have a significant impact on the quality of life. To date, drug therapy is only symptomatic and one third of the patients are drug resistant. This type of epilepsy often originates from a precipitating event followed by a period defined "epileptogenesis", in which multiple pathophysiological phenomena occur, including loss of neurons, neuronal plasticity and aberrant neurogenesis, modifications of glial cells, alteration of the BBB and neuroinflammation. These phenomena may lead, sometimes years later, to the development of the disease.

During my PhD I investigated possible new therapeutic strategies for the of epileptogenesis:

• Evaluation of the hypothesis that 7,8 dihydroxyflavone (7,8- DHF), an antioxidant agent and an agonist of the BDNF receptor TrkB, may exert an anti-epileptogenic role. To pursue this goal, the rat lithium pilocarpine model was used. The effect of 7,8- DHF was evaluated both at the onset of the disease (in terms of frequency and severity of seizures) and on epilepsy comorbidities (through the use of behavioural tests for locomotion, anxiety and memory). In addition, an ex vivo analysis was performed to understand the mechanisms underlying the effects of 7,8- DHF. We found that 7,8- DHF at low (5 mg/kg), but not at high (10 mg/kg) dosage, can exert strong anti-epileptic effects in the lithium-pilocarpine model, and that these different effects are related to differences in TrkB phosphorylation patterns and activation of TrkB-dependent signalling pathways.

• Study of the effect of the Neuronal Regeneration Peptide 2945 (NRP2945) in the pilocarpine model of mTLE, using two different NRP2945 administration paradigms: (i) following pilocarpine-induced status epilepticus, to evaluate its ability to prevent epilepsy development (i.e., a presumed anti-epileptogenic effect) and (ii) in the chronic phase of epilepsy, to evaluate its effect on spontaneous seizures. We found that NRP2945 exerts a strong anti-epileptogenic effect, reducing the frequency of spontaneous seizures, exerting a significant neuroprotective effect, and attenuating anxious behaviours and cognitive impairment. These effects appear to depend on the modulation of the epileptogenesis process and not on seizure suppression, because NRP2945 did not reduce the frequency or duration of spontaneous seizures when administered to already epileptic animals.

Index

Abbreviation index

interleukin; 17
ILAE; 3
International League of Epilepsy; 3
IP3
inositol 1,4,5-triphosphate; 21
JNK
Jun N-terminal kinase; 31
K^+
potassium; 21
KD
KD; 11
KO
knock-out; 36
lTLE
lateral TLE; 13
LVA
low voltage-activated; 8
MAPK
mitogen-activated protein kinase; 32
mitogen-activated protein kinase; 34
mBDNF
mature BDNF; 34
MEK
MAPK/ERK kinase; 32
MES
maxinal electroshock test; 19
MFS
mossy fibers; 27
MRI
magnetic resonance imaging; 7
mTLE
mesial TLE; 13
Na^+
sodium; 7
NDDI-E
Neurological Disorders Depression Inventory for Epilepsy; 17
NFTs
neurotrophic factors; 30
NGF
nerve growth factor; 30
NMDA
N-methyl-D-aspartate; 8
NMDARs
N-methyl-D-aspartate receptors; 8
NRAGE
neuroteophin-receptor-interacting MAGE homologue; 32
NRIF
neurotrophin-receptor interacting factor; 31
NRP
neural regeneration peptide; 73
NRP2945
Neuronal Regeneration Peptide 2945; 39; 73
NT
neurotrophin; 30
NTF
neurotrophic factor; 30
$p75^{NTR}$
p75 neurotrophin receptor; 31
PDK1
phosphoinositide-dependent kinase 1; 32
PET
positron emission tomography; 17
PI3K
phosphatidylinositol-3-OH kinase; 32
PKC
protein kinase C; 34
PLC-γ1
phospholipase C γ1; 32
PN
perineuronal net; 26
PWE
people with epilepsy; 17
QoL
quality of life; 16
RIP 2
receptor-interacting protein 2; 32
ROS
reactive oxigen species; 42
SC1
Schwann cells 1; 32
SCT
saccharin consumption test; 16
SE
status epilepticus; 13; 17
SGZ
subgranular zone; 26
SH2B
Src homology 2-B; 32
Shc
collagen-like adaptor protein; 31
SOS
Son of Sevenless; 32
SRSs
spontaneous recurrent seizures; 14
SUDEP
sudden unexpected death in epilepsy; 11
SVZ
subventricular zone; 26

TLE
temporal lobe epilepsy; 6; 10; 13; 14; 25
TLRs
toll-like receptors; 28
TNF
tumour necrosis factor; 31
TRAF6
tumour necrosis factor receptor-associated factor 6; 32
TrK
tyrosine kinase; 31
VGSCs
voltage gated sodium channels; 7
VNS
vagal nerve stimolation; 10

CHAPTER 1. EPILEPSY

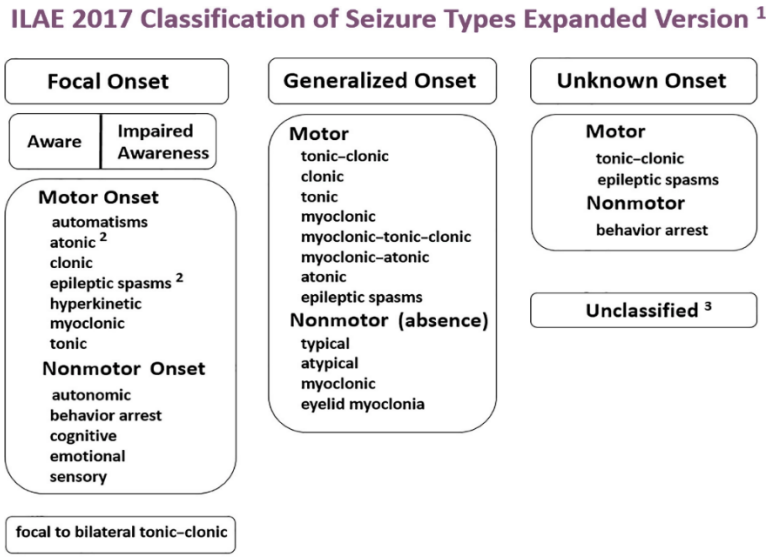
1.1 Definition

The International League Against Epilepsy (ILAE) defined an epileptic seizure as "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain", and epilepsy as "a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition". The diagnosis of epilepsy is made based on the following: (1) At least two unprovoked (or reflex) seizures occurring >24 h apart; (2) 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; (3) diagnosis of an epilepsy syndrome (Fisher et al. 2014). Epilepsy is considered *resolved* for: i) people who had age-dependent epileptic syndrome, and who then exceeded the applicable age limit; ii) people who have been seizure-free for at least 10 years without antiepileptic therapy for the past 5 years (Fisher et al. 2014).

The term epilepsy derives from the Greek term "*epilambanein*", that translates into "attack"(Todman 2008), emphasizing one of the characteristics of this disease: unpredictability. Indeed, in the origin it was believed that this disease affected bad people through the action of the gods. Hippocrates, although still tied to the idea of the "divine" (Todman 2008), was the first to argue that at the base of the epilepsy there was a cerebral dysfunction (Magiorkinis, Sidiropoulou, and Diamantis 2010). However, it was only in the 19th century that John Hughlings Jackson laid the foundations of modern epileptology. He claimed that epileptic discharges are generated in the grey matter of the brain and begin with signs that locate the original discharge lesion (Kaculini, Tate-Looney, and Seifi 2021). Nowadays, around 60 million people worldwide have epilepsy, and this disease affects people of all ages (Fiest et al. 2017).

1.2 Seizures classification

The need for classification for different types of seizures has existed since the days of Hippocrates. Although the initial classifications were primarily anatomical, research has shown that epilepsy is a network disease rather than a symptom of local brain abnormalities (Blumenfeld 2014). In 1969, the first modern classification has been proposed by Gastaut (Gastaut 1969). Over the years, this classification has been progressively improved, to provide a basis for communication for clinical use. ILAE 2017 is the most recent (**fig. 1**) ((Fisher et al. 2017); (Fisher 2017)).



[1]Definitions, other seizure types and descriptors are listed in the accompanying paper and glossary of terms.

[2]Degree of awareness usually is not specified.

[3]Due to inadequate information or inability to place in other categories.

Fig. 1 ILAE classification of seizures (Fisher 2017)

The first level of classification distinguishes seizures based on their onset: focal, generalized and unknown onset seizures.

FOCAL SEIZURES

Focal seizures are characterized by the activation of a localized group of neurons, limited to one hemisphere only. Focal seizures can be divided into:

- *Aware (previously called "simple") forms*: characterized by motor, sensitive or senses, psychic phenomena without disruption of the state of consciousness
- *Impaired awareness (formerly "complex") forms*: the state of consciousness is impaired, exhibiting an inability to respond to environmental stimuli and/or to maintain contact with external events.
- *Focal to bilateral tonic-clonic (secondary generalized) forms*: forms that start as focal seizures and then involve both hemispheres.

GENERALIZED SEIZURES

Generalized seizures involve both hemispheres from the beginning with bilateral, symmetrical and synchronous phenomena; usually there is an alteration of the state of consciousness.
Can be divided into:

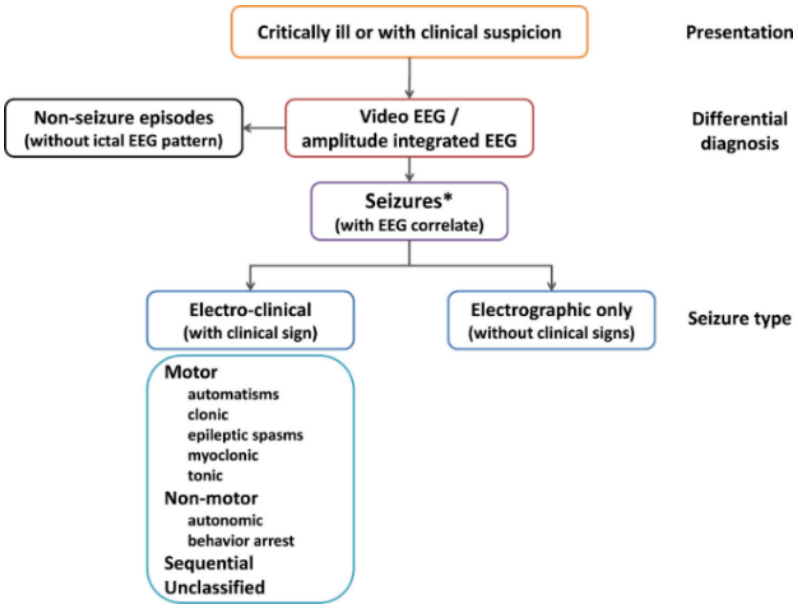
- *Non-motor (absence)*: characterized by a sudden loss of consciousness. They can last from a few seconds to a few minutes and are usually accompanied by tonic, clonic, atonic and vegetative phenomena.
- *Myoclonic seizures:* short and rapid muscle contractions, bilateral and synchronous, usually without alteration of the state of consciousness.
- *Tonic seizures*: characterized by diffuse muscle contraction, accompanied by alteration of state of consciousness.
- *Tonic-clonic seizures*: generalized seizures accompanied by alteration of state of consciousness. The tonic phase is characterized by rigidity, apnea and cyanosis, while during the clonic phase frequent rapid and repetitive bilateral contractions appear.
- *Clonic seizures*: like the tonic-clonic one, but during the clonic phase frequent rapid and repetitive bilateral contractions appear.
- *Atonic seizures:* characterized by a reduction in tone (incomplete or global), followed by the fall of the subject to the ground.

UNCLASSIFIED SEIZURES

All the seizures that cannot be included in the classes described above.

NEONATAL SEIZURES

In addition to ILAE 2017, a further modification regarding neonatal seizures was introduced in 2021 (Pressler et al. 2021). The need to introduce this modification arises from the fact that the peculiarities of neonatal epileptic seizures are that are mostly provoked seizures and are visible only at the electroencephalography (EEG). Indeed, this revision emphasizes the role of EEG in seizure diagnosis and includes a classification of seizure types relevant to this age group (**fig. 2**).



*If no EEG available refer to global alignment of immunization safety assessment in pregnancy levels of diagnostic certainty

Fig. 2 ILAE classification of neonatal seizures (Pressler et al. 2021)

1.3 Diagnosis and treatment

DIAGNOSIS

In order to make a correct diagnosis of seizures and epilepsy, first of all it is important to perform a thorough medical history. Moreover, neurologists can use additional tests like EEG, Neuroimaging, genetic testing or metabolic evaluations (Stafstrom and Carmant 2015).

- *EEG*: The EEG evaluation leads to achieve several goals in terms of diagnosis, depending on the clinical question. In TLE, the EEG remains the gold standard used

in the diagnosis (Rosenow, Klein, and Hamer 2015). It is possible to detect abnormal electrical activities, such as focal spikes or waves (focal epilepsy), or diffuse bilateral spike waves (generalized epilepsy). However, the diagnosis of epilepsy is based on clinical information and the EEG should be considered as confirmatory, not diagnostic. Indeed, although after 3 EEG evaluation the likelihood of identifying an abnormality increases, a normal EEG can be seen in up to 50% of people with epilepsy (Johnson 2019).

- *Neuroimaging:* Computed tomography (CT) and magnetic resonance imaging (MRI) scans are adjuncts to the clinical examination and EEG. MRI is more sensitive than CT and is, thus, preferred.
- *Genetic testing*: when a genetic diagnosis is highly suspect; the clinician could also require genetic testing, up to the entire exome sequencing of the patient and parents.
- *Metabolic evaluations*: used principally in neonatal seizures.

TREATMENT

To date, there is no cure for epilepsy. AEDs are not effective for the underlying cause of seizures, but they limit or prevent seizures (thus, acting on the symptoms). Usually, an antiepileptic therapy is chosen based on the risk-benefit ratio between the AED used and the possible side effects and costs that the patient must bear (Fisher et al. 2014). Considering the side effects (such as sedation and dizziness) is important, because therapy usually lasts for long periods of time. AEDs have a different mechanism of action (**fig. 3**), which result in a decrease in neuronal excitability:

- *Sodium channel blockers*: many AEDs (e.g., Carbamazepine, Lamotrigine, Phenytoin) act on voltage gated sodium channels (VGSCs), which allow influx of sodium (Na^+) in the cells and generate action potentials. Membrane depolarization allows channel opening and then, in a few milliseconds, the channel is rapidly inactivated. During high frequency repetitive firing a slow inactivation occurs caused by a conformational change in the channel that continues to display a residual conductance. Na^+ channel blockers stabilize the channels in the inactive state, reducing the firing rate of the neurons and the spread of seizures (Zuliani, Fantini, and Rivara 2012).
- *Calcium channel inhibitors*: Voltage-gated calcium (Ca^{2+}) channels are localized in the plasma membrane of excitable cells. Following depolarization, the influx of Ca^{2+} leads to further depolarization of the plasma membrane, leading to the activation of

other voltage-gated channels. Moreover, Ca^{2+} leads to the activation of contraction, secretion, gene transcription, and cell death. These channels are divided into low voltage-activated (LVA, or T-type) and high voltage-activated channels (HVA). Evidences from several studies in animal models of generalized absence epilepsy show an over-activity of T-type channels, although T-type channel expression is up regulated in other forms epilepsy as well (Nelson, Todorovic, and Perez-Reyes 2006). Calcium channel inhibitors (such as Ethosuximide) act on these channels by inhibiting the Ca^{2+} influx in the cells.

- *Gamma-aminobutyric acid (GABA) signal enhancers*: GABA type A receptor ($GABA_AR$) is the most crucial inhibitory receptor in the central nervous system (CNS). Upon binding of GABA, the receptor opens and chloride ions diffuse into the cell, leading to an inhibitory postsynaptic potential. Drugs like Barbiturates (Phenobarbital) and Benzodiazepines (Diazepam, lorazepam, Clonazepam) act as positive allosteric modulators of the $GABA_AR$. These drugs are widely employed despite several side effects (such as respiratory depression, sedation and rebound insomnia). Other drugs, like Vigabatrin, inhibit the degradation of GABA, which is mediated by the GABA transaminase. Another mechanism for increasing extracellular GABA concentrations is to act on the GABA transporter (Tiagabine).
- *Glutamate signal inhibitors*: glutamate is the most predominant excitatory neurotransmitter of the adult mammalian brain. It exerts its fast excitatory effect by binding the postsynaptic ionotropic glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs), whereas ***N***-methyl-D-aspartate (NMDA) receptors (NMDARs) mediate part of the slow postsynaptic excitatory potentials (Barker-Haliski and White 2015). Glutamate signal inhibitors include AMPA antagonists (Perampanel) and blockers of presynaptic Ca^{2+} channels in the glutamatergic terminal (Gabapentin).

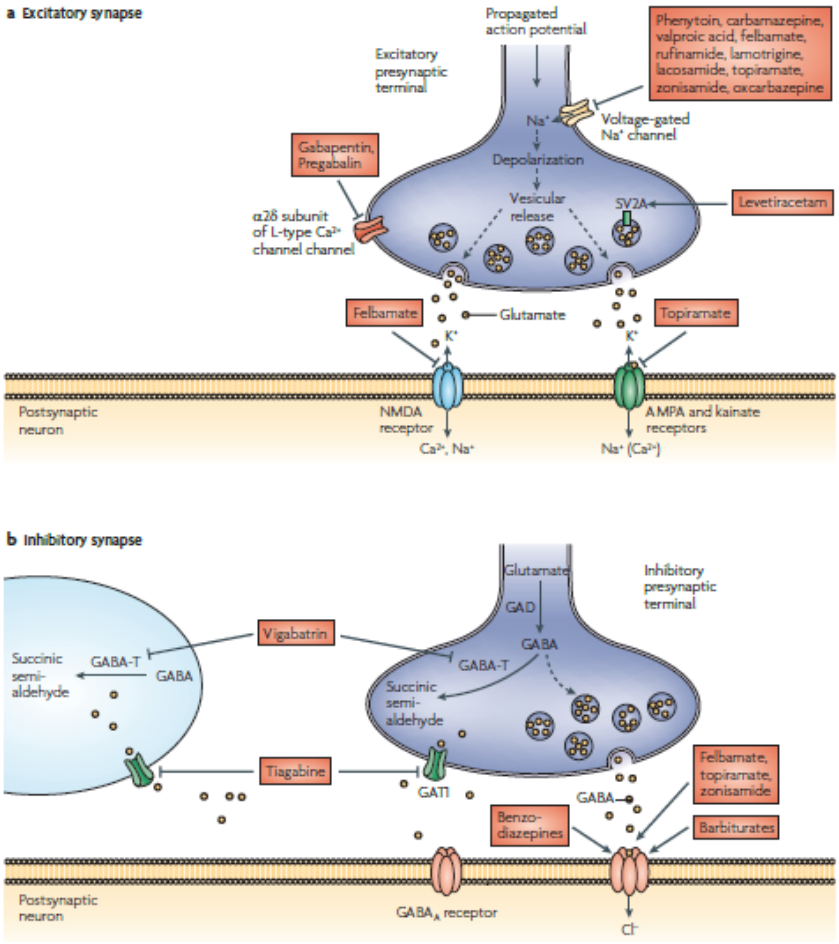


Fig. 3 Proposed mechanisms of action of currently available AEDs at excitatory and inhibitory synapses (Bialer and White 2010).

Despite the wide range of available AEDs, about 30% of patients are drug resistant (Kwan and Brodie 2000). According to ILAE consensus: "It is proposed as a testable hypothesis that drug resistant epilepsy is defined as failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom"(Kwan et al. 2010). Anyhow, there are several alternatives that do not involve the use of AEDs.

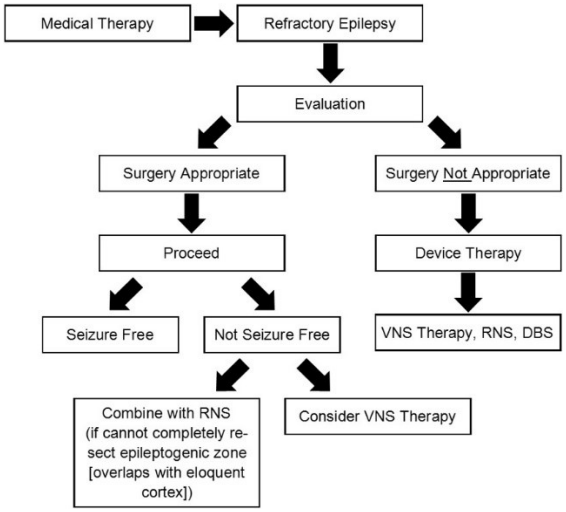


Fig. 4 Treatment sequence for epilepsy surgery and devices (Wheless, Gienapp, and Ryvlin 2018)

Refractory epileptic patients could be candidates for surgery for the resection of the epileptogenic focus or for vagal nerve stimulation (VNS). Surgical resection of the "*epileptogenic focus"* depends on the area involved. Patients with TLE are, often, excellent candidates and, 80% of them undergoing surgical resection, remain long-term seizures-free (41%), and the quality of their lives improves (de Tisi et al. 2011). However, this type of surgery is very invasive. In the 1990s, it was shown that VNS is able to reduce epileptic seizures. VNS is a peripheral surgery, less invasive and expensive than surgical resection. Briefly, VNS consists of two electrodes placed on the cervical vagal nerve and connected to a pulse generator under the collarbone. The stimulator is located on the left vagal nerve to reduce adverse cardiac effects (Sakas et al. 2007). To date, VNS is an approved treatment in more than 70 countries around the world. Moreover, VNS not only improves the quality of life of patients with refractory epilepsy, but the success of the treatment also appears not to depend on age, type of seizure or epileptic syndrome (Wheless, Gienapp, and Ryvlin 2018).

KETOGENIC DIET

Another type of non-drug therapy is the ketogenic diet (KD) (Ulamek-Koziol et al. 2019). It is based on a rich intake of fats and a low intake of carbohydrates. This leads to an increase in the metabolism of fatty acids, which produce ketone bodies, which replace glucose for the brain. Although the mechanisms are not fully understood, the KD appears to be effective on drug-resistant epilepsies, in both children and adults. Ketone bodies in the brain have been observed to turn into acetyl-CoA leading to the production of adenosine triphosphate (ATP) in the mitochondria, improving mitochondrial biogenesis and density and increasing phosphocreatine stores. The brain tissue would thus become more resistant to metabolic stress, elevating the seizure threshold. Furthermore, ATP leads to hyperpolarization of the neuronal membrane, reducing the electrical excitability of the brain. Moreover, the KD can lead to induction of GABA synthesis and inhibition of its degradation (Ulamek-Koziol et al. 2019). Several studies have investigated effects of KD on the composition of the gut microbiome in epilepsy. It seems that alterations in the gut microbiome may contribute to the protective effects of the KD against seizures. Indeed, using a genetic mouse model for sudden unexpected death in epilepsy (SUDEP), the depletion of the gut microbiome promoted spontaneous tonic-clonic seizures, whereas selective enrichment of KD-associated bacterial taxa reduced seizure frequency and duration (Lum, Olson, and Hsiao 2020).

1.4 Temporal lobe

1.4.1 Anatomy

The limbic system is an aggregation of brain structures (located lateral to the thalamus, underneath the cerebral cortex, and above the brainstem) involved in memory and emotion. The limbic system includes the limbic cortex (cingulate gyrus and parahippocampal gyrus), the hippocampal formation (dentate gyrus, hippocampus and subicular complex), amygdala, septal area, and hypothalamus. In this paragraph I will focus on the hippocampal formation, which is the area most involved in mTLE.

The hippocampal formation refers to the dentate gyrus, hippocampus proper, subicular complex, and entorhinal cortex (**fig. 5**).

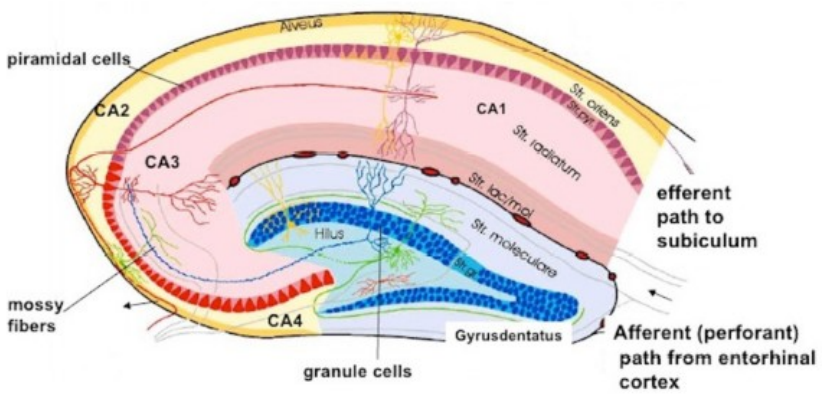


Fig.5 Anatomical organization of the hippocampus

Dentate gyrus (DG): is a structure formed by a trilaminar cortex (archicortex), located in the most medial part of the cerebral cortex. The dentate gyrus has a C-shaped structure separated from cornu ammonis 1 (CA1) and the subiculum by the hippocampal fissure. The spiny dendrites of the neurons that make up the main cell layer of the dentate gyrus branch into its molecular layer. The innermost layer is called the polymorphic layer or the hilum. The cells of the hilum only project to the dentate gyrus. Moreover, the hilum of the dentate gyrus contains the axons of the granular cells (or "mossy fibers") that project to the hippocampus (CA3) (Schultz and Engelhardt 2014).

Hippocampus proper (or “cornu ammonis”, CA): it is sub-divided into 4 fields: CA4, CA3, CA2 and CA1. However, the term CA4 should be omitted, since these neurons should probably belong to the hilar region of CA3. Starting from the ventricular lumen, the following layers are distinguished: the alveus, which is formed by the axons of the pyramidal cells and the stratum oriens (between the alveus and the pyramidal layer) which mainly contains the basal dendrites of the pyramidal cells. The cell bodies of the principal CA neurons form the pyramidal cell layer. The region towards the hippocampal fissure contains the apical dendrites and is divided into the stratum lucidum (absent in CA2 and CA1), the stratum radiatum, and the stratum lacunosum-moleculare (which contains the terminal branches of the apical dendrites). In CA3 stratum lucidum, mossy fibers form synapses with proximal dendrites above the pyramidal cell layer of CA3 (Schultz and Engelhardt 2014).

Subicular area (subiculum, presubiculum, parasubiculum): the subiculum is composed of a molecular layer containing the apical dendrites of the underlying pyramidal cells, the pyramidal layer and the deep polymorphic layer. Projections of the hippocampal formation originate from the pyramidal layer which, through the fornix, reach subcortical structures (to the septal and mammillary nuclei, to the nucleus accumbens, to the anterior thalamus).

Presubiculum and parasubiculum are transition cortices (4-5 layers) towards the entorhinal cortex (Schultz and Engelhardt 2014).

Entorhinal cortex (EC): six entorhinal layers can be distinguished. Layer I is an acellular layer. Layer II is made up of islands of modified pyramidal and stellate cells. Layer III contains pyramidal cells, and their dendrites pass through the space between layer II cell islands. Layer IV is acellular and contains dense fibers (lamina dissecans). Layer V consists of large pyramidal cells and layer VI is distinguishable from V only at the borders of the perirhinal cortex (Schultz and Engelhardt 2014).

1.4.2 Circuitry

The hippocampus receives afferents and spatial information from the entorhinal cortex through two excitatory pathways called "perforant pathways". The direct perforant path originates from the pyramidal neurons of layer III of the entorhinal cortex, which project directly to the most distal apical dendrites of the pyramidal neurons of CA1. The indirect (or trisynaptic) perforant path originates from layer II of the entorhinal cortex, which projects to the granular layer of the dentate gyrus. The axons of the granular cells form the pathway of the muscoid fibers and project in the pyramidal cells of CA3, whose axons, constituting the pathway of the Schaffer collaterals, project to the apical dendrites of the pyramidal cells of CA1. These circuits are involved in the memory consolidation process.

1.5 Temporal lobe epilepsy (TLE)

In this paragraph the attention is focused on temporal lobe epilepsy (TLE), which is the most common, drug-resistant epilepsy in the adult (Blair 2012; Loscher et al. 2020). It is an acquired and focal form of epilepsy, hitting the temporal lobe of the brain. Indeed, it always starts from a localized area in one of the brain hemispheres, and then may spread to the other hemisphere, becoming secondarily generalized. Following an initial epileptogenic event, such as stroke, head trauma, febrile seizures or prolonged status epilepticus (SE), the brain of a healthy individual can undergo a series of neurobiological alterations leading to epilepsy (Pitkanen and Lukasiuk 2011). Indeed, it has been shown in several animal models that inducing SE is sufficient to lead the onset of TLE (Pitkanen 2010). According to ILAE classification, there are 2 main types of TLE: i) lateral TLE (lTLE), in which the onset of seizures is localized in the temporal neocortex; ii) mesial TLE (mTLE), related to hippocampus, parahippocampal gyrus and amygdala alterations. mTLE is more common

than lTLE (Allone et al. 2017). One of the most frequent symptoms associated with TLE is "*aura*", that includes fear, hallucinations, nausea and alteration of consciousness (Gupta et al. 1983). Many patients with TLE suffer from behavioural alterations, such as anxiety, depression, psychosis and deficits in learning and memory, due to the physiological and morphological changes that occur at the level of the limbic system. According to neuropathological research based on human surgical tissues and observation in numerous experimental models of TLE, hippocampal sclerosis (HS) is the most common histological alteration in drug-resistant TLE patients (Thom 2014). HS is primarily characterized by neuronal death. Other common alterations are changes in neuronal excitability and aberrant adaptive plastic modifications (mossy fibers sprouting, neurogenesis, gliosis, neuroinflammation and loss of blood brain barrier (BBB) integrity) (Curia et al. 2014). TLE is often resistant to anti-epileptic drugs (AEDs), especially in patients presenting HS (French 2007; Park et al. 2014).

1.5.1 TLE phisiophatology

mTLE must be considered as a heterogeneous disease but, in general, 3 phases can be identified:

- Acute phase, which follows the epileptogenic insult.
- Latency phase, whose duration is variable, during which the process called "epileptogenesis" takes place.
- Chronic phase, which begins with the onset of the first spontaneous seizure.

A first spontaneous seizure may appear after decades of an initial epileptogenic insult, a period known as *latency*. During this period several cellular, molecular and plastic alterations take place, leading to spontaneous recurrent seizures (SRSs) generation (Curia et al. 2014). The changes involved in the onset of spontaneous seizures are referred to a dynamic process known as "epileptogenesis". These changes include neurodegeneration, aberrant neurogenesis, gliosis, axonal damage, dendritic plasticity, and neuroinflammation, that can continue to progress even after the onset of spontaneous seizures and the diagnosis of epilepsy (Pitkanen and Lukasiuk 2011). Several studies in humans have reported that the latency period ranges from a few weeks to many years and that non-convulsive seizures always precede the first motor seizure. This has led to the hypothesis that humans can remain in the latent periods for many years, often in the presence of unrecognized subclinical seizures (Dudek and Staley 2012). Latency period measurements are difficult because they

require continuous recording from the initial brain insult to the onset of the first seizure. Thus, it can be seen as the initial stage of an ever-changing process.

1.6 Epilepsy comorbidities

The term "comorbidity" indicates the simultaneous presence of more than one pathological condition in the same person. Comorbidities in epilepsy are very common and have an impact on the diagnosis, treatment, cost and quality of life of the patient (Bazil 2004). Epilepsy comorbidities can be classified into:

- *Medical comorbidities*: musculoskeletal disorders and fractures, gastrointestinal disorders, respiratory problems, chronic pain, migraine, neoplasms, obesity, diabetes, infections, allergies.
- *Psychiatric comorbidities*: depression, anxiety, autistic disorders, psychosis.
- *Cognitive comorbidities*: attention deficit hyperactivity disorder (ADHD), learning disorders, dementia.

The relationship between comorbidity and epilepsy can be explained through several mechanisms (**fig. 6**):

- *Chance and artefactual comorbidities*: the prevalence or incidence of a comorbidity is as frequent in people with epilepsy as would be expected in the general population.
- *Causative mechanisms*: the comorbid condition arises first, which then gives rise to epilepsy.
- *Resultant mechanism*: epilepsy takes place first and subsequently gives rise to the comorbid condition.
- *Shared risk factors*: there is no causal relationship between comorbidity and epilepsy, but they share some underlying risk factors, such as genetics or environmental factors.
- *Bidirectional effects*: both conditions cause each other's.

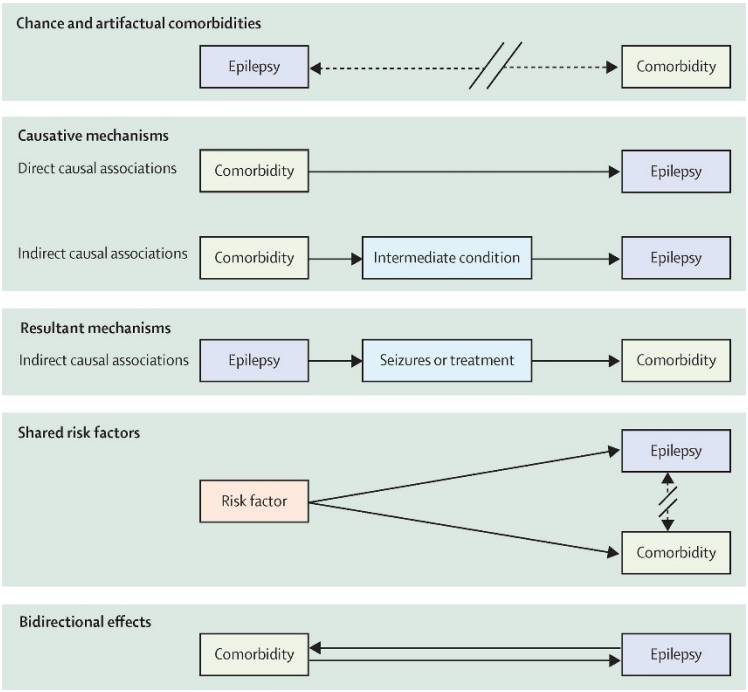


Fig. 6 Mechanisms of association between epilepsy and its comorbidities (Keezer, Sisodiya, and Sander 2016)

1.6.1 Depression

Depression is the most common comorbidity in patients with epilepsy. In 2013, a meta-analysis study reported that depression has an incidence of around 23% of epilepsy patients worldwide and prevalence of 50% among TLE patients (Fiest et al. 2013). Depression has a severe impact on quality of life (QoL) in patients with epilepsy. Indeed, common mechanisms underlying depression and epilepsy, as well as psychosocial limitations imposed by a seizure disorder (such as driving restrictions and/or unemployment) can lead to worsening of the depressive phenotype (Ehrlich et al. 2019). The brain regions involved in both depression and TLE are mainly the hippocampus, amygdala, entorhinal cortex, and neocortical and subcortical regions (Valente and Busatto Filho 2013). Sharing strongly intertwined common pathogenetic mechanisms, the structural and functional changes of one disease can trigger the other, leading to a two-way relationship between them. Several animal models have been useful to demonstrate that the relationship between depression and epilepsy, rather than a psychosocial phenomenon, has a shared neurobiological substrate. Models of TLE such as pilocarpine, kainic acid and kindling are also useful for the evaluation of depression.

The tests used to identify and quantify the main symptoms of depression in humans (anhedonia and despair) in animal models are: i) saccharin consumption test (SCT), which

assesses anhedonia through the consumption of a solution of saccharin by rats (lower consumption indicates anhedonia) (Pucilowski et al. 1993); ii) forced swimming test (FST), which is used to evaluate the symptom of "despair" in rat models; the animal is placed in the water (stressful situation) and the relative immobility time is evaluated (a longer immobility time is an indicator of the state of despair) (Pucilowski et al. 1993). Using these tests, long-lasting anhedonia was observed in mice using the kainate and pilocarpine models. Furthermore, anhedonia appears to be drug resistant, as neither imipramine nor fluoxetine improves the depressive phenotype (Mazarati et al. 2008). Rats subjected to lithium-pilocarpine-induced SE have a longer immobility time in the FST, if compared to controls. These animals also show an impairment in the raphe-hippocampus serotoninergic pathway. Administration of fluoxetine to post-SE rats do not lead to an improvement in the depressive phenotype, although it reverses the SE-induced increase in brain excitability (Klein et al. 2015). Similarly, in a clinical PET (positron emission tomography) study, a decrease in the binding capacity of serotonin at the 5-HT1A receptor in the mesial temporal region was observed in both depressed and epileptic patients (Savic et al. 2004). IL-1β also appears to be involved in both epilepsy and depression. In a cross-sectional study of depressed or non-depressed TLE patients, plasma levels of IL-1β were significantly higher in people with TLE with depression than in controls or in people with TLE without depression (Vieira et al. 2015). Bilateral intra-hippocampal infusion of hippocampal interleukin-1 receptor antagonist ameliorates all depressive symptoms (anhedonia and despair) that develop in rats following induction of status epilepticus (SE) with pilocarpine (Mazarati et al. 2010).

From a clinical point of view, it is important to make a careful diagnosis, as the symptoms of depression may present in the pre-ictal period, apparently with no correlation with the seizures, as well as before, during or after a seizure. Currently, the most commonly validated screening tool used in order to diagnose depression in epilepsy is the “Neurological Disorders Depression Inventory for Epilepsy” (NDDI-E) (Gill et al. 2017). Considering the high prevalence of depression among people with epilepsy (PWE), routine and periodic screening of all PWE and appropriate management of depression is recommended. Additionally, PWE should also be screened for suicidal ideation regularly, and patients should be referred for psychiatric evaluation and treatment if necessary (Mesraoua et al. 2020).

1.6.2 Anxiety

Although it has been described as the "forgotten" psychiatric comorbidity in PWE (Kanner 2011), anxiety disorders (AD) impact the QoL of PWE as much as depression. As with epilepsy, the amygdala and hippocampus play a key role in the neurobiology of anxiety. The amygdala regulates the experience of fear and is responsible for the avoidance behaviour associated with fear, while the hippocampus is associated with reliving fear. Ads are involved in fear circuit activation in these structures (Stahl 2003). For example, electrical stimulation of the amygdala of patients undergoing presurgical evaluation has been shown to induce emotional responses, including anxiety and fear (Lanteaume et al. 2007). In relation to the temporal occurrence to seizures, peri-ictal anxiety symptoms can be: preictal (preceding a seizure), ictal (during a seizure), and postictal (occurring within 72 h of a seizure). ADs may be also due to pharmacologic effects and/or surgical treatment. All types of symptoms can co-occur in the same individual (Hingray et al. 2019). The screening tool used for AD diagnosis is the "Generalized Anxiety Disorder 7" (GAD-7), which is similar to the NDDI-E.

1.6.3 Cognitive disorders

Cognitive disorders are frequent in epileptic patients, particularly in people affected by TLE. Studies on the correlation between epilepsy and cognitive disorders, date back to 1900. Cognitive disorders associated with epilepsy mainly affect memory, attention, language, executive functions and judgment (Zhao et al. 2014). Factors that might contribute to cognitive decline depend on the pathology underlying the epilepsy, the age of onset of the seizures, drug therapy or on other comorbidities. The involvement of the medial structures of the temporal lobe is responsible for deficits in operational memory, both verbal and visuospatial, as well as in executive functions: as already mentioned, the age of onset of seizures and their frequency are important predictors for the decline of these cognitive domains. Another important factor is the hemisphere involved: it seems that there is a lateralisation of the mnemonic processes: patients with left lobe TLE demonstrate reduced performances in verbal working memory, while patients with right lobe TLE show marked deficits in visual working memory. Another factor with a great impact on visuospatial working memory is HS, common in mesial forms of TLE. HS seems to be also involved in

the consolidation deficits of memory traces: the hippocampus plays a crucial role in long-term memory. Therefore, patients with mTLE often have long-term memory deficits, especially episodic ones. Finally, several studies have highlighted that TLE patients have deficits involving executive functions and also language, especially if the dominant hemisphere is involved, as well as deficits in other cognitive domains such as facial recognition and the "theory of mind" (Zhao et al. 2014).

1.7 Experimental modelS of TLE

In order to understand the physio-pathological mechanisms underlying epilepsy and to develop new therapeutic approaches, animal models of the disease that mimic the electro-encephalographic, behavioural and neuropathological characteristics of human epilepsy are essentials. There is, currently, no model that possesses all the features of TLE. Therefore, the choice of the model that best suits the needs of the study represents the first important step. An ideal model is defined “homologous” as it is able to reproduce the human pathology in every aspect; other models are defined “isomorphic” when they reproduce clinical-pathological aspects but not the aetiology or do not allow predictions. The identification of the best experimental model of SE is crucial for researchers who aim to study the mechanisms underlying neuronal death and synaptic reorganization during epileptogenesis, the development of chronic epilepsy and the possibility of identifying more effective AEDs with fewer side effects. For this reason, an ideal epilepsy model should be able to reproduce all the physiopathologic features of human TLE such as the presence of the latency period, epileptogenesis and its neuropathologic characteristics (neuronal death, aberrant neurogenesis, astrogliosis and mossy fibers sprouting). Moreover, epileptic animals should be resistant to AEDs and display comorbidities just like in human TLE.

That having said, acute seizure models are also important. Even if they cannot mimic epilepsy, acute seizure models, for example, the maximal electroshock test (MES), can be very useful for evaluating the anticonvulsant activity of new drugs. Being very rapid tests, they can be used to screen anti-seizure drugs.

In sum, the various animal models can be divided into different categories: models with single seizures or recurrent seizures (i.e., acute or chronic models), screening models, mechanism related models, models with spontaneous seizures versus chemically or electrically induced seizures (Loscher 2011).

Based on the stimulus used in order to induce seizure, models can be classified in:

• *Chemical models*: chemoconvulsants, such as kainic acid and pilocarpine, are used to induce SE which, if maintained for a certain period, can generate the neuronal damage that may represent the initial epileptogenic insult and lead to SRSs in a fashion similar to TLE.
• *Electrical models*: based on the application of electrical stimuli in specific regions of the limbic system.
• *Genetic models*: which involve the manipulation of the animal's genetic material to determine the appearance of spontaneous seizures.
Here, I will focus on the chemical models that I used during my PhD period (pilocarpine and lithium-pilocarpine).

1.7.1 Chemical models

Chemical models can mimic different clinical seizure types and acute or chronic epileptic phenomena (**Table 1**; De Deyn et al. 1992).

Chemical model of epilepsy	**GABA**	Pentylentetrazole
		Bicuculline
		Picrotoxin
		Glutamic Acid Decarboxylase (GAD) inhibitors
		Beta carbolines and convulsant benzodiazepine Ro 5-3663
		GHB (gamma-hydroxy-butyrate)
	Excitatory Amino-Acid	Kainic acid
		Quisqualic acid/alfa-amino-3Hydroxy-5-Methyl-4-Isoxasole Propionic acid
		N-Methyl-D-Aspartic acid (NMDA)
		Homocysteine, homocysteic acid
	Acetylcholine related substances	Pilocarpine and litium-pilocarpine
	Other drugs	Strycnine
		Aminophylline
		Insulin induced hypoglycemia
		Ay-9944
	Inhalants	Fluorothyl
	Topical application	Metals (cobalt, zinc, antimony, aluminia cream, iron
		Antibiotic (penicillins and cephalosporins)
		Tetanus toxin

Table 1 Chemical models of epilepsy (De Deyn et al. 1992)

PILOCARPINE MODEL

Pilocarpine is an alkaloid that produces a powerful cholinergic effect by binding muscarinic receptors M1, M2 and M3. The M1 receptor is located at the level of the CNS; it is coupled to the Gq protein, which activates phospholipase C and increases the production of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The result is an increased level of intracellular Ca^{2+} and K^{+} and the release of the excitatory neurotransmitter from the presynaptic terminal (Segal 1988). Seizures are subsequently maintained by activation of NMDA receptors. M2 and M3 receptors are mainly present at the peripheral level: M2 activation causes a decrease in heart rate, while the activation of M3 causes increased secretions and relaxation of the intestinal muscles.

The pilocarpine model has been described for the first time in 1983 by Turski and colleagues (Turski et al. 1983). Indeed, using high doses (100-400 mg/kg) intraperitoneally (i.p.) administered in rats, pilocarpine led to behavioural alterations and the first signs, such as oral-facial movements, salivation, blinking, yawning appeared. Within 30-90-150 minutes after pilocarpine administration, the first discontinuous seizures appeared, followed by

motor seizures with salivation, lifting of the forelimbs and falling, every 5-15 (SE). Moreover, limbic electrographic alterations and widespread brain damage (24h after SE induction) have been observed. The pilocarpine model is, now, widely used and has been modified in numerous laboratories. Depending on the study needs, researchers use different procedures regarding the animal strain and species, pilocarpine dose, the duration of SE and the administration of drugs which are able to stop it. Generally, systemic administration in rodents of pilocarpine (340-380 mg/kg) leads to: behavioural and EEG changes, which can be divided into an "acute" period which includes limbic SE (inhibited, at specific time points, with benzodiazepines), a "silent" or "latency" period (4-44 days) and a "chronic" period characterized by humanlike SRSs (Cavalheiro 1995). Usually, SE is reached by 60% of the animals and disappears spontaneously after 5-6 h. Weight loss is frequent (about 20% of cases) and the mortality rate is around 30%. The duration of the latency period varies depending on the protocol used. In rats Wistar it lasts from 1 to 6 weeks. The pilocarpine dose does not appear to significantly affect the onset of SRS. On the other hand, both the duration of the SE and the drugs used to block it may affect the duration of latency (Curia et al. 2008). It has been demonstrated that 30–60 min of SE is sufficient to induce hippocampal damage and epilepsy if SE is blocked only with a single administration of diazepam (Cavalheiro 1995). However, early inhibition of SE may either reduce the intensity or delay the onset of SRSs (Sharma et al. 2007). Methyl-scopolamine can be administered to prevent the peripheral effects induced by pilocarpine (piloerection, salivation, diarrhoea, tremor) due to the interaction with the M2 and M3 receptors. Its methylated form has the advantage of not crossing the BBB, exerting its effect only at the peripheral level and increasing the central bioavailability of pilocarpine (Clifford et al. 1987).

LITHIUM-PILOCARPINE MODEL

Lithium is a mood stabilizer used to treat bipolar disorders. Moreover, it can be used in the treatment of acute brain injuries (e.g., ischaemia) and chronic neurodegenerative diseases (Wada et al. 2005). In 1983, Honchar and colleagues (Honchar, Olney, and Sherman 1983) demonstrated for the first time that the administration of lithium (127 mg/kg) 24 hours before pilocarpine is able to reduce the seizure threshold, allowing the induction of SE with a 10-fold lower dose of pilocarpine (30 mg/kg). This drug combination result in a higher percentage of rats developing SE, but not an increase in mortality (Clifford et al. 1987; Honchar, Olney, and Sherman 1983). SE and neuronal damage from this model are similar compared to the administration of high dose pilocarpine alone (Clifford et al. 1987). Through

the use of MRI, it was observed that the first changes in the brains of rats treated with lithium-pilocarpine were detected in EC, thalamus and amygdala 6 hours after SE, while the hippocampus appeared to be affected only 36-48 hours later (Curia et al. 2008). It appears that lithium pre-treatment is effective only when pilocarpine is administered within 24 h (Clifford et al. 1987).

It has been shown that the administration of diazepam alone (10 mg/kg, i.p.) to block SE is not sufficient, if the duration of SE is longer than 30 minutes: the severity of behavioural seizures is reduced but SE is not completely blocked (electrical seizures are not stopped by diazepam alone). The combination of two anticonvulsants, diazepam and phenobarbital, is much more effective in blocking SE than diazepam alone and the use of a muscarinic antagonist (scopolamine) favours the anticonvulsant activity of the first two drugs by blocking the activity of pilocarpine present on the muscarinic receptors in the brain (Brandt et al. 2015).

Although the mechanisms underlying the potentiation of the pilocarpine effect are still unclear, some studies show that lithium may be able to activate T lymphocytes and monocytes. This induces the production of inflammatory cytokines, such as IL-1β, which lead to altered permeability of the BBB, leading to a decrease in the epileptogenic threshold (Nirwan, Vyas, and Vohora 2018). Another hypothesis is that lithium induces an alteration in the binding sites of muscarinic receptors (Gibbons et al. 2016) and an increase in the release of acetylcholine in the hippocampus, with consequent hyperactivation of M1 receptors (Hillert et al. 2014). Furthermore, lithium leads to an increase in IP3 (more dramatic than that induced by pilocarpine) (Honchar, Olney, and Sherman 1983). IP3 increases intracellular Ca^{2+}, which promotes the exocytosis of acetylcholine at the pre-synaptic level, activation of post-synaptic M1 receptors, oxidative stress and cell damage.

CHAPTER 2. EPILEPTOGENESIS

The term epileptogenesis is associated with acquired epilepsy more than genetic ones. Traditionally, epileptogenesis was represented by the latency period, specifically the period between the first insult and the first spontaneous seizure. Initially, research focused mostly on the neuronal mechanisms responsible for generating epileptic seizures (ictogenesis), rather than the causative processes of the initial brain damage that lead to chronic epilepsy. Indeed, experimental models able to induce acute seizures, like kindling, were largely used (Goddard 1967). The kindling model, however, is not a model capable of reproducing chronic epilepsy, because animals have seizures when stimulated but do not develop SRSs (Stafstrom and Sutula 2005). The creation of animal models in which epilepsy develops over time after an induced SE led to a redefinition of the term epileptogenesis. Several studies have shown that epileptogenesis is characterized by morphological and functional modifications, which do not stop at the onset of the first spontaneous seizure, but contribute to the progression of the epileptic condition (**fig. 7**) (Pitkanen et al. 2015).

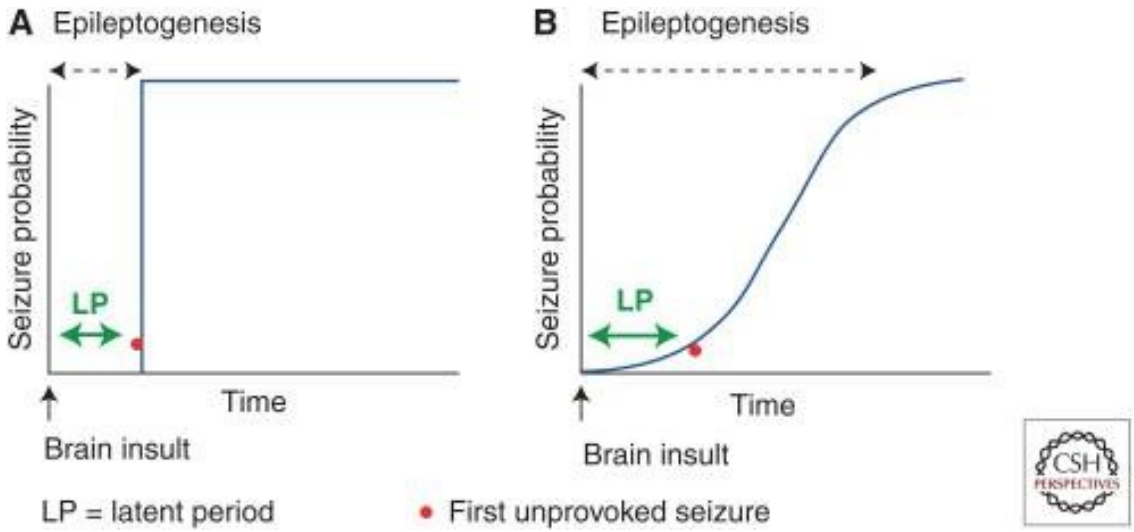


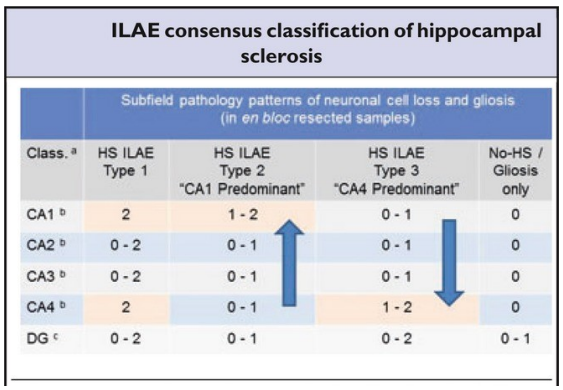
Fig. 7 Definitions of epileptogenesis (Pitkanen et al. 2015).

This evolution in the concept of epileptogenesis is coherent with the clinical evidence that human epilepsy has a progressive course. The new concept is also relevant from a therapeutic point of view, as epileptogenesis can be a potential target of treatment even after the onset of SRSs. Consequently, antiepileptogenic interventions could not only prevent disease onset but also be disease-modifying treatments after diagnosis.

2.1 PatHological hallmarks of epileptogenesis

NEURODEGENERATION

The term "neurodegeneration" refers to the complex alterations in neuronal functions that progressively lead to neuronal death. Recently (**fig. 8**), ILAE has proposed a classification system of semiquantitative models of hippocampal cell loss: i) HS ILAE type 1: severe neuronal cell loss and gliosis in CA1 and CA4; ii) HS ILAE type 2: loss of neuronal cells in CA1 and gliosis; iii) HS ILAE type 3: loss of neuronal cells in CA4 and gliosis. Surgical hippocampal specimens obtained from TLE patients may also show normal neuron content with reactive gliosis only (no-HS) (Blumcke et al. 2013).



ILAE consensus classification of hippocampal sclerosis

	Subfield pathology patterns of neuronal cell loss and gliosis (in *en bloc* resected samples)			
Class. [a]	HS ILAE Type 1	HS ILAE Type 2 "CA1 Predominant"	HS ILAE Type 3 "CA4 Predominant"	No-HS / Gliosis only
CA1 [b]	2	1 - 2	0 - 1	0
CA2 [b]	0 - 2	0 - 1	0 - 1	0
CA3 [b]	0 - 2	0 - 1	0 - 1	0
CA4 [b]	2	0 - 1	1 - 2	0
DG [c]	0 - 2	0 - 1	0 - 2	0 - 1

Fig. 8 ILAE consensus classification of hippocampal sclerosis

The use of animal models of acquired epilepsy allows researchers to better understand which are the neurodegenerative pathways that can contribute to excitotoxicity and cell death. In animal models of TLE neuronal loss is mainly observed in the hilus of DG, in CA1 and CA3 pyramidal cells and GABAergic interneurons. CA2 appears to be less affected (Pitkanen and Sutula 2002). The extra-hippocampal areas involved are the amygdala, the entorhinal cortex and the subiculum, although little attention has been given to brain areas other than the hippocampus in preclinical research (Arellano et al. 2004). Neurodegeneration leads to impaired function of the neuronal network and can contribute to the process leading to epilepsy and associated comorbidities. Preclinical and clinical studies have shown that HS and cell death are not always associated with epileptogenesis and TLE, just as the reduction of neurodegeneration does not always prevent the development of SRSs. Hence, it is still

unclear which interventions will be helpful in preventing epileptogenesis in patients (Naegele 2007).

ABERRANT NEUROGENESIS

Neurogenesis occurs mainly in the embryonic stages in the mammalian CNS, but persists throughout adulthood (adult neurogenesis), in the Subgranular Zone (SGZ) of the hippocampal DG and the Subventricular Zone (SVZ) of the forebrain lateral ventricles. Adult neurogenesis is implicated in a variety of diseases of the CNS, including epilepsy (Chen, Wang, and Chen 2020). After pilocarpine-induced SE in rats, dentate gyrus cell proliferation increases 5–10 fold after a latent period of several days and persists for several weeks (Parent et al. 1997). Using epileptic mice in which newly born hippocampal dentate granule cells (DGC) were labelled with GFP, seizure frequency was observed to increase in animals in which the percentage of neonate DGC was higher, confirming the hypothesis that abnormal DGC promotes epileptogenesis (Hester and Danzer 2013). The use of cytosine-b-D-arabinofuranoside after SE induction in rats led to a reduction in the number of abnormal granule cells, and also to a reduction in the frequency of seizures (Jung et al. 2004). Moreover, it has been shown that seizures of different severity, cause different alterations in neurogenesis (Uemori, Toda, and Seki 2017).

DENDRITIC PLASTICITY AND CHANGES IN EXTRACELLULAR MATRIX

The extracellular matrix (ECM) has an important role in regulating use-dependent synaptic plasticity. ECM molecules surround cell bodies and proximal dendrites of neurons, forming perineuronal nets (PN). These nets are composed of molecules like hyaluronic acid (HA), chondroitin sulphate, proteoglycans of the aggrecan family, tenascin-R and link proteins. The ECM appears to contribute to synaptic plasticity during the period of epileptogenesis, as mature ECM can inhibit activity-dependent plasticity. However, injury to adult tissue (for example SE) can reactivate the mechanisms that operate during development. SE has been shown to lead to increased HA levels, aberrant lectican expression, and decreased PN support structures. An increase in unbound HA in the ECM may make the system more susceptible to increased neurite outgrowth and synaptic plasticity after SE (McRae and Porter 2012).

MOSSY FIBERS SPROUTING (MFS)

MF arises from dentate granule cells, located in the dentate gyrus, and project their axons through CA4 and to dendrites of CA3 pyramidal cells. CA3 projects Schaffer collaterals to CA1 which, in turn, sends projections to the EC through the subiculum (**fig. 9**; Wiera and Mozrzymas 2015).

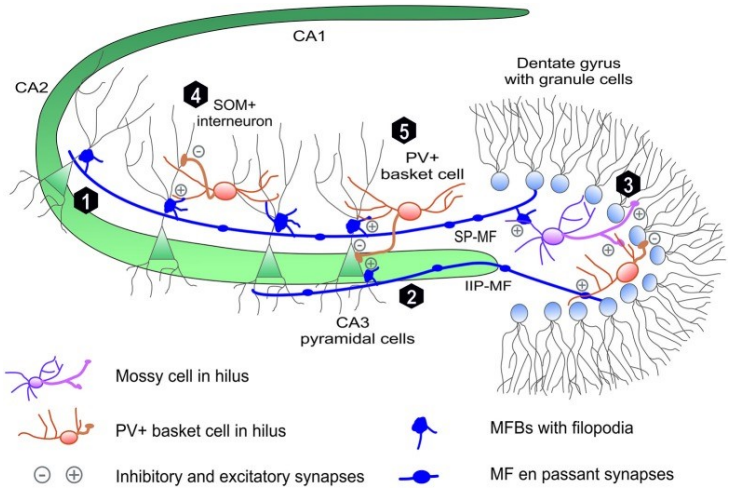


Fig. 9 MFs projection in hippocampal formation (Wiera and Mozrzymas 2015)

In CA3, there are 2 projections of MF: one is suprapyaramidal (stratum lucidum) and the other one is infrapyramidal (IIP). IIP axons are involved in hippocampus-dependent learning (Wiera and Mozrzymas 2015). In 1979, Messenheimer and Steward suggested for the first time a correlation between lesion-induced sprouting with epilepsy (Messenheimer, Harris, and Steward 1979). However, it was not yet clear if the sprouting was a consequence of neurotoxic damage, of SE, or of both factors. The first real evidence that axonal sprouting was seizure-correlated came from the observation in kindled rats, in which axons of mossy fibers labelled by Timm histochemistry were found to expand their terminal field to the supragranular region of the dentate gyrus, in the absence of cell damage (Cavazos, Golarai, and Sutula 1991; Represa, Le Gall La Salle, and Ben-Ari 1989). To date, MFS has been observed in many animal models of chronic epilepsy, as well as in the human epileptic temporal lobe (Sutula 2002).

NEUROINFLAMMATION AND GLIOSIS

Neuroinflammation occurs following the release of proinflammatory molecules in a context of innate immunity activation. Astrocytes provide structural support to neurons, but also participate in the formation and maturation of synapses, receptor trafficking, control of ion homeostasis, clearance of neurotransmitters, regulation of the extracellular volume and modulation of synaptic plasticity (Curia et al. 2014). Astrogliosis, that occurs throughout the temporal lobe in both human and animal models of TLE, has led in recent decades an increase in the number of studies aimed at understanding the role of astrocytes in this pathology. Acute seizures and neuronal damage are able to activate astrocytes that establish a state of reactive gliosis, characterized by hypertrophy of primary processes, an increase in the expression of intermediate filament proteins, such as the glial acid fibrillar protein (GFAP) , and a reduction in glutamine synthetase (GS) expression (Wilcox et al. 2015). This process is most evident in HS often associated with mTLE. When gliosis is moderate, there is no proliferation of new astrocytes and a regression often occurs with the resolution of the initial insult; more severe forms of gliosis, on the other hand, lead to an increase in astrocyte proliferation and the formation of a scar that derives from a close interaction between astrocytes and surrounding cells. These changes persist even after the end of the initial insult (Dossi, Vasile, and Rouach 2018). Astrocytes play also an important role in maintaining the balance between excitation and inhibition, removing glutamate and GABA from the synaptic cleft via the glutamate transporters GLAST and GLT-1 and the GAT3 GABA transporter, respectively. Preclinical studies have shown that in animal models of epilepsy there is a downregulation of GLT-1 after the induction of SE (Clarkson et al. 2020). It has been observed that mTLE patients have five times higher extracellular glutamate levels in the epileptic sclerotic hippocampus than in non-HS and non-epileptic hippocampal formation (Cavus et al. 2008).

Microglia make up the main component of the immune system of the CNS. In an animal model of TLE, inhibition of microglia with minocycline administration for two weeks led to a reduction in the frequency, duration and severity of spontaneous seizures in animals (Abraham et al. 2012; Wang et al. 2015).

Finally, animal models of epileptogenesis have made it possible to identify the activation of toll-like receptors (TLRs) as a possible cause of neuroinflammation. These receptors can be activated by endogenous ligands, such as the High Mobility Box 1 (HMGB1) group, released by brain cells and leukocytes following tissue damage (Vezzani et al. 2011). Moreover, the

increase in the production of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, has an impact on epileptogenesis by increasing neuronal excitability (Vezzani et al. 2013).

CHAPTER 3. BDNF

3.1 Neurotrophins (NTs)

NTs belong to the family of neurotrophic factors (NTFs), which are endogenous proteins that play an important role in neuronal proliferation, survival and differentiation. Furthermore, they are involved in both excitatory and inhibitory synaptic modulation (Simonato 2018). In the adult CNS, NTs also trigger fast synaptic responses and cause changes in the function of synapses and in the morphology of neurites, influencing behaviour, learning, memory formation and cognition. The first neurotrophin, the nerve growth factor (NGF), was identified and isolated by Rita Levi Montalcini and colleagues in the 1950s (Cohen, Levi-Montalcini, and Hamburger 1954). At the time it was a revolutionary discovery, both considering the limited tools available and the concept that a protein released from one cell could control the differentiation of neighbouring cells (Bothwell 2014). In the next decades, other members of the NTs family were identified: brain derived neurotrophic factor (BDNF) (Barde, Edgar, and Thoenen 1982), neurotrophin-3 (NT3) (Rosenthal et al. 1990) and neurotrophin-4/5 (NT4/5) (Hallbook, Ibanez, and Persson 1991).

NTs STRUCTURE

The 4 NTs are structurally very similar. The NTs precursors (or pro-neurotrophins) are cleaved intracellularly by furin or pro-convertases to produce the mature proteins. The mature form of the protein is formed by two monomers linked by non-covalent chemical bonds to form homodimers (about 120 amino acids for each peptide chain). Highly conserved pairs of cysteine residues are present within each monomer, forming a "cysteine knot" pattern, which stabilizes the proteins. The domain in which NTs differ is within the peptide loops that protrude from the nucleus of the molecules. These loops mediate the contacts between neurotrophins and their specific receptors on the cell surface (Dechant and Neumann 2002).

Neurotrophins have different biological functions, dictated by the selective binding to 4 receptors: the p75 neurotrophin receptor ($p75^{NTR}$), belonging to the tumour necrosis factor (TNF) receptor family, and the tyrosine kinase (Trk) receptors A, B and C. Specifically, all 4 neurotrophins (both in the "pro" and mature form) bind and activate $p75^{NTR}$. Regarding Trk receptors, NGF preferentially binds and activates TrkA, NT3 preferentially binds and activates TrkC, and BDNF and NT4/5 preferentially bind and activate TrkB. Although these interactions have been considered to be of high affinity, in reality the situation appears to be more complex than that. Indeed, the NT-Trk binding can be regulated by various factors, such as structural modifications or associations with the $p75^{NTR}$ receptor. Both the affinity of the receptor and the specificity for NTs can depend on the association with $p75^{NTR}$. Furthermore, both receptors (Trk and $p75^{NTR}$) independently activate downstream signals that contribute to the specific physiological responses (Chao 2003) (**fig. 10**).

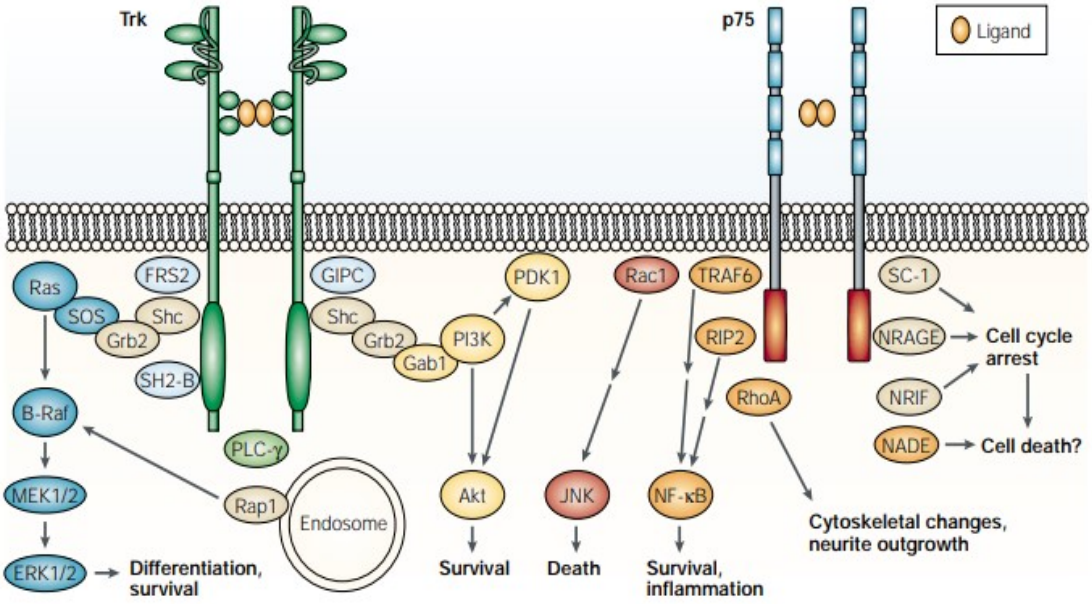


Fig. 10 ***NTs receptor signalling from*** (Chao 2003): Trk receptors mediate differentiation and survival signalling through extracellular signal regulated kinase (ERK), phosphatidylinositol-3-OH kinase (PI3K) and phospholipase C-γ1 (PLC-γ1) pathways. Trk family members recruit and increase the phosphorylation of PLC-γ1 and Src homologous and collagen-like adaptor protein (Shc), which leads to the activation of PI3K and ERK. Rap1 exerts its actions from an endosomal location. The p75 receptor predominantly signals to activate NF-kb and Jun N-terminal kinase (JNK) and modulates RhoA activity. These responses are mediated through adaptor proteins that bind the cytoplasmatic domain of p75, including neurotrophin-receptor interacting factor (NRIF), neurotrophin-associated death executor (NADE), neurotrophin-receptor-interacting MAGE

homologue (NRAGE), Schwann cells 1 (SC1) and receptor-interacting protein 2 (RIP 2), which can exsert actions on apoptosis, survival, neurite elongation and growth arrest. Akt, protein kinase B; FRS2, fibroblast growth factor receptor substrate 2; Gab-1, Grb2-associated binder-1; Grb2, growth factor receptor- bound protein 2; GIPC, GAIP interacting protein, C-terminus; MEK, mitogen-activated protein kinase (MAPK)/ERK kinase; PDK1, phosphoinositide-dependent kinase 1; SH2B, Src homology 2-B; SOS, Son of Sevenless; TRAF6, tumour necrosis factor receptor-associated factor 6.

Trk receptors consist of five extracellular domains (2 cysteine-rich regions, 1 leucine-rich region, and 2 immunoglobulin-like domains) a transmembrane region, and the intracellular kinase domain (Chao 2003). There are 10 conserved tyrosines in the cytoplasmic domain of each Trk receptor. Phosphorylation of these residues promotes signalling by activating intracellular signalling cascades, including the Ras/ERK protein kinase pathway, the PI3K/Akt kinase pathway and PLC-γ1.

In addition, the interaction of neurotrophins with their receptors can be influenced by the splicing variants of Trk receptors. It has been observed that truncated forms of the TrkB and TrkC receptors lack the tyrosine kinase domain and are thought to act as dominant negative modulators of Trk signalling, counteracting their full-length counterparts (Bucci, Alifano, and Cogli 2014).

$p75^{NTR}$, unlike Trk receptors, has no intrinsic catalytic activity, but it works with Trk receptors and with other non-neurotrophic receptors (i.e., sortilin), affecting a wide range of cellular functions. When $p75^{NTR}$ is complexed with Trk receptors, it enhances its affinity for the mature neurotrophins and boosts pro-survival and pro-growth signalling, while pro-neurotrophins bind with higher affinity to the $p75^{NTR}$/sortilin complex, which leads to the activation of apoptotic pathways and death (Meeker and Williams 2015). In fact, it was observed in vitro that the activation of $p75^{NTR}$ by pro-neurotrophins induces a pro-apoptotic effect on neurons, while the cleaved proteins promotes recovery through Trk receptors (Lee et al. 2001).

NTs FUNCTIONS

Based on the above findings, it is not possible to generalize the function of NTs. The question is much more complex, as NTs can mediate different and, in some cases, opposite cellular responses, based on the different patterns of receptor activation (Bothwell 2014). Not surprisingly, the implication of NTs in epilepsy is also very controversial: on the one hand, several studies have shown their preventive effect in the development of spontaneous

seizures and their ability to reduce cognitive decline caused by neuronal loss; on the other hand, they seem to have a crucial role in prompting epileptogenesis. Indeed, it appears that a single NT can have opposite effects depending on the conditions in which it is expressed. For example, BDNF, the most studied NT, seems to support the generation of epileptic seizures after the induction of the SE but, on the other hand, it may have a neuroprotective effect (Simonato, Tongiorgi, and Kokaia 2006).

3.2 BDNF

BDNF modulates the survival and differentiation of specific cell populations during development, but it is also involved in many functions in adulthood, including neuronal homeostasis and the processes related to neuronal plasticity involved in memory and learning. In addition, it also acts by preventing neuronal apoptosis through the expression of Bcl-2, an anti-apoptotic protein that inhibits both caspases and pro-apoptotic proteins such as Bax and Bad (Marosi and Mattson 2014). The human BDNF gene (**fig. 11**) is located on chromosome 11 and it spans ~70 kb. It consists of eight exons 5' (exons I-VIII), with each respective promoter, and an exon 3' (exon IX) which encodes the BDNF protein in both humans and rodents (Liu et al. 2006; Pruunsild et al. 2007). Moreover, two more human exons have been recently identified: Vh and VIIIh, with and without specific promoters respectively (Pruunsild et al. 2007).

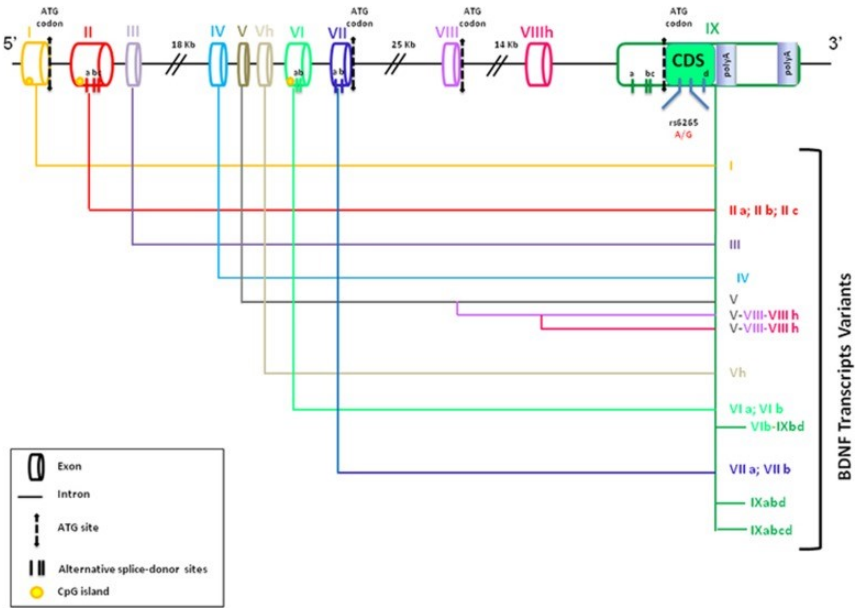


Fig. 11. Human BDNF gene structure (Cattaneo et al. 2016).

BDNF is synthesized in the endoplasmic reticulum as pre-pro-BDNF, and then cleaved to produce pro-BDNF (~32 kDa). The latter is transported to the Golgi complex, where it is cleaved intracellularly to generate mature BDNF (mBDNF) (~13 kDa) or secreted as pro-BDNF (which is also biologically active), and then cleaved extracellularly to generate mBDNF. The pro-BDNF/mBDNF ratio varies during development stages; in the neonatal and adolescent phase, both pro-BDNF and mBDNF are present, while in adulthood mBDNF prevails (Cattaneo et al. 2016).

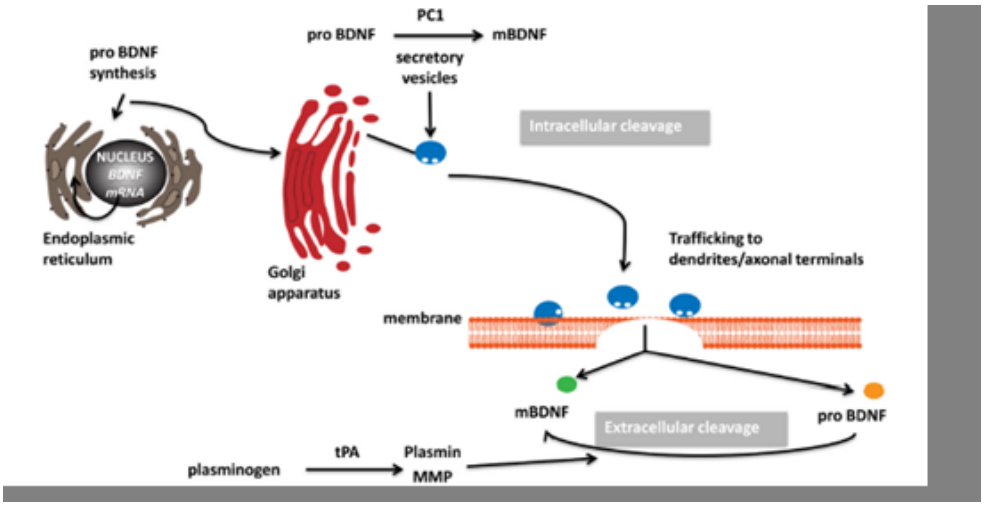


Fig. 12. Mechanism of production and release of pro-BDNF and mBDNF (Marosi and Mattson 2014)

mBDNF binds with high affinity to TrkB receptors, which are synthesized in the neuronal cell body, sorted into vesicles and anterogradely transported to the axon terminals by kinesin 1 (Segal 2003). The binding of BDNF to the high affinity receptor TrkB leads to autophosphorylation of tyrosines in the cytoplasmic domain. Phosphorylation of the tyrosine residue Y816 promotes the association of PLCγ1 and signalling mediated by IP3 and DAG. Instead, the Y515 residue promotes the association of TrkB with the Shc adapter protein, and activation of the PI3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK) signalling pathways (Reichardt 2006). Specifically:

- The phosphorylation of the Tyr816 tyrosine domain determines the recruitment and activation of PLCγ1 pathway, which leads to the production of DAG and IP3. DAG activates protein kinase C (PKC) while IP3 causes an intracellular calcium release. As a consequence, there is an increase in the activity of calmodulin kinase (CamK) and of the factor cAMP-response element binding protein (CREB) which, in addition

to promoting neuronal survival and neuroplasticity, is a key factor in regulating the expression of BDNF itself (Wu et al. 2014).

- The phosphorylation of the intracellular domain Tyr515 recruits the Shc protein, which interacts with the Grb2 adapter protein. Together, these proteins activate the SOS factor. SOS determines the activation of Ras and therefore of the MEK-MAPK/Erk pathway, which regulates the synthesis of proteins involved in synaptic plasticity and the activation of transcription factors such as CREB (Yoshii and Constantine-Paton 2010).
- The recruitment of Shc also allows the activation of PI3K, which modifies the composition of phospholipids in the membrane: this involves the activation of Akt, which is involved in cell survival. It also determines the activation of the mTOR complex, an important regulator of protein synthesis. This signalling pathway is involved in the long-term maintenance of synaptic plasticity in regulating the trafficking of synaptic proteins (Yoshii and Constantine-Paton 2010).

BDNF mRNA in rats, as well as in humans, is expressed during development and in a site-specific manner. In adults, it is expressed throughout the brain, but especially in the hippocampus. The structure is similar to that in humans: the various BDNF transcripts have the coding region and 3' in common, while they differ in the 5' region. All the exons identified in humans are also expressed in rats, with the exception of exons VIIB and VIII. Within the hippocampus, granule cells, pyramidal cells, and some hilar GABAergic neurons express mRNA for BDNF and TrkB. There is evidence that BDNF is anterogradely transported into the brain (Conner et al. 1997).

BDNF RELEASE

Several studies suggest that neurons target NTs to the regulated secretory pathway for release at presynaptic nerve terminals. As an example, hippocampal neurons infected with a herpes simplex virus vector expressing BDNF release the protein following depolarization (Goodman et al. 1996). Despite this evidence, however, the molecular basis of NTs release from neurons is poorly understood.

Released BDNF has been shown to promote GABAergic maturation: long-term treatment with BDNF of neuronal cultures facilitated high-K^+-induced GABA release, upregulated the expression of glutamic acid decarboxylase (GAD) and $GABA_A$ receptors and enlarged the soma of GABAergic neurons (Yamada et al. 2002). It has been shown that BDNF colocalizes with the calcium-dependent activator protein for secretion 2 (CAPS2), both synaptically and

extrasynaptically, in axons of hippocampal neurons (Sadakata et al. 2004; Shinoda et al. 2011). Together with CAPS1, CAPS2 belongs to the CAP family proteins. These are essential components of the synaptic vesicle priming machinery. CAPS2 is vesicle priming proteins and contains a sequence stretch with homology to the priming domain of Munc13s. The regulation of this priming process controls the strength and plasticity of synaptic transmission between neurons, which in turn determines many complex brain functions (Jockusch et al. 2007). In a study conducted on CAPS2 knock-out (KO) mice, it has been shown that, while the excitatory neurons remain unaltered in terms of morphology and excitatory postsynaptic potential, the secretion of BDNF is reduced and the GABAergic systems are compromised. Overexpression of exogenous CAPS2 in hippocampal neurons of CAPS2-KO mice enhanced BDNF exocytosis (Shinoda et al. 2011). Moreover, it has been found that CAPS2 with deletion of exon3 (dex3) is overrepresented in some patients with autism. A severe impairment in axonal Caps2-dex3 localization has been observed in Caps2-dex3 mice (*Caps2* $^{dex3/dex3}$mice, created using a Cre/loxP system to generate a mouse line carrying a deletion of exon 3 of the Caps2 gene), contributing to a reduction in BDNF release from axons (Sadakata et al. 2012).

3.2.1 BDNF and epilepsy

Considering the many effects that BDNF is capable of exerting, it is not surprising that studies on its contribution to epilepsy led to conflicting results. If, on the one hand, BDNF may be a causal mediator of the epileptogenic processes (“demon”), on the other hand, its trophic properties could be used to prevent the development of seizures in syndromes such as TLE and to reduce the cognitive decline associated with progressive neuronal loss (“angel”) (Simonato, Tongiorgi, and Kokaia 2006). Starting from the "bad side", there are several reports supporting the notion that BDNF might aggravate epilepsy (proepileptogenic effects). First of all, acute seizures increase BDNF expression in neurons (Nawa, Carnahan, and Gall 1995; Simonato et al. 1998) and, as a consequence, enhance activation of TrkB (Binder, Routbort, and McNamara 1999). Moreover, examining the acute actions of BDNF in the rat dentate gyrus after pilocarpine-induced seizures, it has been shown that BDNF boosts excitatory synaptic transmission (Scharfman, Goodman, and Sollas 1999) and reduces inhibitory synaptic transmission (Tanaka, Saito, and Matsuki 1997). MF axons of dentate granule cells, which innervate dendrites of CA3 pyramidal cells, contain high levels of BDNF, which is further up regulated after seizures (Rudge et al. 1998). Application of

BDNF on hippocampal slices leads to increase in amplitude spikes evoked in the CA3 area and paired-pulse stimulation of the granule cell layer causes multiple population spikes in CA3, which are not observed in the absence of BDNF. Thus, when up-regulated in epileptic brains, BDNF may induce an abnormal enhancement of excitability of CA3 pyramidal cells (Scharfman 1997). In fact, transgenic over-expression of BDNF is sufficient to cause TLE in adult mice (Isgor et al. 2015).

In contrast with these findings, it has been demonstrated that BDNF may also exert antiepileptogenic effects. Indeed, infusion of BDNF delays the development of kindling in rats (Reibel et al. 2000) even if it is still unclear if this effect depends on hyperactivation or desensitization of the TrkB signalling pathway cause by the prolonged BDNF infusion (in the latter case, the finding would be consistent with a pro-epileptogenic effect). However, viral vector-mediated supplementation of BDNF attenuates epileptogenesis in the pilocarpine model (Paradiso et al. 2009). In addition, BDNF reduces the frequency of generalized seizures, improves cognitive performance, and reverts many histological alterations associated with chronic epilepsy (Falcicchia et al. 2018).

The conflicting effects of BDNF do not allow to reach a firm conclusion whether the inhibition or enhancement of BDNF signalling is needed for the prevention of TLE, and the question is still open. Noteworthy, cellular responses to BDNF diverge dramatically depending on how it is delivered. In cultured neurons, acute and gradual increases in BDNF elicited, respectively, transient and sustained activation of TrkB receptor and its downstream signalling, leading to differential expression of the immediate-early genes Homer1a and Arc. Transient TrkB activation promoted neurite elongation and spine head enlargement, whereas prolonged TrkB activation facilitated neurite branch and spine neck elongation. In hippocampal slices, fast and slow increases in BDNF enhanced basal synaptic transmission and LTP, respectively. Thus, the kinetics of TrkB activation seem critical for cell signalling and functions. This temporal dimension in cellular signalling may also have implications for a therapeutic drug design (Ji et al. 2010).

DOWNSTREAM SIGNALLING PATHWAY HYPOTHESIS

Recently, researchers have tried to understand more deeply the mechanisms behind these opposite effects. In 2005, Chen and colleagues generated transgenic mice with a modification in TrkB locus ($TrkB^{F616A}$) domain (Chen et al. 2005). This mutation makes mice sensitive to transient TrkB inhibition by a BBB-permeable small molecule, 1-(1,1-dimethylethyl) -3-(1-naphthalenylmethyl) -1H-pyrazolo[3,4-d] -pyrimidine-4-amine

(1NMPP1), while no such TrkB inhibition is observed in wild-type neurons. Using this approach, it has been shown that the treatment with 1NMPP1 for two weeks after SE prevents development of TLE and comorbid anxiety like-behaviour in mice (Liu et al. 2013). The same group showed that 1NMPP1 treatment of $TrkB^{F616A}$ mice produced three- to ten-fold increase in the number of degenerating (FJC-positive) cells in the CA3 and CA1 pyramidal cell layers of the hippocampus, as compared to vehicle treated controls (Gu et al. 2015). In sum, under experimental conditions in which a complete inhibition of TrkB kinase is obtained, the blockade of epileptogenesis associates with an exacerbation of SE-induced neuronal death. This prompted the hypothesis that the different signalling pathways downstream TrkB activation could mediate undesirable (epileptogenesis) and desirable (neuroprotective) consequences.

Thus, a novel strategy was implemented, aimed at a selective inhibition of diverse TrkB-activated signalling pathways. First, PLCγ1, the signalling effector that promotes excitability and, therefore, could be hypothesized to facilitate epilepsy development. The post-SE treatment with a novel peptide (pY816), that uncouples TrkB from PLCγ1, led to the inhibition of TLE and the prevention of anxiety-like disorder while preserving the neuroprotective effects of endogenous TrkB signalling (Gu et al. 2015). Second, and counterproof of the first, the Shc signalling, that is thought to play a key role in TrkB-induced neuroprotection. After disrupting TrkB-mediated Shc signalling, intra-amygdala kainic acid evokes similar SE in wild-type and $TrkB^{Shc/Shc}$ mice (in which phenylalanine is substituted for tyrosine at residue 515) but exacerbates hippocampal neuronal death in $TrkB^{Shc/Shc}$ mice (Huang et al. 2019).

PHARMACOLOGICAL PROFILE OF BDNF

Although BDNF could be a potential target for epilepsy treatment, the development of effective therapies has been hindered in large part by its inability to cross the BBB and reach the target sites of therapeutic action in a stable, controlled and continuous manner (Rubin and Staddon 1999). There are approaches for a direct and localized supplementation of BDNF, for example the use of micro-devices of engineered cells producing and secreting BDNF (Falcicchia et al. 2018) or the use of gene therapy delivery systems (Paradiso et al. 2009). Even if effective, these methods are invasive and cannot be proposed for use in individuals that are at risk, but not certain, to develop epilepsy (Bollen et al. 2013; Thoenen and Sendtner 2002).

CHAPTER 4. RESEARCH WORK

4.1 Aim of the thesis

A primary focus of epilepsy research is the identification of the mechanistic basis of the disease. Addressing the need to discover effective antiepileptic agents or disease-modifying therapies is a direct implication of this research focus. Post-SE animal models offer the opportunity to investigate epileptogenic changes and analyse factors responsible for disease progression, and thereby identifying and testing potential therapeutic targets for the secondary prevention of epilepsy (Clossen and Reddy 2017).

During my PhD I investigated possible novel therapeutic strategies to modulate epileptogenesis, in an attempt to prevent development of epilepsy and of its comorbidities. The principal aim of this work was to test the hypothesis that the antioxidant agent and TrkB agonist 7,8 dihydroxyflavone (7,8- DHF) could exert an antiepileptogenic action. Preclinical data show that this compound is well tolerated and effective in many neurological disorders (Emili et al. 2022). To pursue this goal, we employed the rat lithium-pilocarpine model. The efficacy of 7,8- DHF was evaluated both in terms of frequency and severity of seizures and on behaviour (through the use of tests for locomotion, anxiety and spatial memory). In addition, ex-vivo analysis was performed in order to understand the mechanisms underlying the effects of 7,8- DHF.

A second strategy was based on the Neuronal Regeneration Peptide 2945 (NRP2945). To test the hypothesis that NRP2945 may exert antiepileptogenic and/or anti-epileptic effect, we administered it: (i) following pilocarpine induced SE, to assess its ability to prevent epilepsy development (i.e., a putative anti-epileptogenic effect), and (ii) in the chronic phase of epilepsy, to assess its effect on spontaneous seizures.

4.2 7,8- DHF

The need to identify compounds with a better pharmacological profile, mimicking the biological actions of BDNF, led to the identification of flavonoid 7,8-dihydroxyflavone (7,8 DHF), a selective agonist of the TrkB receptor (Jang et al. 2010; Liu et al. 2010). Flavonoids are plant metabolites that are known to produce many favourable effects on human health. Indeed, they are known to have antioxidant properties, acting as scavengers of various reactive species (Pietta 2000). The catechol moiety in the B-ring is the main factor controlling the efficiency of O_2 physical quenching of flavonoids and the presence of a 3-hydroxyl determines the efficiency of their chemical reactivity with O_2. It seems that a carbonyl group at C-4 and a double bond between C-2 and C-3 are also important features for high antioxidant activity in flavonoids (Harborne and Williams 2000). Structurally, 7,8-DHF (**fig. 13**) is a flavonoid derivative, whose pharmacophore is a 1-4 benzopyrone (also called chromone) substituted with 2 hydroxyl groups in position -7 and -8 and a phenyl (ring B) in position 2.

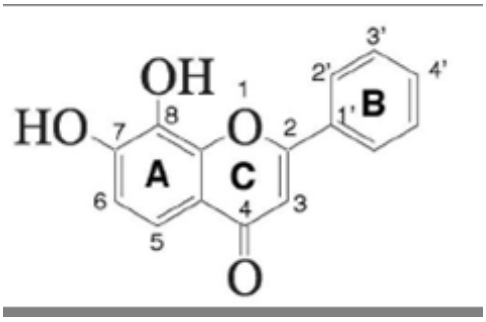


Fig. 13. 7,8- DHF structure (Liu et al. 2010)

The catechol group (ring A) (Liu, Chan, and Ye 2016) and the oxygen atom present in the C ring are fundamental for the purpose of binding to the trkB receptor. Ring B, on the other hand, can undergo modifications, without leading to loss of activity (Liu et al. 2010). 7,8-DHF is selective for the TrkB receptor (Kd ≈ 15.4 nM) (Liu et al. 2014), and does not seem to activate the apoptotic processes induced by binding to $p75^{NTR}$ (Wurzelmann, Romeika, and Sun 2017). The main advantages of using 7,8- DHF as a possible therapeutic approach are the small size of the molecule (27 kDa) compared to BDNF (254 Da), which allows it to easily cross the BBB and allows oral or intra-peritoneal administration (Wurzelmann, Romeika, and Sun 2017). Furthermore, compared to BDNF (whose half-life is about 10 minutes), 7,8- DHF has a much longer half-life (134 minutes), which leads to a more prolonged effect: in fact, the molecule can be identified in plasma up to eight hours after administration (Liu, Chan, and Ye 2016; Zhang et al. 2014). 7,8- DHF binds to the

extracellular domain (ECD) of the TrkB receptor, inducing its dimerization and auto-phosphorylation (Jang et al. 2010). The activation modality of the receptor is not yet clear, as the binding site is different from that of BDNF (Jang et al. 2010). The signalling pathways activated by 7,8- DHF (**fig.14**) are the same as those of BDNF. Once activated, however, the receptor internalization process is much slower than with BDNF.

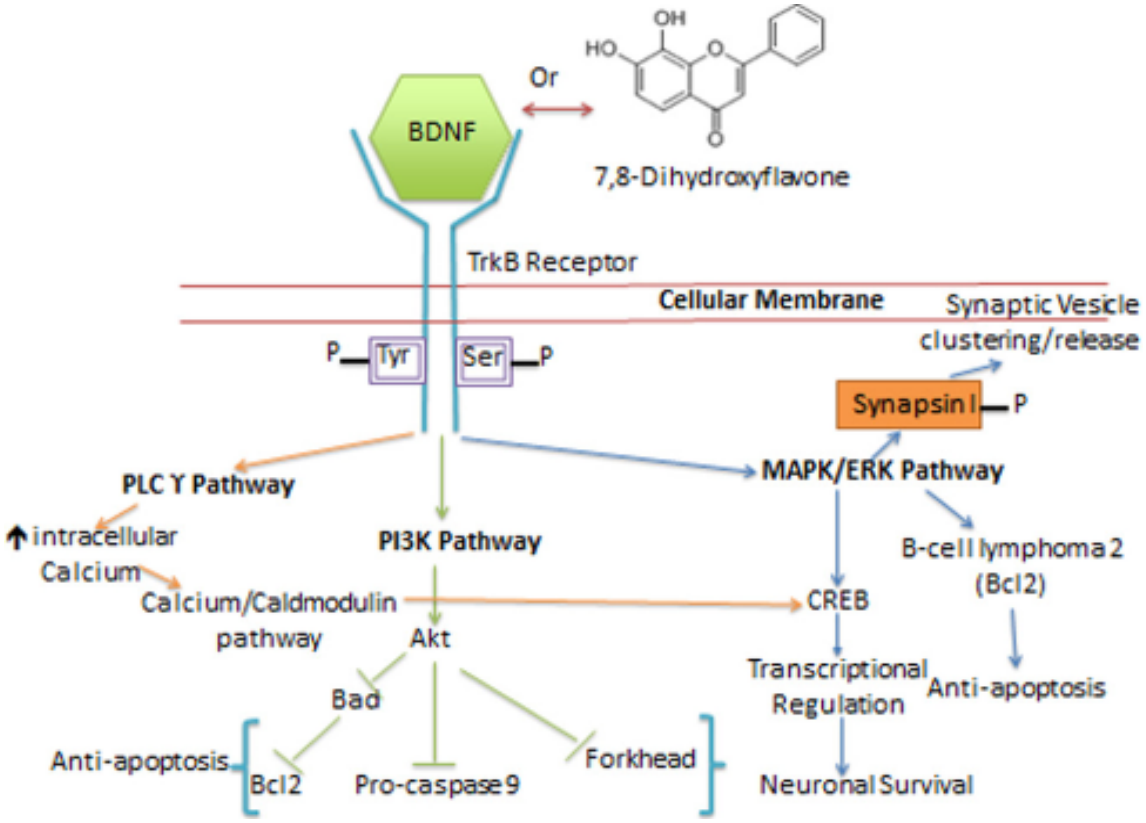


Fig. 14. 7,8- DHF signalling pathways (Wurzelmann, Romeika, and Sun 2017)

It seems that 7,8- DHF does not induce significant toxicity after long-term administrations. However, it is necessary to carefully determine the dosage in relation to the duration of treatment (Liu, Chan, and Ye 2016). The use of high doses could result in adverse effects, such as a decrease in neuronal vitality. This phenomenon can be explained by a downregulation of the TrkB-mediated response, and a decrease in neuroprotection (Wu et al. 2014). There are conflicting results regarding the activation power of signal pathways induced by 7,8- DHF. In vitro studies on hippocampal neurons show that 7,8- DHF causes both Erk and Akt activation in a dose-dependent manner (Jang et al. 2010) while, in studies on experimental models of Huntington's disease it has been observed, both in vitro and in vivo, an increase only in PLCγ levels (Garcia-Diaz Barriga et al. 2017). These discrepancies may depend on the complexity of the signalling cascades (Marongiu, Imbrosci, and Mittmann 2013).

It has been observed that 7,8- DHF promotes neuronal survival and synaptogenesis, improving memory and learning (Liu, Chan, and Ye 2016) and reducing apoptosis in cortical neurons. Furthermore, like BDNF, it has been shown to exert a positive effect on spatial memory in aged rats, as well as an improvement in synaptic plasticity and an increase in the number of dendritic spines that enhance cognitive functions (Zeng et al. 2012).

Preclinical data show that 7,8- DHF is effective in many neurological disorders (Emili et al. 2022). For example, the administration of 7,8- DHF led to reduction of cell damage in hippocampal neurons and attenuation of cognitive decline in experimental models of traumatic brain injury (Agrawal et al. 2015; Chen et al. 2015). In an animal model of Alzheimer disease, 7,8- DHF attenuated cognitive deficits in association with an increase in the density of synaptic spines in CA1 pyramidal neurons (Castello et al. 2014). 7,8- DHF also led to an improvement in motor function and brain atrophy in an animal model of Huntington disease (Jiang et al. 2013).

As anticipated, non-TrkB mediated effects have also been identified, in particular antioxidant activity. Oxidative stress-induced neuronal death plays a pivotal role in the pathogenesis of neurodegenerative disorders. In PC12 cells, which lack TrkB, 7,8- DHF prevented cell death, apoptosis and mitochondrial dysfunction induced by 6-hydroxydopamine (6-OHDA). Moreover, 7,8- DHF also elevated total superoxide dismutase activity in 6-OHDA-treated cells, indicating that it protects PC12 cells against cytotoxicity through its powerful antioxidant activity (Han et al. 2014). In in vitro studies on HT-22 cells, another non-TrkB expressing cell line, the administration of 7,8- DHF protected from reactive oxygen species (ROS) and toxicity induced by glutamate (Chen et al. 2011). In a study designed to confirm the cytoprotective effects of 7,8-DHF against oxidative

stress-induced cellular damage, 7,8-DHF attenuated hydrogen peroxide (H_2O_2) -induced growth inhibition and exhibited scavenging activity against intracellular ROS induced by H_2O_2. 7,8-DHF also significantly attenuated H_2O_2-induced DNA damage and cell apoptosis and increased the levels of heme-oxygenase-1 (HO-1), a potent antioxidant enzyme. It seems that 7,8-DHF augments the cellular antioxidant defence capacity through activation of the Nrf2/HO-1 pathway, which also involves the activation of the PI3K/Akt and ERK pathways (Kang et al. 2015) .

All these promising results, led us to test the therapeutic efficacy of 7,8- DHF also in experimental models of epilepsy and in comorbidities associated with the development of the disease. In our study, we found that low- (5 mg/kg), but not high-dose 7,8- DHF (10 mg/kg) can exert strong anti-epileptogenic effects in the lithium-pilocarpine model (i.e., highly significant reduction in the frequency of spontaneous seizures and in the time to first seizure after status epilepticus), and that these different effects correlate with differences in TrkB phosphorylation patterns and in the activation of TrkB-dependent signaling pathways.

4.2.1

LOW-DOSE 7,8- DIHYDROXYFLAVONE ADMINISTRATION AFTER STATUS EPILEPTICUS PREVENTS EPILEPSY DEVELOPMENT IN THE PILOCARPINE MODEL

Original article

Low-dose 7,8-Dihydroxyflavone Administration after Status Epilepticus Prevents Epilepsy Development

Annunziata Guarino,[1†] Barbara Bettegazzi,[2†] Nimra Aziz,[1] Mario Barbieri,[1] Daniela Bochicchio,[1] Lucia Crippa,[2] Pietro Marino,[1] Maddalena Sguizzato,[1] Marie Soukupova,[1] Silvia Zucchini,[1,3] Michele Simonato[1,4]

[1] Department of Neuroscience and Rehabilitation, University of Ferrara, via Fossato di Mortara 70, 44121 Ferrara, Italy

[2] University Vita-Salute San Raffaele, via Olgettina 58, 20132 Milan, Italy

[3] Laboratory of Technologies for Advanced Therapy (LTTA), Technopole of Ferrara, Ferrara, Italy

[4] Division of Neuroscience, IRCCS San Raffaele Hospital, via Olgettina 60, 20132 Milan, Italy

[†] These authors contributed equally to this work.

Address correspondence to

Silvia Zucchini
Department of Neuroscience and Rehabilitation
University of Ferrara
via Fossato di Mortara 70
44121 Ferrara
Italy
Email: silvia.zucchini@unife.it

Running title

Anti-epileptogenic effect of 7,8-DHF

Abstract

Temporal lobe epilepsy often manifests months or even years after an initial epileptogenic insult (e.g., stroke, trauma, status epilepticus) and, therefore, may be preventable. However, no such preventive treatment is currently available. Aim of this study was to test an antioxidant agent, 7,8-dihydroxyflavone (7,8-DHF), that is well tolerated and effective in preclinical models of many neurological disorders, as an anti-epileptogenic drug. However, 7,8-DHF also acts as a TrkB receptor agonist and, based on the literature, this effect may imply an anti- or a pro-epileptogenic effect.

We found that low- (5 mg/kg), but not high-dose 7,8-DHF (10 mg/kg) can exert strong anti-epileptogenic effects in the lithium-pilocarpine model (i.e., highly significant reduction in the frequency of spontaneous seizures and in the time to first seizure after status epilepticus), and that these different effects correlate with differences in TrkB phosphorylation patterns and in activation of TrkB-dependent signaling pathways.

These data support the possibility to develop drugs with desirable effects on the TrkB receptor, based on the selective activation of therapeutically relevant signaling pathways. In this respect, and also considering its excellent safety profile and antioxidant properties, 7,8-DHF represents a promising candidate for a preventive, anti-epileptogenic therapy, or at least a template for the development of effective and well-tolerated anti-epileptogenic drugs.

Keywords

Brain-derived neurotrophic factor; TrkB receptor; epileptogenesis; neuronal death

Abbreviations

BDNF = brain-derived neurotrophic factor; DAPI = 4,6-diamidino-2-phenylindole dihydrochloride; 7,8-DHF = 7,8-dihydroxyflavone; DMSO = dimethylsulfoxide; EPM = elevated plus maze; FGF-2 = fibroblast growth factor-2; GFAP = glial fibrillary acidic protein; KA = kainic acid; mTLE = mesial temporal lobe epilepsy; OF = open field; OLT = object location task; PBS = phosphate-buffered saline; PLCγ1 = phospholipase Cγ1; SE = status epilepticus; SRSs = spontaneous recurrent seizures; TBI = traumatic brain injury.

Introduction

Some forms of epilepsy, for example mesial temporal lobe epilepsy (mTLE), can originate months or even years after a brain-damaging event, e.g., injury, stoke, status epilepticus (SE), infection.{Simonato, 2021 #1} While patients can recover from this initial event, it may set in motion a series of alterations at molecular, cellular and circuitry level that, in time, lead to the transformation of a normal brain into epileptic, i.e., to the appearance of spontaneous seizures. This process is called epileptogenesis. In principle, these forms of epilepsy should be preventable. In practice, this is extremely difficult, because only a subset of the individuals who experience an epileptogenic insult will later become epileptic, and no reliable biomarker is currently available to predict who will and who will not. {Simonato, 2021 #1} This implies the practical impossibility of clinically testing potential preventive therapies. {Simonato, 2012 #2; Simonato, 2014 #3}

The question posed by the epileptogenesis process can be viewed as an example of a general neuroscience question: how a fleeting event (a life experience in general, an epileptogenic event in our case) can lead to permanent changes in the structure and function of the brain. One hypothesis may be the accumulation in the lesion area of endogenous molecules that can be responsible for the structural and functional alterations mentioned above. Such molecules should therefore 1) increase after the epileptogenic insult, and 2) have a profile of actions coherent with an involvement in neural circuitry plastic changes ultimately resulting in hyperexcitability. {McNamara, in press #4} One molecule that meets these criteria is the brain-derived neurotrophic factor (BDNF).

Epileptogenic insults increase BDNF signaling. For example, SE increases BDNF expression, {Gall, 1993 #5; Nawa, 1995 #6; Simonato, 1998 #7} and causes enhanced activation of the BDNF high-affinity receptor, TrkB.{Binder, 1999 #9; He, 2002 #10} Less clear is whether TrkB hyper-activation plays a pro- or anti-epileptogenic role. A local supplementation of BDNF together with another neurotrophic factor, fibroblast growth factor-2 (FGF-2), has been reported to attenuate SE-induced cell damage, increase hippocampal neural stem cell proliferation and neuronal differentiation, and reduce the aberrant aspects of epileptogenesis-associated neurogenesis, thereby ameliorating the epilepsy pathology and reducing the frequency and severity of spontaneous seizures.{Paradiso, 2009 #11} In addition, a partial agonist of the TrkB receptor (LM22A-4) has been reported to enhance structural and functional measures of GABAergic inhibition and to suppress post-traumatic epileptogenesis when administered after cortical injury.{Gu, 2018 #13}

However, other lines of evidence suggest that BDNF is instead pro-epileptogenic: transgenic overexpression of BDNF is sufficient to cause mTLE in adult mice,{Croll, 1999 #15; Isgor, 2015 #17} and BDNF heterozygotes exhibit impairments in kindling epileptogenesis,{Kokaia, 1995 #19} an effect observed also after intraventricular administration of proteins that selectively scavenge BDNF.{Binder, 1999 #8} The most compelling evidence of a pro-epileptogenic role of BDNF, however, comes from transgenic mice carrying a genetic modification in the TrkB kinase domain ($TrkB^{F616A}$), that renders the receptor sensitive to inhibition by an otherwise inert blood-brain barrier-permeable small molecule, 1NMPP1. Treatment with 1NMPP1 for two weeks after intra-amygdala kainate (KA)-induced SE prevents development of mTLE and comorbid anxiety-like behavior in $TrkB^{F616A}$ transgenic animals. {Liu, 2013 #20} However, this also exacerbates SE-induced neuronal degeneration. {Gu, 2015 #22} In brief, converging evidence supports a neuroprotective effect of BDNF, but data are conflicting on its effect on epileptogenesis.

Aim of the present study was to test a promising BDNF agonist, 7,8-dihydroxyflavone (7,8-DHF), that proved to be well tolerated and effective in preclinical models of many neurological disorders.{Emili, 2020 #23} In addition to its effect on TrkB, this compound is known to exert antioxidant effects.{Zhang, 2009 #27} Because reactive oxygen species are rapidly induced in the brain after epileptogenic insults, and antioxidant drugs have been reported to exert anti-epileptogenic effects,{Terrone, 2020 #28} 7,8-DHF is expected to exert favorable effects. However, its actions on BDNF may represent a double-edged sword.

Materials and Methods

Animals

All experiments were performed in male Sprague-Dawley rats (Envigo, Udine, Italy) weighing 200–250 g. Animals were kept under standard housing conditions: room temperature 22–24°C, 12 h light/dark cycle and free access to food and drinking water. They were allowed to adapt to laboratory conditions for at least 1 week before starting the experiments. All experimental protocols were approved by the University of Ferrara Committee for Animal Welfare and by the Italian Ministry of Health (D.M. 90/2021-PR) and were carried out in accordance with the guidelines of the National Institute of Health and the European Community (EU Directive 2010/63/EU) on the Use and Care of Animals. In addition, all

experimental procedures have been performed following the ARRIVE (Animal Research: Reporting in Vivo Experiments) and the NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animal Research) guidelines.{Kilkenny, 2011 #29; Lidster, 2016 #30}

Lithium-pilocarpine model

Rats were administered 127 mg/kg lithium chloride by gastric gavage. After approximately 14 h, they received a subcutaneous injection of methyl-scopolamine (1 mg/kg, Sigma-Aldrich, Saint Louis, MO, USA) to reduce the undesirable peripheral effects of pilocarpine. SE was induced 30 min later by administration of pilocarpine (50 mg/kg i.p., Sigma-Aldrich). The intensity of motor seizures was classified according to Racine's scale:{Racine, 1972 #32} stage 1, immobility, eyes closed, and facial clonus; stage 2, head nodding and more severe facial clonus; stage 3, clonus of one forelimb; stage 4, rearing with bilateral forelimb clonus; and stage 5, generalized tonic-clonic seizures with rearing and falling. Within 30 min after pilocarpine injection, animals develop continuous, long-lasting generalized seizure activity (stage 4 and higher), i.e., convulsive SE. Animals that did not enter SE within 30 min were administered a second, lower dose of pilocarpine (25 mg/kg).

SE was interrupted 2 h after onset by i.p. administration of a cocktail of drugs: diazepam (10 mg/kg), phenobarbital (25 mg/kg) and scopolamine (1 mg/kg). This cocktail was administered again after 4 h. Finally, after another 4 h, rats received an i.p. administration of diazepam and scopolamine only. This procedure allows a complete stop of seizure activity. {Brandt, 2015 #34} To facilitate animal's recovery and reduce the weight loss that follows SE, hydration was promoted by daily s.c. administration of 0.9% saline (1 ml) and palatable food was provided to support feeding for the next 5 days. Of the 134 rats that underwent this procedure, 31 (i.e., 22%) did not enter SE and 12 (9%) died during SE or within 24 h. The remaining 91 rats were assigned to the 3 experimental groups: vehicle, 7,8-DHF 5 mg/kg and 7,8-DHF 10 mg/kg. Allocation to groups was performed randomly on the basis of the baseline performance in the behavioral tests (see below) and on the severity of SE. Animals were killed 28 days after SE.

Drug treatments

7,8-DHF (Tokyo Chemical Industry, TCI, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS 1x) containing 50% dimethylsulfoxide (DMSO). Rats received i.p. injections of 5 or 10 mg/kg 7,8-DHF or vehicle once daily for 7 consecutive days, beginning the day after SE. These regimen and doses were chosen based on previous in vivo studies demonstrating that they produce activation of central TrkB receptors, increase neurogenesis, and evoke behavioral changes in models of neurodegenerative diseases. {Jang, 2010 #36; Andero, 2011 #37}

Assessment of spontaneous recurrent seizures

After SE induction, animals were placed in individual cages and video monitored (24 h/day, 7 days/week) for 21 days (Videostar, Misterbianco, Catania, Italy). Frequency and severity of motor spontaneous recurrent seizures (SRSs) were recorded and scored using the scale of Racine,{Racine, 1972 #32} by investigators that were blind of the treatment administered to the different rats.

Electrode implantation

A separate subgroup of 15 animals was implanted with a bipolar electrode (PlasticsOne, Roanoke, VA, USA) in the right dorsal hippocampus 2 weeks prior to SE induction. Rats were first anesthetized using ketamine/xilazine (87 mg/kg and 15 mg/kg i.p., respectively) and anesthesia was then maintained with 2% isoflurane. Ophthalmic ointment was used for eyes lubrification. A midline incision was made in the scalp and a hole was drilled in the skull. The coordinates for electrode implantation were AP –3.9, ML –1.7 from bregma and P –3.5 from dura. {Paxinos, 2013 #38} A ground wire was connected to four screws secured to the skull, and the electrode was fixed with dental cement. Animals received an antibiotic prior to and after surgery (enrofloxacin, 5 mg/kg s.c.), to avoid possible infections and an analgesic drug (tramadol, 7 mg/kg s.c. daily) for 3 days after surgery.

Video-EEG monitoring

SE was evoked in electrode-implanted animals as described above, and rats were then randomly assigned to the different experimental groups (vehicle n=5, 5 mg/kg 7,8-DHF n=5, 10 mg/kg 7,8-DHF n=5). The electrode was connected through a tripolar cable (PlasticsOne) to an EEG100C amplifier/MP160 Data Acquisition system (Biopac Systems, Goleta, CA, USA), paired with video cameras to record animal behavior. EEG signals were analyzed using the AcqKnowledge 5.0 software (Biopac). An EEG seizure has been defined as a paroxysmal electrical activity characterized by i) 3-times higher amplitude than baseline, ii) duration of at least 5 s, iii) more than 5 spikes/s. {Paradiso, 2011 #39} Video-EEG monitoring was performed 24h/7d for 3 weeks after SE.

Behavioral tests

The effects of the 7,8-DHF treatment on comorbidities associated with epilepsy such as anxiety and cognition were investigated using different behavioral tests: open field (OF), elevated plus maze test (EPM) and object location task (OLT). All tests were carried out at the following time points: i) 8-6 days before the induction of SE, at baseline; ii) 8-10 days after SE, i.e., early phase of the disease process and iii) 21-23 days after SE, i.e., late phase. The early phase corresponds to the time of onset of SRSs in epileptic control animals, whereas the late phase corresponds to the chronic period, when epileptic control animals regularly experience SRSs. Tests were performed in a soundproof room, where animals were transferred 30 min before the test for acclimatization. All procedures were conducted, and data analyzed by 2 investigators that were blind of the experimental conditions.

The OF test was carried out in an apparatus consisting of a square-shaped arena (82 × 82 × 40 cm). Each rat was placed in the center area and its behavior was video monitored using an infra-red video camera (DSS1000 video recording system V4.7.0041FD, AverMedia Technologies, USA) for 20 min. Recorded parameters included: the total distance run by the rat, the distance run in the center quadrant of the arena (41 × 41 cm), the number of entries in the central quadrant and the immobility time. Data were automatically measured using the ANY-Maze software (Ugo Basile, Gemonio, Varese, Italy). The OF apparatus was carefully cleaned with 70% ethanol after each test session.

The EPM test was performed as previously described. {Tchekalarova, 2015 #40} The maze consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 cm) connected through a central platform (10 × 10 cm). The apparatus was 80 cm above the floor. At the start of the test, animals were placed in the central square, facing an open arm. The observation lasted 5 min. The calculated measures were the following: number of entries in open arms; number

of entries in closed arms; time spent in open arms; time spent in closed arms. The EPM apparatus was carefully cleaned with 70% ethanol after each test.

The OLT was performed in the arena used for the OF test. The test consisted of three phases: habituation, training and test. The OF test, conducted the day before OLT, was used as habituation phase. The day after habituation, the training phase was conducted by placing the rat in the arena, in which two identical objects were positioned in two adjacent corners, at 10 cm from the wall. The time of interaction of the animal with each object was recorded for 5 min. Interaction was defined as sniffing or observing the object at less than two cm distance.{Ennaceur, 2005 #41} After 2 h, rats were placed again in the arena, where one of the objects was moved to a different corner. Again, the time that each animal spent exploring each object was recorded for 5 min. The OLT apparatus was carefully wiped clean with 70% ethanol after each test.

Immunofluorescence

Animals were killed 4 weeks after SE (vehicle n=11, 5 mg/kg 7,8-DHF n=12, 10 mg/kg 7,8-DHF n=9) together with a control group of naïve animals (n=7). Brains were removed and immersed in 10% neutral formalin solution (Sigma-Aldrich) for 48 h, before undergoing tissue processing (VTP 300, Bio-Optica, Milan, Italy) and paraffin-embedding. Coronal, 6 µm thick tissue sections were cut using a Leica RM2125RT microtome across the hippocampus, {Paxinos, 2013 #38} and mounted onto polarized slides (Superfrost slides, Diapath Martinengo, Bergamo, Italy). Sections were dewaxed and rehydrated as previously described: {Paradiso, 2009 #11} two 10 min washes in xylene (Sigma-Aldrich), 5 min in 100% ethanol, 5 min in 95% ethanol, 5 min in 80% ethanol, 5 min in distilled water.

All antigens were unmasked using a solution of citric acid and sodium citrate in a microwave oven at 750 W (5 cycles of 5 min) for NeuN; 750 W (1 cycle of 5 min) and then 350 W (2 cycles of 3 min) for glial fibrillary acidic protein (GFAP). After a wash in PBS, sections were incubated at room temperature with Triton X-100 (0.3% in 1×PBS; Sigma-Aldrich) for 10 min, washed twice in PBS, and then incubated for 30 min with 5% bovine serum albumin and 5% serum of the species in which the secondary antibody was produced. Sections were incubated overnight at 4°C in a humid atmosphere with a primary antibody as follows: anti-NeuN (mouse monoclonal, Immunological Science, Rome, Italy), 1:100 dilution; anti-GFAP (rabbit polyclonal, Sigma-Aldrich), 1:100. After 5 min washing in PBS, sections were incubated with Triton (as described above; 30 min), washed in PBS, and incubated with the secondary antibody, goat anti-mouse Alexa Fluor 594 (Invitrogen, Waltham, MA, USA) 1:500 for mouse primary antibodies, or goat anti-rabbit,

Alexa Fluor 594 (Invitrogen) 1:500, at room temperature for 3 h. After staining, sections were washed in PBS, counterstained with 0.0001% 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific, Waltham, MA, USA) for 15 min, and washed again before mounting. Coverslips were mounted using an aqueous antifading mounting gel (Sigma). The primary antibody was omitted on a subset of slices for detection of non-specific staining.

We analyzed 6 sections at 3 levels throughout the dorsal hippocampus, –2.3, –2.8 and –3.3 relative to bregma. {Paxinos, 2013 #38} Images were captured using a 20× objective at the level of the DG, CA3 and CA1 region using a Leica microscope (DMRA2, Leica). NeuN- and GFAP-positive pixel was measured using the Fiji (ImageJ) open-source software,{Schindelin, 2012 #42} and an algorithm tailored to measure percent of supra-threshold pixels according to the IsoData method.{Lovisari, 2021 #43} Data were expressed as percent of positive pixels within the hilus of the DG (the region situated between the granule layer and the CA3 pyramidal neurons and remaining between the boundaries of the DG,{Paradiso, 2011 #39} or within a rectangular frame (400 × 180 pixels) along the pyramidal layer of the CA3 and CA1 regions. Data obtained from the 6 sections examined for each rat were averaged to obtain a single estimate for each animal. The investigator who performed quantification was blinded to the experimental condition.

Western Blot analysis

Tissue homogenization was performed as previously described. {Bettegazzi, 2021 #44} Briefly, hippocampi from naïve and 7,8 DHF treated rats 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). About 50 µg of proteins was 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. In the cases where stripping was not possible, the same lysates were run simultaneously on duplicate gels, and probed with phospho- and total antibodies. 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. Phosphorylated TrkB (Y516 and Y816), AKT, ERK and PLCγ levels were normalized against the corresponding total protein, then for loading (GAPDH). Details on the antibodies employed in Western Blot analysis are reported in **Table 1**.

Data availability

This study does not include data deposited in public repositories. Data are available on request to the corresponding author.

Results

Development of spontaneous seizures

To test the effect of 7,8-DHF on epileptogenesis, the drug was administered daily at two different doses (5 and 10 mg/kg i.p.) for one week, beginning the day following SE induction (**Fig. 1A**). Vehicle-treated animals began experiencing EEG, non-motor seizures 6±1 day after SE (n=5) and motor seizures 10±2 days after SE (n=14, not including 3 animals that did not display any motor seizure in the 21 days of observation: **Fig. 1B**). Therefore, this dosing regimen covered the latency period and a very initial chronic epileptic phase with only non-generalized, non-motor seizures.

The lower dose of 7,8-DHF almost completely prevented the occurrence of SRSs in the observation period of 21 days. Whereas, as noted above, 14 of 17 vehicle-treated rats displayed spontaneous motor seizures, only 2 of 18 rats treated with 5 mg/kg 7,8-DHF experienced each a single motor seizure, and this happened much later than in the vehicle group, i.e., 18 and 20 days after SE (**Fig. 1B**). Therefore, the average number of motor seizures per day was significantly lower (**Fig. 1C**) and the time to first seizure was highly prolonged (**Fig. 1D**) in 5 mg/kg 7,8-DHF-treated animals. Non-motor, EEG seizures were also dramatically attenuated by 5 mg/kg 7,8-DHF (**Fig. 2**). All vehicle-treated rats displayed EEG seizures. In contrast, EEG seizures were recorded in only 3 of 5 animals treated with 5 mg/kg 7,8-DHF; moreover, 2 of these 3 animals experienced a single EEG seizure, and the third just had two. Overall, the daily number of seizures was significantly reduced (**Fig. 2C**).

In contrast with the lower dose regimen, the higher dose did not produce any significant effect as compared with vehicle. Spontaneous motor seizures (**Fig. 1B** and **1C**), time to first motor seizure (**Fig. 1D**) and spontaneous EEG seizures (**Fig. 2C**) were not significantly different in these two groups. Taken together, these data show that 7,8-DHF produces a robust and long lasting antiepileptogenic effect when administered at low but not at high doses.

Behavioral testing

We also evaluated possible effects of 7,8-DHF on epilepsy co-morbidities by employing behavioral tests that explore anxiety and cognition. The OF test is used for the evaluation of both motor activity and anxiety-like behavior in rodents. Under physiological conditions, rodents spend more time in peripheral spaces than in the center of the arena. Conversely, pilocarpine-treated rats alternated periods of hyperactivity (**Fig. 3A**) and of freezing (**Fig. 3B**), spending significantly more time in the central part of the testing arena (**Fig. 3C** and **3D**). This anxiety-like phenotype was partially reverted by low-dose 7,8-DHF, that normalized the total distance walked by the rats and the time spent immobile in the late phase (**Fig. 3A** and **3B**), but was not affected by 10 mg/kg DHF (**Fig. 3**).

EPM is another test aimed at the evaluation of anxiety. This test is based on the rodent preference for dark and closed spaces and their fear of elevated and open spaces. Under physiological conditions, rats tend to spend more time in the closed arms of the apparatus (see baseline in **Fig. 4**). Epileptic animals display a restless, anxiety-like behavior, because they spend equal or more time in the open and in the closed arms and enter the open arms much more frequently than under control, baseline conditions (**Fig. 4**). 7,8-DHF did not modify this phenotype, neither at the early or at the late phase, at 5 or at 10 mg/kg (**Fig. 4**).

Finally, we explored cognitive abilities using the OLT, that evaluates short-term, hippocampal-dependent spatial memory. {Denninger, 2018 #45} At baseline, all rats displayed a clear exploratory preference for the re-located novel object, but this preference disappeared with the development of spontaneous seizures (**Fig. 5**), indicating loss of spatial memory. However, the low (but not the high) dose of DHF reinstated the ability to distinguish the re-located object in the late phase (**Fig. 5B**). Taken together, these data suggest that 7,8-DHF can attenuate some epilepsy co-morbidities reinstating a more physiological behavior, when administered at low but not at high doses.

Immunofluorescence

To evaluate effects on epilepsy-associated neuronal death, we used NeuN immunofluorescence. {Paradiso, 2009 #11} A loss in NeuN-positive cells was observed in the hilus of the dentate gyrus and in the CA3 pyramidal layer of animals experiencing SRSs, one month after pilocarpine-induced SE (**Fig. 6A-6E**). Five, but not 10 mg/kg 7,8-DHF was found to partially protect from damage (**Fig. 6A-6G**). In fact, i) the loss of NeuN signal in the hilus of the dentate gyrus was highly significant in untreated and 10 mg/kg 7,8-DHF treated pilocarpine animals, but not in 5 mg/kg 7,8-DHF treated pilocarpine animals (**Fig. 6A**); ii) a nearly complete protection from CA3 neuronal loss was observed in animals treated with 5, but not with 10 mg/kg 7,8-DHF (**Fig. 6B** and **6D-6G**).

Epilepsy-associated astrocytosis was evaluated using GFAP immunofluorescence. Consistent with previous findings, {Bovolenta, 2010 #46} one month after pilocarpine SE the percentage of GFAP-positive pixels in the hippocampus increased in the CA3 area and displayed a clear tendency to increase also in the hilus of the dentate gyrus and in CA1 (**Fig. 6H-6L**). In addition, many of the GFAP-positive cells in epileptic controls displayed short, thick processes, an indication of activated astrocytes (**Fig. 6L**). Once again, 5, but not 10 mg/kg 7,8-DHF prevented all these effects (**Fig. 6H-6N**).

Taken together, these data suggest that low, but not high doses of 7,8-DHF can attenuate epilepsy-associated histological alterations.

TrkB receptor phosphorylation and TrkB-activated intracellular pathways

All the data described above converge on the apparently paradoxical concept that a low dose of 7,8-DHF can produce beneficial effects that disappear at a higher dose. We hypothesized that these effects may depend on a dose-dependent differential activation of TrkB signaling pathways. In fact, activation of TrkB by BDNF leads to receptor dimerization and auto-phosphorylation of selected tyrosines in the cytoplasmic domain. Phosphorylation of tyrosine 515 promotes association of TrkB with the Shc adaptor and activation of the PI3-kinase (PI3K)/AKT and of the Raf-MEK-ERK (i.e., MAPK/ERK) signaling pathways; phosphorylation of tyrosine 816, conversely, leads to the recruitment of phospholipase Cγ1 (PLCγ1).{Reichardt, 2006 #47} Whereas the former pathway has been reported to exert neuroprotective effects, the latter has been suggested to produce pro-epileptogenic effects (**Fig. 7A**).{Huang, 2019 #48}

Therefore, we decided to test whether TrkB phosphorylation and the AKT/ERK and PLCγ1 signaling pathways were differentially activated by 7,8-DHF as a function of the dose. Hippocampal homogenates isolated from vehicle or 7,8-DHF-treated rats were analyzed by western blot, as shown in **Fig. 7B**. Coherently with our hypothesis, we found that,

whereas 7,8-DHF increased TrkB Y515, AKT and ERK phosphorylation to similar levels at both doses (**Fig. 7C-7E**), only the dose of 10 mg/kg increased (by ~2 fold) the levels of phosphorylated TrkB Y816 and of PLCγ1 (**Fig. 7F** and **7G**).

Discussion

The main finding of this study is that low-, but not high-dose 7,8-DHF can exert strong anti-epileptogenic effects in the pilocarpine model. By using the two most commonly used 7,8-DHF treatment regimens,{Emili, 2020 #23} we found highly significant dose-dependent differences in many respects (in particular SRSs and cell death) that may depend on differences in the activation of TrkB-dependent signaling pathways.

Flavonoids are plant metabolites that are known to produce many favorable effects in human health, due mainly, but not only, to their antioxidant and anti-inflammatory actions.{Panche, 2016 #49} In fact, 7,8-DHF is a flavonoid originally known for its antioxidant properties,{Zhang, 2009 #27} and more recently recognized as a high-affinity and selective TrkB receptor agonist. 7,8-DHF is orally bioavailable, crosses the blood brain barrier, and has a relatively long half-life (3 h in mice, >6 h in monkeys).{Emili, 2020 #23} Its profile of actions and its favorable pharmacokinetics have prompted a very large number of preclinical studies that highlight it as a promising treatment for many, diverse neurological and psychiatric disorders.{Emili, 2020 #23} However, no study was performed thus far in epilepsy models.

As described in the Introduction, it was difficult to predict the effect of 7,8-DHF in epileptogenesis because, whereas antioxidant drugs are well known to exert anti-epileptogenic effects, {Terrone, 2020 #28} conflicting data are available on pro- or anti-epileptogenic implications of the BDNF/TrkB system.{McNamara, in press #4} A possible explanation of this conundrum has been recently found to lay in the different TrkB receptor signaling pathways. A membrane permeable peptide comprising the HIV-1 Tat domain and a TrkB sequence, able to block PLCγ1 binding to residue 816 of TrkB, has been shown to prevent epilepsy development following intra-amygdala KA while preserving the neuroprotective effects of BDNF.{Gu, 2015 #22} In contrast, intra-amygdala KA administration in mice carrying a mutation blocking the Shc-Akt signaling pathway (phenylalanine substituted for tyrosine at residue 515, $TrkB^{Shc/Shc}$ mice) evokes similar grade SE as in WT animals, but exacerbates hippocampal neuronal death.{Huang, 2019 #48}

Coherent with these findings, we obtained beneficial effects on epileptogenesis with the low dose (5 mg/kg) of 7,8-DHF. At this dose regimen, we found selective phosphorylation of the Y515 residue of TrkB (i.e., no phosphorylation at Y816) and selective activation of the Shc-Akt pathway (i.e., no activation of the PLCγ1 pathway). These effects may explain the neuroprotective effect on hippocampal neurons and, together with the expected antioxidant action, contribute to the robust antiepileptogenic effect. In contrast, the higher dose of 7,8-DHF (10 mg/kg) induces TrkB phosphorylation at both Y515 and Y816, recruits the PLCγ1 pathway and, by doing so, it may oppose the neuroprotective and anti-epileptogenic antioxidant effects. It is unclear how 7,8-DHF may differentially activate different TrkB signaling pathways in a dose-dependent manner. Because it is known to bind the extracellular domain of the receptor at a different site in comparison with BDNF,{Liu, 2013 #20} it may be hypothesized that the conformational changes induced by low 7,8-DHF doses in the intracellular domain prompt a preferential phosphorylation of tyrosine 515.

It seems instead unlikely that the effects of 7,8-DHF depend on TrkB receptor internalization. First, as compared with BDNF, 7,8-DHF has been shown to induce a much slower TrkB internalization and a much longer-lasting phosphorylation, not inducing its ubiquitination or degradation. {Liu, 2014 #50} Incidentally, these findings suggest that 7,8-DHF and BDNF activate TrkB with different mechanisms, supporting the above hypothesis that the patterns of activation of intracellular pathways may also differ. Second, internalization would lead to an antagonist-like effect, switching off all TrkB-activated signaling pathways and all TrkB-dependent effects, a condition under which one would expect an anti-epileptogenic effect. {Liu, 2013 #20} Internalization would be stronger with the high dose of 7,8-DHF, which would therefore produce a more robust anti-epileptogenic effect than the low dose. However, we observed the opposite. Third, a reduced activation of signaling pathways would be expected in case of internalization. Not only this was not the case, but the higher dose proved even more effective than the lower dose in activating signaling pathways.

The primary outcome measure in this study was the frequency and severity of spontaneous seizures. However, we also investigated the impact of the treatment on co-morbidities, in particular anxiety and cognition. Based on OF (but not EPM) and on OLT, we observed an attenuation of these co-morbidities in animals treated with 7,8-DHF at low, but again not at high doses. However, these effects were partial for anxiety, because only a few parameters of the OF were corrected, and all appeared only in the late, chronic phase of the disease, while anxiety traits and cognitive impairments were observed in all animals (including those treated with low dose 7,8-DHF) in the early phase, i.e., at the time when vehicle-treated animals begin experiencing SRSs. Several behavioral alterations have been observed to follow epileptogenic insults (SE or traumatic brain injury, TBI) in animal models, but the majority of these alterations cannot predict which animals will subsequently become epileptic (i.e., will display SRSs) and which will not.{Broer, 2015 #55; Pascente, 2016 #54; Lapinlampi, 2020 #53; Nizinska, 2021 #52} In the chronic course of epilepsy, these behavioral

alterations are generally maintained,{Pascente, 2016 #54; Lapinlampi, 2020 #53; Nizinska, 2021 #52} and this was the case also in the present study. Therefore, the observation that animals treated with low-dose 7,8-DHF had improvements at late time points may be attributed to the fact that they did not (or very marginally did) experience SRSs. In other words, SRSs seem to sustain anxiety-like behavior and cognitive impairment in our experimental settings, because these behavioral alterations tend to attenuate in time in animals receiving a treatment that prevents seizure occurrence.

In conclusion, considering its pharmacological properties and context of use (excellent pharmacokinetics and tolerability, antioxidant effects, profile of actions on the TrkB receptor, and prospective short-term administration following an epileptogenic insult), 7,8-DHF represents a promising candidate for a preventive, anti-epileptogenic therapy, or at least a template for developing an effective and well-tolerated anti-epileptogenic drug. In the former, translational prospect, the present data suggest that, although an accurate dose titration would be needed, the treatment would not be associated with significant side effects even at higher doses. In the latter, drug development prospect, our data indicate that it may be possible to develop drugs with desirable effects on the TrkB receptor (i.e., selectively activating the "good" Shc-Akt pathway or selectively inhibiting the "bad" PLCγ1 pathway). In this respect, peptides represent an alternative approach.{Gu, 2015 #22} The peptide approach is clearly more refined in terms of targeting, but holds other problems, especially in terms of pharmacokinetics (absent or limited absorption after oral administration) and costs of large-scale production.{Muttenthaler, 2021 #56} Continuing research on both small molecules and peptides is therefore warranted.

Acknowledgments

The authors thank Drs. Enrico Tongiorgi and Gabriele Baj for generously providing pTrkB antibodies.

Funding

This work was supported by funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement 602102 (EPITARGET).

Competing interests

The authors declare that they have no competing interests.

References

1. Simonato M, Agoston DV, Brooks-Kayal A*, et al*. Identification of clinically relevant biomarkers of epileptogenesis - a strategic roadmap. *Nat Rev Neurol*. Apr 2021;17(4):231-242. doi:10.1038/s41582-021-00461-4

2. Simonato M, Loscher W, Cole AJ*, et al*. Finding a better drug for epilepsy: preclinical screening strategies and experimental trial design. *Epilepsia*. Nov 2012;53(11):1860-7. doi:10.1111/j.1528-1167.2012.03541.x

3. Simonato M, Brooks-Kayal AR, Engel J, Jr.*, et al*. The challenge and promise of antiepileptic therapy development in animal models. *Lancet Neurol*. Sep 2014;13(9):949-60. doi:10.1016/S1474-4422(14)70076-6

4. McNamara JO, Simonato M. Neurotrophic factors. In: Engel JJ, Moshe SL, eds. *Epilepsy: A Comprehensive Textbook 3rd edition*. Wolters Kluwer; in press.

5. Gall CM. Seizure-induced changes in neurotrophin expression: implications for epilepsy. *Exp Neurol*. Nov 1993;124(1):150-66. doi:10.1006/exnr.1993.1186

6. Nawa H, Carnahan J, Gall C. BDNF protein measured by a novel enzyme immunoassay in normal brain and after seizure: partial disagreement with mRNA levels. *Eur J Neurosci*. Jul 1 1995;7(7):1527-35. doi:10.1111/j.1460-9568.1995.tb01148.x

7. Simonato M, Molteni R, Bregola G*, et al*. Different patterns of induction of FGF-2, FGF- 1 and BDNF mRNAs during kindling epileptogenesis in the rat. *Eur J Neurosci*. Mar 1998;10(3):955-63. doi:10.1046/j.1460-9568.1998.00105.x

8. Binder DK, Routbort MJ, McNamara JO. Immunohistochemical evidence of seizure induced activation of trk receptors in the mossy fiber pathway of adult rat hippocampus. *J Neurosci*. Jun 1 1999;19(11):4616-26. Page 22 of 41 ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 Support (434) 964 4100 Brain For Peer Review 23

9. He XP, Minichiello L, Klein R, McNamara JO. Immunohistochemical evidence of seizure-induced activation of trkB receptors in the mossy fiber pathway of adult mouse hippocampus. *J Neurosci*. Sep 1 2002;22(17):7502-8.

10. Paradiso B, Marconi P, Zucchini S*, et al*. Localized delivery of fibroblast growth factor- 2 and brain-derived neurotrophic factor reduces spontaneous seizures in an epilepsy model. *Proc Natl Acad Sci U S A*. Apr 28 2009;106(17):7191-6. doi:10.1073/pnas.0810710106

11. Gu F, Parada I, Yang T, Longo FM, Prince DA. Partial TrkB receptor activation suppresses cortical epileptogenesis through actions on parvalbumin interneurons. *Neurobiol Dis*. May 2018;113:45-58. doi:10.1016/j.nbd.2018.01.018

12. Croll SD, Suri C, Compton DL*, et al*. Brain-derived neurotrophic factor transgenic mice exhibit passive avoidance deficits, increased seizure severity and in vitro hyperexcitability in the hippocampus and entorhinal cortex. *Neuroscience*. 1999;93(4):1491-506. doi:10.1016/s0306-4522(99)00296-1

13. Isgor C, Pare C, McDole B, Coombs P, Guthrie K. Expansion of the dentate mossy fiber- CA3 projection in the brain-derived neurotrophic factor-enriched mouse hippocampus. *Neuroscience*. Mar 12 2015;288:10-23. doi:10.1016/j.neuroscience.2014.12.036

14. Kokaia M, Ernfors P, Kokaia Z, Elmer E, Jaenisch R, Lindvall O. Suppressed epileptogenesis in BDNF mutant mice. *Exp Neurol*. Jun 1995;133(2):215-24. doi:10.1006/exnr.1995.1024

15. Binder DK, Routbort MJ, Ryan TE, Yancopoulos GD, McNamara JO. Selective inhibition of kindling development by intraventricular administration of TrkB receptor body. *J Neurosci*. Feb 15 1999;19(4):1424-36. Page 23 of 41 ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 Support (434) 964 4100 Brain For Peer Review 24

16. Liu G, Gu B, He XP, *et al*. Transient inhibition of TrkB kinase after status epilepticus prevents development of temporal lobe epilepsy. *Neuron*. Jul 10 2013;79(1):31-8. doi:10.1016/j.neuron.2013.04.027

17. Gu B, Huang YZ, He XP, Joshi RB, Jang W, McNamara JO. A Peptide Uncoupling BDNF Receptor TrkB from Phospholipase Cgamma1 Prevents Epilepsy Induced by Status Epilepticus. *Neuron*. Nov 4 2015;88(3):484-91. doi:10.1016/j.neuron.2015.09.032

18. Emili M, Guidi S, Uguagliati B, Giacomini A, Bartesaghi R, Stagni F. Treatment with the flavonoid 7,8-Dihydroxyflavone: a promising strategy for a constellation of body and brain disorders. *Crit Rev Food Sci Nutr*. Sep 11 2020:1-38. doi:10.1080/10408398.2020.1810625

19. Zhang R, Kang KA, Piao MJ, *et al*. Preventive effect of 7,8-dihydroxyflavone against oxidative stress induced genotoxicity. *Biol Pharm Bull*. Feb 2009;32(2):166-71. doi:10.1248/bpb.32.166

20. Terrone G, Balosso S, Pauletti A, Ravizza T, Vezzani A. Inflammation and reactive oxygen species as disease modifiers in epilepsy. *Neuropharmacology*. May 1 2020;167:107742. doi:10.1016/j.neuropharm.2019.107742

21. Kilkenny C, Browne W, Cuthill IC, *et al*. Animal research: reporting in vivo experiments-the ARRIVE guidelines. *J Cereb Blood Flow Metab*. Apr 2011;31(4):991-3. doi:10.1038/jcbfm.2010.220

22. Lidster K, Jefferys JG, Blumcke I, *et al*. Opportunities for improving animal welfare in rodent models of epilepsy and seizures. *J Neurosci Methods*. Feb 15 2016;260:2-25. doi:10.1016/j.jneumeth.2015.09.007

23. Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol*. Mar 1972;32(3):281-94. doi:10.1016/0013- 4694(72)90177-0 Page 24 of 41 ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 Support (434) 964 4100 Brain For Peer Review 25

24. Brandt C, Tollner K, Klee R, Broer S, Loscher W. Effective termination of status epilepticus by rational polypharmacy in the lithium-pilocarpine model in rats: Window of opportunity to prevent epilepsy and prediction of epilepsy by biomarkers. *Neurobiol Dis*. Mar 2015;75:78-90. doi:10.1016/j.nbd.2014.12.015

25. Jang SW, Liu X, Yepes M, *et al*. A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. *Proc Natl Acad Sci U S A*. Feb 9 2010;107(6):2687- 92. doi:10.1073/pnas.0913572107

26. Andero R, Heldt SA, Ye K, Liu X, Armario A, Ressler KJ. Effect of 7,8- , a small-molecule TrkB agonist, on emotional learning. *Am J Psychiatry*. Feb 2011;168(2):163-72. doi:10.1176/appi.ajp.2010.10030326

27. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates, 7th edition*. Academic Press; 2013.

28. Paradiso B, Zucchini S, Su T, *et al*. Localized overexpression of FGF-2 and BDNF in hippocampus reduces mossy fiber sprouting and spontaneous seizures up to 4 weeks after pilocarpine-induced status epilepticus. *Epilepsia*. Mar 2011;52(3):572-8. doi:10.1111/j.1528-1167.2010.02930.x

29. Tchekalarova J, Moyanova S, Fusco AD, Ngomba RT. The role of the melatoninergic system in epilepsy and comorbid psychiatric disorders. *Brain Res Bull*. Oct 2015;119(Pt A):80-92. doi:10.1016/j.brainresbull.2015.08.006

30. Ennaceur A, Michalikova S, Bradford A, Ahmed S. Detailed analysis of the behavior of Lister and Wistar rats in anxiety, object recognition and object location tasks. *Behav Brain Res*. Apr 30 2005;159(2):247-66. doi:10.1016/j.bbr.2004.11.006

31. Schindelin J, Arganda-Carreras I, Frise E, *et al*. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. Jun 28 2012;9(7):676-82. doi:10.1038/nmeth.2019 Page 25 of 41

ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 Support (434) 964 4100 Brain For Peer Review 26

32. Lovisari F, Guarino A, Soukupova M, *et al*. Anti-epileptogenic effect of NRP2945 in the pilocarpine model of temporal lobe epilepsy. *Eur J Pharmacol*. Jun 15 2021;901:174068. doi:10.1016/j.ejphar.2021.174068

33. Bettegazzi B, Sebastian Monasor L, Bellani S, *et al*. Casein Kinase 2 dependent phosphorylation of eIF4B regulates BACE1 expression in Alzheimer's disease. *Cell Death Dis*. Aug 4 2021;12(8):769. doi:10.1038/s41419-021-04062-3

34. Denninger JK, Smith BM, Kirby ED. Novel Object Recognition and Object Location Behavioral Testing in Mice on a Budget. *J Vis Exp*. Nov 20 2018;(141)doi:10.3791/58593

35. Bovolenta R, Zucchini S, Paradiso B, *et al*. Hippocampal FGF-2 and BDNF overexpression attenuates epileptogenesis-associated neuroinflammation and reduces spontaneous recurrent seizures. *J Neuroinflammation*. Nov 18 2010;7:81. doi:10.1186/1742-2094-7-81

36. Reichardt LF. Neurotrophin-regulated signalling pathways. *Philos Trans R Soc Lond B Biol Sci*. Sep 29 2006;361(1473):1545-64. doi:10.1098/rstb.2006.1894

37. Huang YZ, He XP, Krishnamurthy K, McNamara JO. TrkB-Shc Signaling Protects against Hippocampal Injury Following Status Epilepticus. *J Neurosci*. Jun 5 2019;39(23):4624-4630. doi:10.1523/JNEUROSCI.2939-18.2019

38. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. *J Nutr Sci*. 2016;5:e47. doi:10.1017/jns.2016.41

39. Liu X, Obianyo O, Chan CB, *et al*. Biochemical and Biophysical Investigation of the Brain-derived Neurotrophic Factor Mimetic 7,8-Dihydroxyflavone in the Binding and Activation of the TrkB Receptor. *J Biol Chem*. Oct 3 2014;289(40):27571-27584. doi:10.1074/jbc.M114.562561 Page 26 of 41 ScholarOne, 375 Greenbrier Drive, Charlottesville, VA, 22901 Support (434) 964 4100 Brain For Peer Review 27

40. Broer S, Loscher W. Novel combinations of phenotypic biomarkers predict development of epilepsy in the lithium-pilocarpine model of temporal lobe epilepsy in rats. *Epilepsy Behav*. Dec 2015;53:98-107. doi:10.1016/j.yebeh.2015.09.028

41. Pascente R, Frigerio F, Rizzi M, *et al*. Cognitive deficits and brain myo-Inositol are early biomarkers of epileptogenesis in a rat model of epilepsy. *Neurobiol Dis*. Sep 2016;93:146-55. doi:10.1016/j.nbd.2016.05.001

42. Lapinlampi N, Andrade P, Paananen T, *et al*. Postinjury weight rather than cognitive or behavioral impairment predicts development of posttraumatic epilepsy after lateral fluidpercussion injury in rats. *Epilepsia*. Sep 2020;61(9):2035-2052. doi:10.1111/epi.16632

43. Nizinska K, Szydlowska K, Vouros A, *et al*. Behavioral characteristics as potential biomarkers of the development and phenotype of epilepsy in a rat model of temporal lobe epilepsy. *Sci Rep*. Apr 21 2021;11(1):8665. doi:10.1038/s41598-021-88088-9

44. Muttenthaler M, King GF, Adams DJ, Alewood PF. Trends in peptide drug discovery. *Nat Rev Drug Discov*. Apr 2021;20(4):309-325. doi:10.1038/s41573-020-00135-8

Table 1. Antibodies employed in Western Blot.

Antibody	Product no.	Concentration	Blocking	Producer
S473-AKT	4060	1:1000	5% BSA in TBST	Cell Signaling Technology
AKT	4691	1:5000	5% BSA in TBST	Cell Signaling Technology
Phospho-ERK	4370	1:1000	ERK 5% BSA in TBST	Cell Signaling Technology
ERK	9102	1:1000	5% BSA in TBST	Cell Signaling Technology
alpha Tubulin	T9026	1:6000	5% Milk in TBST	Merck
GAPDH (14C10)	2118	1:1000	5% Milk in TBST Cell	Cell Signaling Technology
PLCγ	5690	1:1000	5% BSA in TBST	
Phospho- PLCγ Tyr783	2821	1:1000	5% BSA in TBST	Cell Signaling Technology
TrkB	4603	1:1000	5% BSA in TBST	Cell Signaling Technology
TrkB Y816	ABN1381	1:1000	5% Milk in TBST	Merck
TrkB Y515	LS-C336054-50	1:1000	5% Milk in TBST	LSBio

Figure legends

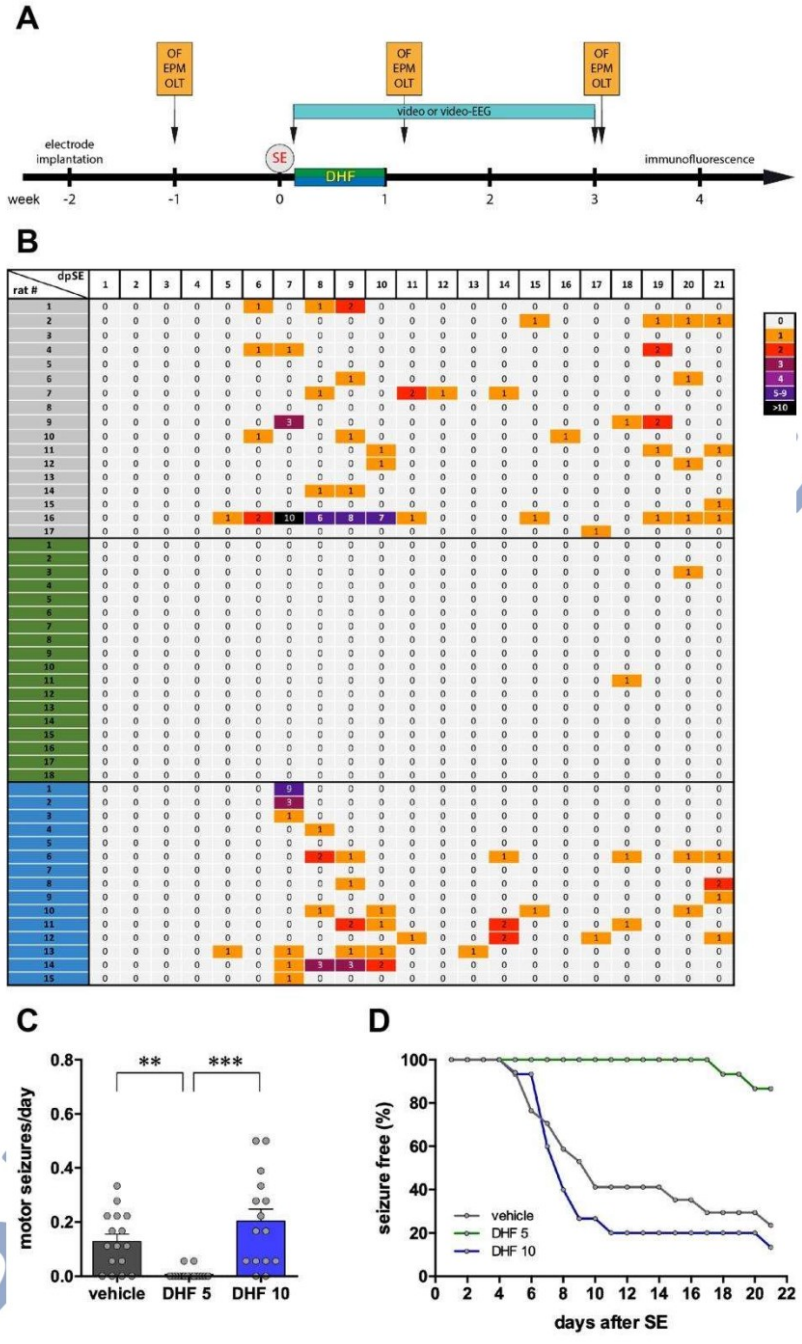


Figure 1. **Spontaneous motor seizures.** (**A**) Experimental plan. (**B**) Heat map (one rat per line) of the number of spontaneous motor seizures (class 4 or 5 according to Racine {Racine, 1972 #32}) detected each day during weeks 1–3 after SE. The number of seizures per day is represented using the color code shown at the right of the panel. (**C**) Average number of spontaneous motor seizures per day in the 3 weeks after SE. Bars represent the mean ± SEM and gray dots

represent data from individual animals. ** p<0.01; *** p<0.001, Kruskal-Wallis one-way ANOVA and *post-hoc* Tukey's test. (**D**) Kaplan-Meier estimates for time to first seizure. Vehicle-treated animals are represented in gray, 7,8-DHF 5 mg/kg (DHF 5) in green, 7,8-DHF 10 mg/kg (DHF 10) in blue. Abbreviation: EPM: elevated plus maze; OF: open field; OLT: object location task; SE: status epilepticus.

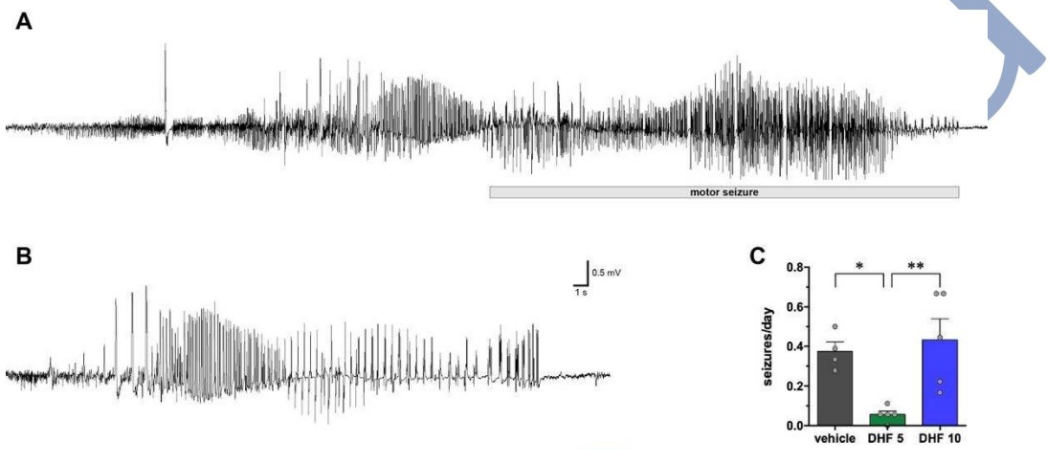


Figure 2. **EEG.** Representative EEG patterns in the hippocampus during motor (A) and nonmotor (B) seizures in vehicle-treated animals. Identical patterns were observed in animals treated with 7,8-DHF 5 or 10 mg/kg. The horizontal bar in A indicates the motor part of the seizure. (C) Average number of spontaneous recurrent seizures (motor and nonmotor) per day in the 3 weeks after SE. Bars represent the mean ± SEM and gray dots represent data from individual animals. * p<0.05; ** p<0.01, Kruskal-Wallis one-way ANOVA and *post-hoc* Tukey's test. Vehicle-treated animals are represented in gray, 7,8-DHF 5 mg/kg (DHF 5) in green, 7,8-DHF 10 mg/kg (DHF 10) in blue.

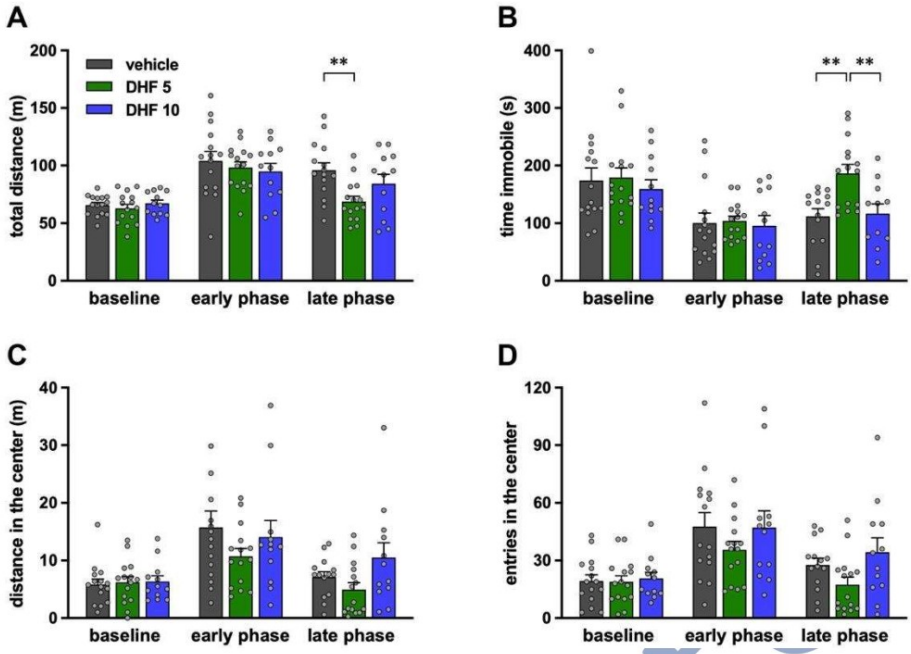


Figure 3. **Open field (OF) test**, performed before SE (baseline), 8 days (early phase) or 21 days (late phase) after SE. (**A**) Total distance run by each rat. (**B**) Time spent immobile. (**C**) Distance run in center quadrants. (**D**) Number of entries in the center quadrants of the arena. Bars represent the mean ± SEM and gray dots represent data from individual animals. ** $p<0.01$, Kruskal-Wallis one-way ANOVA and *post-hoc* Tukey's test. Vehicle-treated animals are represented in gray, 7,8-DHF 5 mg/kg (DHF 5) in green, 7,8-DHF 10 mg/kg (DHF 10) in blue.

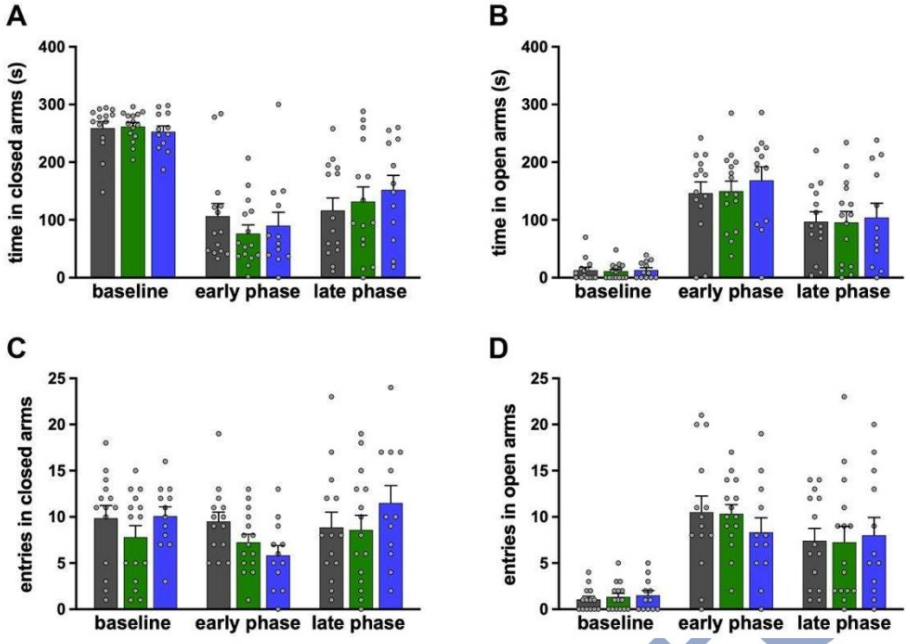


Figure 4. **Elevated plus maze (EPM) test**, performed before SE (baseline), 8 days (early phase) or 21 days (late phase) after SE. (**A**) Time spent in closed arms. (**B**) Time spent in open arms. (**C**) Number of entries in closed arms. (**D**) Number of entries in open arms. Bars represent the mean ± SEM and gray dots represent data from individual animals. Vehicle-treated animals are represented in gray, 7,8-DHF 5 mg/kg (DHF 5) in green, 7,8-DHF 10 mg/kg (DHF 10) in blue.

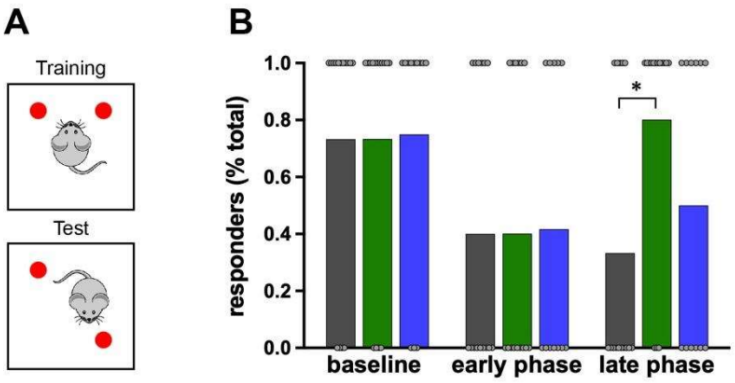


Figure 5. **Object location task (OLT).** (**A**) Schematic representation of the test (see Materials and Methods for additional details). Twenty-four hours after the habituation phase in the empty arena, animals were allowed to explore two identical objects for 5 min (training phase). After a 2h interval, animals were re-entered in the arena, where one object was moved in a different location, and allowed to explore the objects for another 5 min (testing phase). (**B**) Percent of animals spending more time exploring the re-positioned object. Gray dots represent individual animals. Vehicle-treated animals are in gray, 7,8-DHF 5 mg/kg (DHF 5) in green, 7,8-DHF 10 mg/kg (DHF 10) in blue. * $p<0.01$; Fisher's exact test.

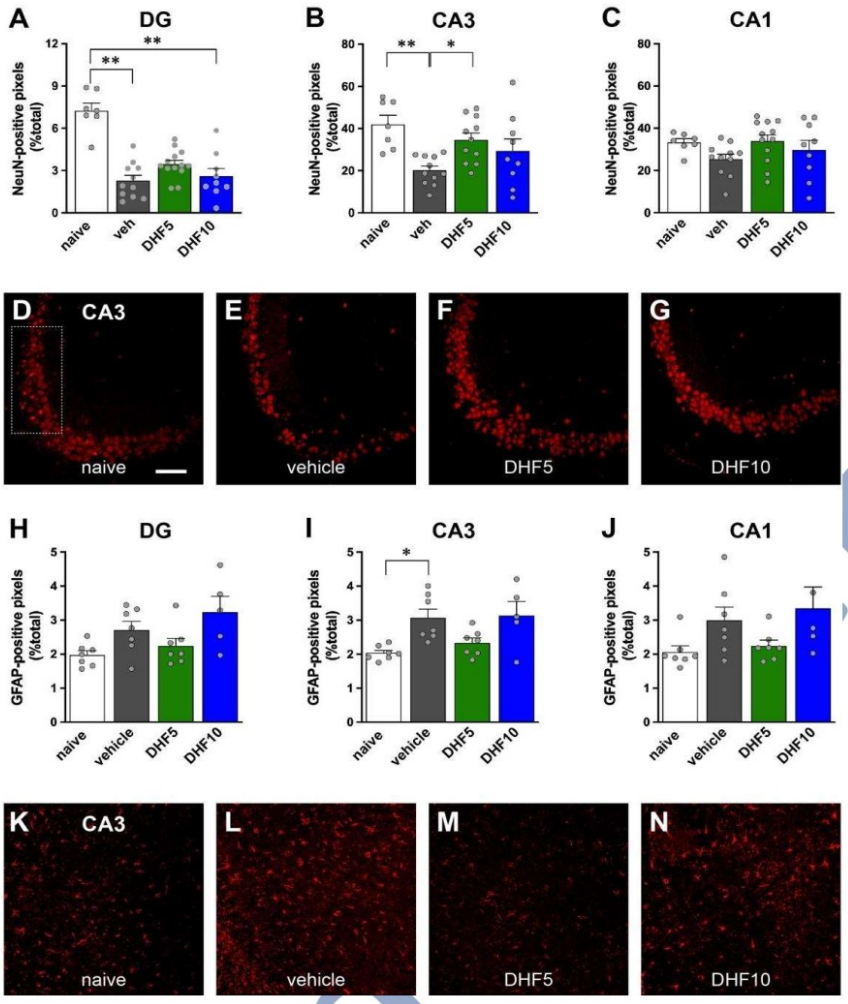


Figure 6. **Immunohistochemical analysis.** Quantification of NeuN-positive pixels in the dentate gyrus (DG, panel **A**), CA3 (**B**) and CA1 area (**C**). Data are expressed as percent of positive pixels within the hilus of the DG or within a rectangular region along the pyramidal layer of the CA3 and CA1 regions, as shown in panel D. See Materials and Methods for details. Bars represent the mean ± SEM and gray dots represent data from individual animals. Naïve animals are in white, vehicle-treated animals in gray, 7,8-DHF 5 mg/kg (DHF 5) in green, 7,8-DHF 10 mg/kg (DHF 10) in blue. * $p<0.05$, ** $p<0.01$, Kruskal-Wallis one-way ANOVA and *post-hoc* Tukey's test. Representative sections at CA3 level of naïve (**D**), vehicle-treated (**E**), DHF 5-treated (**F**) and DHF 10-treated animals (**G**), showing neurons labeled in red with a NeuN antibody. Quantification of GFAP-positive pixels in the DG (**H**), CA3 (**I**) and CA1 area (**J**). Data were generated and represented like in A-C. Statistical analysis was performed like in A-C. Representative sections at CA3 level of naïve (**K**), vehicle-treated (**L**), DHF 5-treated (**M**) and DHF 10-treated animals (**N**), showing astrocytes labeled in red with a GFAP antibody. Horizontal bar in panel D (for all image panels) = 100 μm.

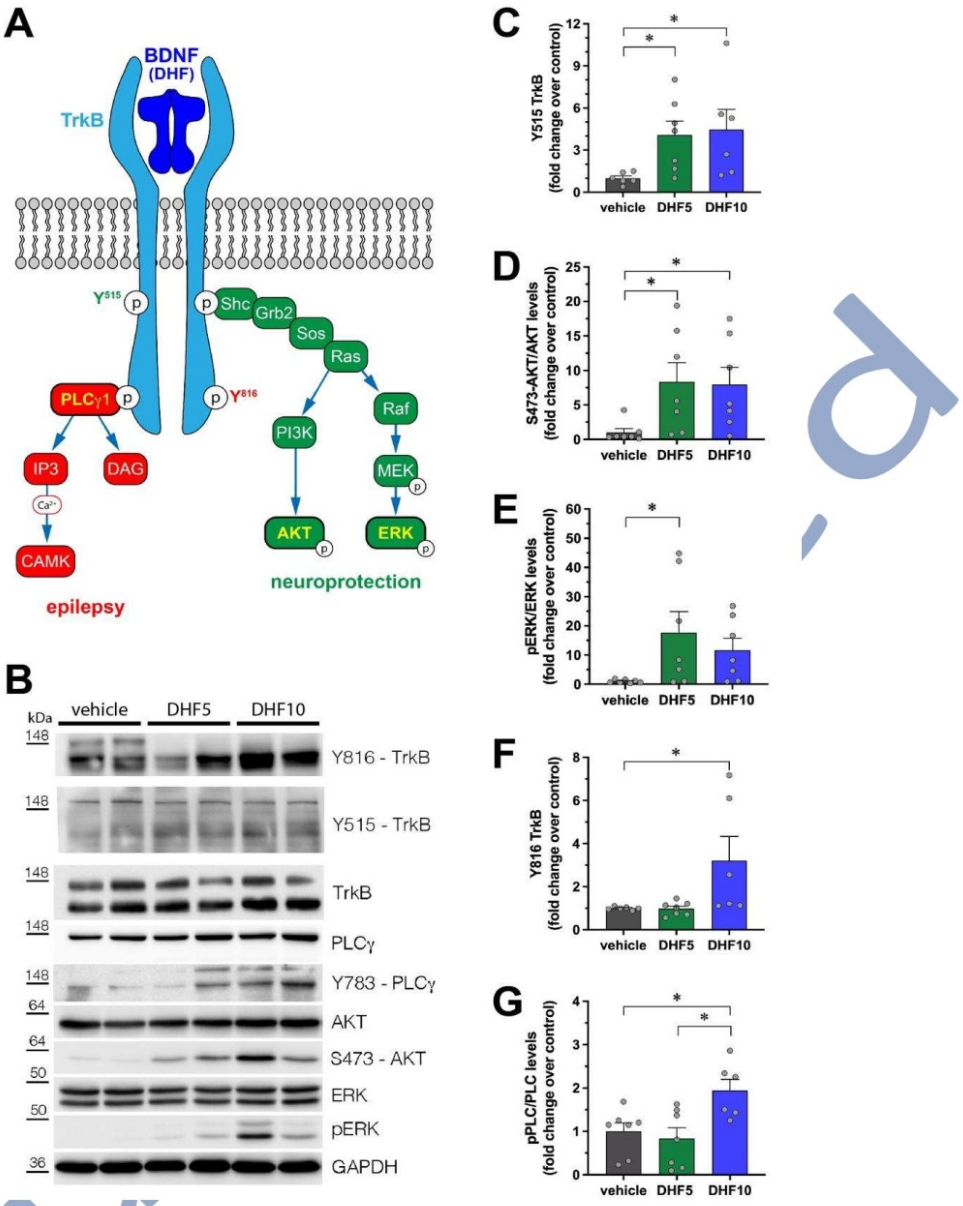


Figure 7. Phosphorylation of TrkB, AKT, ERK and PLCγ proteins in the hippocampi of 7,8-DHF treated rats. (**A**) Schematic representation of the different signaling pathways activated by BDNF (or 7,8-DHF) upon binding to TrkB receptor. (**B**) Representative western blot of the indicated proteins in extracts from hippocampi of DHF treated rats. (**C-G**) Quantification of Y515 TrkB (C), S473-AKT (D), ERK (E), Y816 TrkB (F) and Y783-PLCγ (G) phosphorylation. Protein levels are shown as fold change over control (vehicle-treated rats). Levels of phosphorylated proteins are normalized against the corresponding total protein, then for loading (GAPDH). * $p<0.05$, Kruskal-Wallis one-way ANOVA and *post-hoc* Tukey's test.

4.3 Neural regeneration peptide (NRP) 2945

The Neural Regeneration Peptide (NRP) molecule belongs to a family of proteins which are found in all mammals. NRPs are ultra-potent in in vitro neuronal survival assays. They exert their actions by binding as agonists to a plasma membrane receptor complex at subpicomolar concentrations to the chemokine receptor CXCR4 (Gorba et al 2006). CXCR4 activation by NRP leads to upregulation of $GABA_A$ receptor alpha and beta subunit expression and of increase GABA signalling (Sajadian et al. 2015). The NRP gene family is directly involved in "building" embryonic brain architecture by exerting a chemoattractive effect on migrating and developing neuroblasts. A new NRP peptide was recently developed, NRP2945, whose sequence occurs within the N-terminal region of calcium-dependent secretion activator 2 (CAPS-2). NRP2945 is a synthetic 11-mer peptidomimetic with the sequence H-GRRAAPGRAIBGG-NH2 that has been chemically modified at position four and position nine (alanine switched with the non-natural amino acid α-aminoisobutyric acid), in order to enhance physicochemical shelf-life stability (Dezsi et al. 2017). The large vesicular docking protein CAPS-2 is responsible for the secretion of BDNF and NT-3 (Sadakata et al. 2004).
We aimed at evaluating the effects NRP2945 in the pilocarpine model of mTLE, using two different paradigms of administration: (i) following pilocarpine induced SE, to assess its ability to prevent epilepsy development (i.e., a putative anti-epileptogenic effect), and (ii) in the chronic phase of epilepsy, to assess its effect on spontaneous seizure.
We found that NRP2945 exerts a robust anti-epileptogenic effect, reducing the frequency of spontaneous seizures, exerting a significant neuroprotective effect and attenuating anxiety-like behaviours and cognitive impairment. These effects appear to depend on the modulation of the epileptogenesis process and not on seizure suppression, because NRP2945 did not reduce the frequency or duration of spontaneous seizures when administered to already epileptic animals.

4.3.1

ANTI-EPILEPTOGENIC EFFECT OF NRP2945 IN THE PILOCARPINE MODEL OF TEMPORAL LOBE EPILEPSY

European Journal of Pharmacology 901 (2021) 174068

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Anti-epileptogenic effect of NRP2945 in the pilocarpine model of temporal lobe epilepsy

Francesca Lovisari [a,*,1], Annunziata Guarino [a,1], Marie Soukupova [a], Chiara Falcicchia [a], Selene Ingusci [a], Pietro Marino [a], Mark Thomas [b], Frank Sieg [b], Michele Simonato [a,c]

[a] Department of Neuroscience and Rehabilitation, University of Ferrara, Via Fossato di Mortara 17-19, 44121, Ferrara, Italy
[b] CuroNZ Ltd, Ruakura Research Centre, 10 Bisley Road, Hamilton, 3214, New Zealand
[c] IRCCS San Raffaele Scientific Institute, Via Olgettina 60, 20132, Milan, Italy

ARTICLE INFO

Keywords:
Epilepsy
Cognition
Anxiety
Neural regeneration peptide
Epileptogenesis

ABSTRACT

Innovative therapeutic strategies are highly needed to tackle the major medical needs of epilepsy, like prevention of epilepsy development in at-risk individuals, treatment of severe and drug-resistant forms, control of co-morbidities. The Neural Regeneration Peptide NRP2945 (a peptidomimetic analogue of the human CAPS-2 protein) has been recently found to exert many potentially anti-epileptic effects, for example increased neuronal survival and differentiation. In the present study, we tested the effects of NRP2945 on the development of epilepsy (epileptogenesis) and on chronic, spontaneous seizures, by using the pilocarpine model of temporal lobe epilepsy. We found that NRP2945 exerts a robust anti-epileptogenic effect, reducing the frequency of spontaneous seizures, exerting a significant neuroprotective effect and attenuating anxiety-like behaviors and cognitive impairment. These effects appear to depend on modulation of the epileptogenesis process and not on seizure suppression, because NRP2945 did not reduce frequency or duration of spontaneous seizures when administered to already epileptic animals. These findings may form the basis for a preventive therapy for individuals at-risk of developing epilepsy.

1. Introduction

The term "epilepsy" indicates a heterogeneous group of diseases, all characterized by the occurrence of spontaneous seizures, that is, signs and/or symptoms due to abnormal excessive or synchronous neuronal activity (Fisher et al., 2005). Almost 60 million people worldwide suffer from epilepsy, making it one of the most common neurological diseases (WHO[2]). There is currently no cure and only symptomatic relief can be provided. Patients are treated with antiepileptic drugs (AEDs) for their seizures, but about one third are or become pharmaco-resistant. In addition, many patients (20–50%) develop comorbidities like psychiatric and cognitive disorders that heavily compromise quality of life, and are not controlled by AEDs (Johnson et al., 2004; Boylan et al., 2004). Thus, epilepsy and its associated comorbidities represent urgent unmet medical needs.

One the most common forms of epilepsy in adults is characterized by focal seizures originating in the mesial structures of the temporal lobe (thus, mesial temporal lobe epilepsy, mTLE). mTLE often occurs secondary to epileptogenic events, like a trauma, a stroke, or an episode of prolonged seizures (status epilepticus, SE) occurring in a healthy brain. These damaging insults may set in motion a cascade of neurobiological events that can lead to epilepsy (Pitkanen and Lukasiuk, 2011). In other words, patients may recover from these initial events and be apparently well for months or even years, but then a subset of them will begin experiencing spontaneous seizures and receive a diagnosis of epilepsy (Klein and Tyrlikova, 2020). In principle, these forms of mTLE may be preventable. Unfortunately, however, none of the currently available AEDs proved effective in this respect (Pitkanen and Lukasiuk, 2011) (Temkin, 2001). This indicates that the mechanisms underlying the transformation of a normal brain tissue into an epileptic one (epileptogenesis) differ from those generating seizures (ictogenesis), only the latter being tackled by AEDs (Pitkanen and Lukasiuk, 2011; Temkin,

* Corresponding author. Department of Neurosciences and Rehabilitation, Section of Pharmacology, University of Ferrara, Via Fossato di Mortara 17-19, 44121, Ferrara, Italy.
E-mail address: francesca.lovisari@unife.it (F. Lovisari).
[1] Both authors contributed equally to this work.
[2] https://www.who.int/mental_health/neurology/epilepsy/report_2019/en/.

https://doi.org/10.1016/j.ejphar.2021.174068
Received 28 December 2020; Received in revised form 19 March 2021; Accepted 25 March 2021
Available online 31 March 2021

2001). As an implication, conceptually new drugs should be identified for the prevention of epilepsy.

Innovative, clinically translatable therapeutic strategies are pursued by studying the mechanisms underlying epileptogenesis and identifying novel, druggable therapeutic targets. Many molecular, cellular and/or circuitry alteration(s) have been shown to occur during epileptogenesis, including cell death; axonal and dendritic plasticity; increased, largely aberrant neurogenesis neuroinflammation; and functional alterations in ion channel and synaptic properties (Pitkanen and Lukasiuk, 2011). In the attempt to identify therapeutic approaches to counteract epileptogenesis, a key question remains which of the aforementioned alteration (s) may lead to hyperexcitability and to spontaneous seizures. Although the modulation of some of these events proved effective in preventing epilepsy development in animal models, to date none of these putative treatments has been translated into clinical studies (Pitkanen and Lukasiuk, 2011).

Neural Regeneration Peptides (NRPs) were found to provide lasting survival and regeneration of committed neural stem cells in *ex vivo* thalamocortical tissue (Landgraf et al., 2005). Recently, it has been found that a short (11 amino acid), blood-brain barrier (BBB) penetrable peptide sequence (NRP2945), belonging to the family of NRPs, exerts very potent neuronal survival and differentiation effects and, via activation of the chemokine receptor CXCR4 (Gorba et al., 2006), is also capable of differentially regulating expression of $GABA_A$ receptor subunits (Dezsi et al., 2017; Sajadian et al., 2015; Sieg, 2016). In most mammals, the NRP2945 peptide sequence occurs within the N-terminal region (region 40–50) of calcium-dependent secretion activator 2 (CADPS2, also known as CAPS-2). The large vesicular docking protein CAPS-2 is responsible for the secretion of the neurotrophic factors neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) (Sadakata et al., 2004). Because neuroprotection and proper neurogenesis (Jessberger and Parent, 2015), increased GABA signaling (Bialer and White, 2010) as well as increased BDNF availability can produce antiepileptogenic (Falcicchia et al., 2018) and antiepileptic (Paradiso et al., 2009) effects, it can be hypothesized that NRP2945 may exert positive effects on epileptogenesis and/or on chronic, spontaneous seizures. Supporting evidence for this working hypothesis derives from loss of function mutations within the CAPS-2 gene that result in intellectual disability and drug-resistant seizures (Bonora et al., 2014). Moreover, NRP2945 proved capable of suppressing pentylenetetrazol (PTZ)-induced seizures in Wistar rats as well as absence seizures in the adult GAERS rat model after peripheral (subcutaneous) administration (Sajadian et al., 2015).

To test this hypothesis, we studied the effect of NRP2945 in the pilocarpine model of mTLE. In this model, similar to the human condition, an episode of SE induced by the systemic administration of the chemoconvulsant is followed, after about ten days of apparent well-being, by the occurrence of spontaneous recurrent seizures, i.e. epilepsy. We used two different paradigms of NRP2945 administration: (i) following pilocarpine-induced SE, to assess its ability to prevent epilepsy development (i.e. a putative anti-epileptogenic effect), and (ii) in the chronic phase of epilepsy, to assess its effect on spontaneous seizures.

2. Materials and methods

2.1. Animals

Sprague Dawley male rats (250 g) were purchased from Envigo SRL (Bresso, MI), and were housed under standard conditions: constant temperature (22–24 °C) and humidity (55–65%), 12 h light/dark cycle, water and food at *ad libitum conditions*. They were habituated for a week before starting of the experimental procedures.

2.1.1. Ethics

The number of animals was kept as small as possible to ensure 80% powered results. We followed the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and the recommendations of the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) for improving animal welfare in rodent models of epilepsy and seizures (Lidster et al., 2016). Procedures involving animals and their care were carried out in accordance with European Community, national and local approved guidelines, laws and policies. All experimental protocols were approved by the University of Ferrara Ethics Committee for Animal Experimentation and by the Italian Ministry of Health (authorization: 823/2018-PR).

2.2. Behavioral tests

Animals were tested for epilepsy comorbidities through a battery of behavioral tests, to assess anxiety-related behavior and cognitive impairment. These tests were conducted at several time points, i.e. before the induction of status epilepticus (SE) (baseline), at 7 days post SE (Latency phase), at 18 days post SE (Early Chronic phase) and at 30 days post SE (Chronic phase). Behavioral tests were performed in laboratories of the facility where animals were moved 1 h before the start of the behavioral testing. These rooms were isolated from any source of distraction and noise, and illuminated by suffused red light.

2.2.1. Elevated plus maze (EPM)

This behavioral test is largely used in preclinical research to assess levels of anxiety. The test was run on a 100 cm elevated apparatus, consisting of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 50 cm), connected in a central area (10 × 10 cm), thereby forming a plus shape. Every rat was moved from its cage to the center of the apparatus and allowed to explore its environment for 5 min. Different parameters were taken into account: time spent in the open and closed arms, number of entries recorded in the open and closed arms, number of *rearings*, number of *stretched-attended postures* (SAP, i.e. how many times the rat looked at the open arms while its body being in a closed arm) and number of *head dippings* (HD, i.e. how many times the test subject looked down from the open arms of the EPM apparatus). In addition, we decided to also consider the total time spent on the apparatus, because epileptic rats frequently jumped out of the apparatus before the end of the 5 min testing paradigm.

2.2.2. Novel object recognition (NOR)

This test assesses cognitive abilities of rodents, and specifically recognition memory. The test was performed inside a squared arena (75 × 75 × 50 cm), and was composed of three different phases. 1) Habituation, namely when the animal was left undisturbed to explore the arena for 10 min, and then moved back to its cage. 2) Familiarization (24 h after habituation), when the rat was moved inside the arena, where two identical objects were placed on symmetrical corners. The test subject was allowed to explore the two objects for 5 min, while the operator kept note of the time spent with each of them. 3) Testing period (2 h after familiarization), involved the replacement of one familiar object with a new one. This phase, like the previous one, was set to last 5 min and again the operator took note of the exploration time dedicated to each object.

2.3. Pilocarpine

The day after the baseline behavioral tests, status epilepticus (SE) was induced through the administration of pilocarpine, an agonist of cholinergic muscarinic receptors. A muscarinic antagonist incapable of crossing the blood-brain barrier (methyl-scopolamine, 1 mg/kg, s.c.) was injected 30 min before pilocarpine administration to prevent peripheral adverse effects. Pilocarpine was then administered (340 mg/kg, i.p.) and each rat was cautiously monitored, taking notes of seizure activity according to the Racine scale (Racine et al., 1972). Usually continuous, long-lasting behavioral seizure activity (i.e. convulsive SE) begins within 30 min of pilocarpine administration. If this did not

happen, a second half dose of pilocarpine (170 mg/kg, i.p.) was administered 30 min after the first. Two h after its onset, SE was stopped using the GABAA receptor agonist diazepam (10 mg/kg, i.p.).

A total of 40 rats underwent this procedure. Two did not enter SE and 10 died during SE or at the following day. Therefore, 28 rats that developed and survived SE were randomly divided into two groups for the anti-epileptogenesis protocol: NRP2945-treated (n = 12) and vehicle-treated controls (n = 16). For the anti-seizure protocol, the former 16 control rats were further subdivided in two subgroups, NRP2945-treated (n = 7) and vehicle-treated (n = 9). NRP2945 or vehicle were injected daily, from day 1–7 post SE in the anti-epileptogenesis protocol and from day 20–27 post SE in the anti-seizure protocol. All rats were continuously video-monitored 24/7, using a video-surveillance acquisition system (BioPac, Goleta, CA). Videos were reviewed by researchers that were blind of the group to which the animals belonged. These researchers recorded occurrence and duration of each individual generalized motor seizure (class 4–5 according to Racine, 1972). NRP2945 (20 μg/kg, s.c. with the batch displaying 99% purity) was administered once daily. NRP2945 has a plasma half-life of 22–25 min, Tmax of 7 min and Cmax of 1.5–2 ng/ml. The dose choice of 20 μg/kg NRP2945 was made according to the most efficacious pharmacodynamic range in rodents, as previously established (Sajadian et al., 2015; Dezsi et al., 2017).

2.4. NeuN immunofluorescence

Vehicle and NRP2945-treated rats enrolled in the anti-epileptogenesis protocol were killed by decapitation after an anesthetic overdose, one month after pilocarpine-induced SE. In addition, a group of 5 naïve rats that did not receive pilocarpine was used as control. Immunofluorescence was performed as previously described (Lovisari et al., 2020). Brains were removed and immersed in 10% formalin for 48 h, then processed using a standard protocol (VTP 300, Bio-Optica, Milan, Italy) and paraffin-embedded. Groups of 20 coronal sections (6 μm thick) were collected at 250 μm intervals within the dorsal hippocampus (−3.8, −4.05 and −4.3 mm from bregma (Paxinos and Watson, 1982)) and mounted onto polarized slides (Superfrost slides, Diapath Martinengo, BG, Italy). Sections were dewaxed with 2 washes in xylol (10 min each), 5 min in ethanol 100%, and rehydrated in ethanol 95%, ethanol 80% and phosphate buffered saline (PBS) 1× (5 min each).

The NeuN antigen was unmasked with a solution of citric acid and sodium citrate in a microwave oven at 750 W (5 cycles of 5 min). Sections were incubated in 0.3% Triton X-100 for 10 min and then with 5% bovine serum albumin (BSA) and 5% goat serum, for 30 min at room temperature. Thereafter, they were incubated in a humid plastic box with the primary antibody, overnight at 4 °C. The primary antibody was anti-NeuN/Fox-3 1:100 (mouse monoclonal, #MAB-90228 Immunological Science, Rome, Italy), that labels neuronal nuclei and cytoplasm. Two washes in 1× PBS and a 30 min incubation in 0.3% Triton X-100 were performed before applying the secondary antibody, 594 Alexa-Fluor anti-mouse (diluted 1:500 in 1× PBS). Sections were left in a dark chamber under controlled humidity conditions for 3 h before proceeding with DAPI staining (0.0001% in 1xPBS for 15 min; Santa Cruz, Texas, USA) to label nuclei. Coverslips were mounted using an aqueous anti-fading mounting gel (Sigma).

Images at the level of the dentate gyrus, CA3 and CA1 region were captured using a Leica microscope (DMRA2, Leica) using a 20× objective. NeuN-positive cells in the hilus of the dentate gyrus were counted in 6 sections (2 per level) from each animal. Hilar cells were those situated between the granule layer and the CA3 pyramidal neurons, remaining between the boundaries of the dentate gyrus (inner CA3 field or CA3c) (Scharfman and Myers, 2012). Cell counting was performed by a researcher that was blind of the animal group to which slices belonged. Average values of the 6 sections were employed for statistical analysis, that is, data obtained from the multiple sections examined for each rat were averaged to obtain a single estimate for each animal.

3. Results

3.1. Effects of NRP2945 on epileptogenesis

NRP2945 (20 μg/kg, s.c.) was administered for 7 days during the latency period, starting 24 h after cessation of SE (n = 12). This dose regimen was previously reported to ensure adequate drug concentrations in the picomolar range in the rat brain (Dezsi et al., 2017). Animals treated with vehicle were used as controls (n = 16). A graphic outline of the protocol is shown in Fig. 1.

3.1.1. Spontaneous recurrent seizures (SRS)

Video-monitoring (24/7) was performed from day 3–20 post SE, and generalized seizures (class 4 and 5 according to Racine) were recorded. NRP2945 significantly reduced the cumulative number of seizures (Fig. 2A), and this effect continued after discontinuation of treatment (Fig. 2B). However, duration of individual seizures was not modified (vehicle: 35 ± 2 s; NRP2945: 36 ± 4 s).

3.1.2. Elevated plus maze (EPM)

The EPM was employed for an initial assessment of anxiety-related behavior after SE. Under normal conditions, rats explore for a significantly longer time the closed arms of the apparatus, i.e. the area perceived as safer. Epileptic animals display a restless, anxiety-like behavior because 1) often fail to complete the task and jump out of the apparatus before completion of the testing paradigm set at 300 s; 2) spend equal or more time in the open than in the closed arms. When analyzing the results by considering only the animals that stayed in the apparatus for more than 30 s, no significant difference emerged between the groups (Fig. 3A and 3B). However, whereas many vehicle-treated rats jumped out of the apparatus within 30 s both during latency (50% of the animals) and the early chronic phase (44%), none of those treated with NRP2945 displayed such behavior (Fig. 3C).

3.1.3. Novel object recognition (NOR)

No change in NOR outcome was observed at the end of the treatment week (latency), but a significant improvement was instead observed later, when animals were in the chronic period (Fig. 4A). In fact, whereas control animals displayed a progressive decline in the time of exploration of the novel object, which became significant in the Chronic period ($P < 0.05$, Mann-Whitney U test), when the novel object was indistinguishable from the familiar one, NRP2945-treated animals appeared to maintain cognitive abilities, continuing to distinguish the novel from the familiar object (Fig. 4A). Control animals displayed an apparent tendency to increase the total time of exploration during latency, which may be attributed to their "restless" behavior observed in the EPM test, but then progressively reduced it (Fig. 4B), as previously reported (Falcicchia et al., 2018; Paolone et al., 2019). In contrast, NRP2945-treated rats displayed a constant time of exploration across the different phases on the disease (Fig. 4B).

3.1.4. Immunofluorescence

To evaluate effects on epilepsy-associated neuronal death, we used NeuN immunofluorescence (Fig. 5). A loss in NeuN-positive cells can be observed in the hilus of the dentate gyrus of animals experiencing spontaneous recurrent seizures (SRSs), one month after pilocarpine-induced SE, but NRP2945-treated animals were partially protected from damage (Fig. 5A-D). In addition, loss of CA3 pyramidal neurons (defined as NeuN counts lower than average minus two standard deviations of the counts in naïve animals) was observed in all pilocarpine animals treated with vehicle, but only in 4 of 7 of those treated with NRP2945 (Fig. 5E-G), and loss of CA1 pyramidal neurons was observed in 3 of 9 pilocarpine animals treated with vehicle, but only in 1 of 7 of those treated with NRP2945 (Fig. 5E-G).

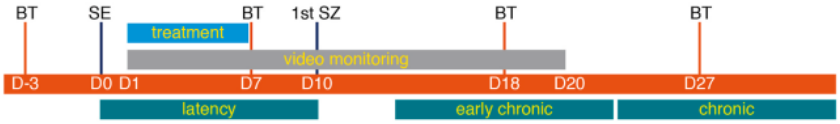


Fig. 1. Timeline of the anti-epileptogenesis protocol. BT = behavioral tests; SZ = seizure; D = day.

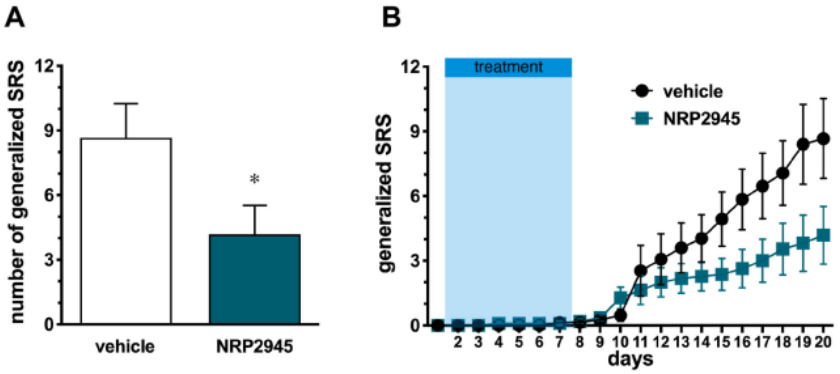


Fig. 2. Seizure quantification in the anti-epileptogenesis protocol. (A) Cumulative number of generalized spontaneous recurrent seizures (SRS) in the 20 days after SE. (B) Number of generalized SRS in time (days after SE). Data are the means ± S.E.M. of 16 vehicle- and 12 NRP2945-treated animals. *P < 0.05; Mann Whitney *U* test.

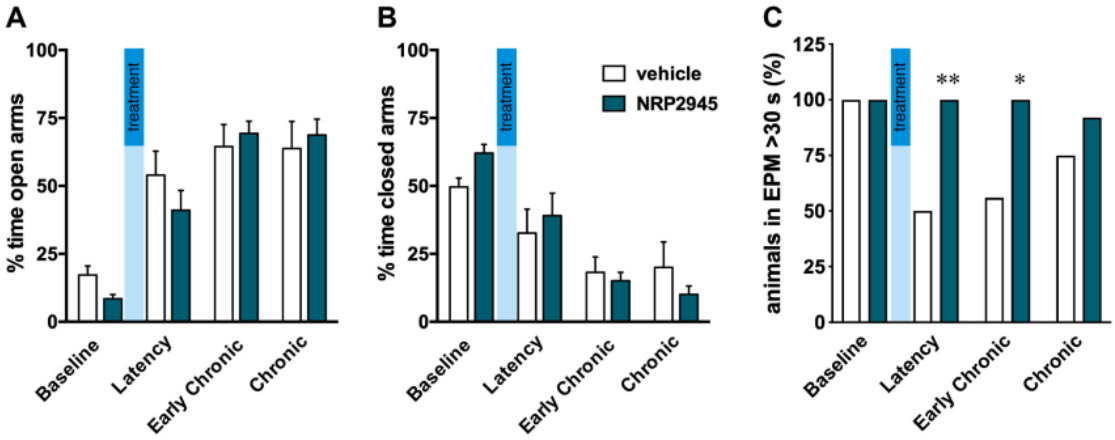


Fig. 3. EPM testing in the anti-epileptogenesis protocol. Time (% total) spent in the open arms (A) and in the closed arms (B) of the EPM apparatus at the different time points. Data are the means ± S.E.M. of 16 vehicle- and 12 NRP2945-treated animals. (C) Percentage of rats staying on the apparatus for more than 30 s at Baseline (vehicle 16 of 16 rats; NRP2945 12 of 12), Latency (vehicle 8/16; NRP2945 12/12), Early chronic (vehicle 9/16; NRP2945 12/12) and Chronic stage (vehicle 12/16; NRP2945 11/12). **P < 0.01; *P < 0.05; Fisher's exact test.

3.2. *Effects of NRP2945 on spontaneous recurrent seizures*

In this protocol, animals have been first monitored for occurrence of SRSs to verify epilepsy development. The first spontaneous seizure occurred 10 ± 1 days after SE. After another 10-day "stabilization" period, i.e. about 20 d after SE, rats were treated with NRP2945 (20 μg/kg, s.c.) or vehicle once daily for 7 days. Frequency and severity of SRSs were measured during this week of treatment, and behavioral tests were performed at the end of it. An outline of the experimental design is provided in Fig. 6A.

3.2.1. *Spontaneous recurrent seizures (SRS)*

No difference was observed in the frequency (Fig. 6B) and duration (Fig. 6C) of SRSs between epileptic rats treated with NRP2945 and rats receiving vehicle.

3.2.2. *Elevated plus maze (EPM)*

Again, no significant differences were observed between groups in the time spent in the open (Fig. 6D) or closed arms (Fig. 6E). All animals in all groups completed the task (none jumped off the apparatus) and no difference was observed on other EPM-related behaviors (rearings, stretched-attend postures and head dipping; data not shown).

3.2.3. *Novel object recognition (NOR)*

As described above in the anti-epileptogenesis treatment paradigm, control animals displayed a progressive decline in novel object exploration, which became significant in the Chronic period (P < 0.05, Mann-Whitney *U* test), when the novel object was indistinguishable from the

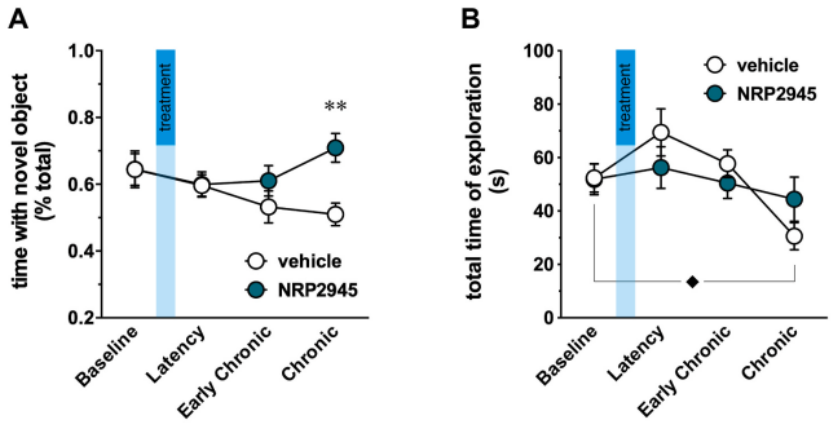


Fig. 4. NOR testing in the anti-epileptogenesis protocol. (A) Time with the novel object; (B). Data are expressed as the time spent exploring the novel object divided by the total exploration time (A) and total time of exploration (B). Data are the means ± S.E.M. of 16 vehicle- and 12 NRP2945-treated animals. **P < 0.01; NRP2945 vs. vehicle, Mann Whitney *U* test. ◆ P < 0.05; vehicle Chronic vs. vehicle Baseline, Mann Whitney *U* test.

familiar one (same time spent exploring the familiar and the novel object). The NRP2945 treatment in already epileptic animals displayed a tendency to restore cognitive abilities, but this effect did not reach statistical significance (Fig. 6F).

4. Discussion

The main finding of the present study is that NRP2945 can exert an anti-epileptogenic effect when administered during latency, as documented by an approximately 50% reduction of SRSs. In parallel, NRP2945 also seems to attenuate anxiety-like behaviors and epilepsy-induced cognitive impairment. This finding is important to the field, because there is so far no clear clinical or preclinical evidence that any of the currently available AEDs, when administered during the latency period, would prevent the development of epilepsy in at risk individuals. Only some experimental treatments proved effective in animal models (Pitkanen and Lukasiuk, 2011), and cannabidiol (CBD) displays effects against SRS only when applied before SE (Patra et al., 2019). In contrast, NRP2945 produced anti-epileptogenic effects under clinically-relevant conditions, i.e. when administered in the latency period after SE.

Interestingly, these effects do not appear to be dependent on seizure suppression, because NRP2945 did not reduce frequency and duration of SRS when administered within a short-term dosing regimen of a week to already epileptic animals, nor did NRP2945 produce in these animals any significant effect on anxiety-related behaviors or cognitive impairment. The lack of effect on SRS was unexpected, because NRP2945 proved previously effective in blocking seizures in models of acute (Sajadian et al., 2015) and genetic (Dezsi et al., 2017) epilepsy, namely the PTZ and the GAERS rat models. However, acute seizures in an otherwise normal brain, like in the PTZ model, are likely different from spontaneous seizures in an epileptic brain, and absence seizures in the GEARS model are obviously very different from the convulsive seizures we monitored in the chronic phase of the pilocarpine model. However, it remains to be determined if a more repetitive dosing profile with NRP2945, lasting several weeks, may generate a SRS frequency reduction.

The mechanism(s) of this specific SRS frequency lowering effect of NRP2945 on epileptogenesis remains to be investigated. As stated in the Introduction, NRP peptides, and NRP2945 in particular, have been reported to exert multiple, pleiotropic effects: increased neurogenesis and neuroprotection, up-regulation of GABA signaling, release of BDNF (Sieg, 2016). Up-regulation of GABA signaling should primarily produce an anti-seizure effect, because a number of mechanistically heterogeneous pharmacological interventions capable of potentiating GABA can attenuate seizures not only in acute, but also in chronic models, and are in fact at the basis of the mechanism of action of many anti-epileptic drugs (Bialer and White, 2010). The alternative hypothesis of increased BDNF release also cannot satisfactorily explain the results. Increased availability of BDNF can exert anti-seizure effects (Falcicchia et al., 2018), while its effect on epilepsy development is still debated, with data supporting anti-epileptogenesis (Paradiso et al., 2009), others pro-epileptogenesis (Lin et al., 2020), most likely depending on the TrkB signaling pathway that gets activated (Gu et al., 2015; Huang et al., 2019).

Therefore, the remaining hypothesis relates to the neurotrophic effects of NRP2945. Indeed, we found that the neuronal loss associated with the development of epilepsy in the pilocarpine model was attenuated by NRP2945 treatment. In addition to that, NRP2945 may also improve neurogenesis. Not only NRPs can increase neuronal survival, but may also increase neuronal differentiation of committed adult neural stem cells and may attenuate those aberrant traits of epileptogenesis-associated neurogenesis that are thought to contribute to epilepsy development (Danzer, 2018). In fact, it has been recently shown that CXCR4 deletion in mice reduces adult neurogenesis and leads to the appearance of ectopic granule cells (Sakai et al., 2018). Because aberrant neurogenesis is associated with epileptogenesis but is not observed in chronic epilepsy, when instead neurogenesis is depressed, a prominent effect of NRP2945 at this level would explain our current observations.

We performed some initial experiments to evaluate the potential effect of NRP2945 on common epilepsy comorbidities, like anxiety and cognitive impairment. The former was examined by using the EPM test. The achieved results turned out to be difficult to interpret because many of the control animals were hyperactive and restless, to the point of deliberately jumping out of the apparatus within seconds after test start. This may, however, be considered as an anxiety-like behavior, that was prevented by NRP2945. When restricting the conventional analysis (time in open arms vs. time in closed arms) only to those controls that remained in the apparatus for a sufficiently long time, no difference was observed between vehicle- and NRP2945-treated animals. However, the comparison here was between NRP2945 and the "less stressed" subset of control animals, indicating that the treatment can indeed attenuate anxiety, leading all NRP2945 treated animals to behave like the less hyperactive controls.

Cognitive impairment was analyzed using the NOR test. Indeed, pilocarpine leads to a progressive impairment in this test, up to the point that animals become incapable of distinguishing the novel from the familiar object in the chronic period, i.e. spent identical time exploring

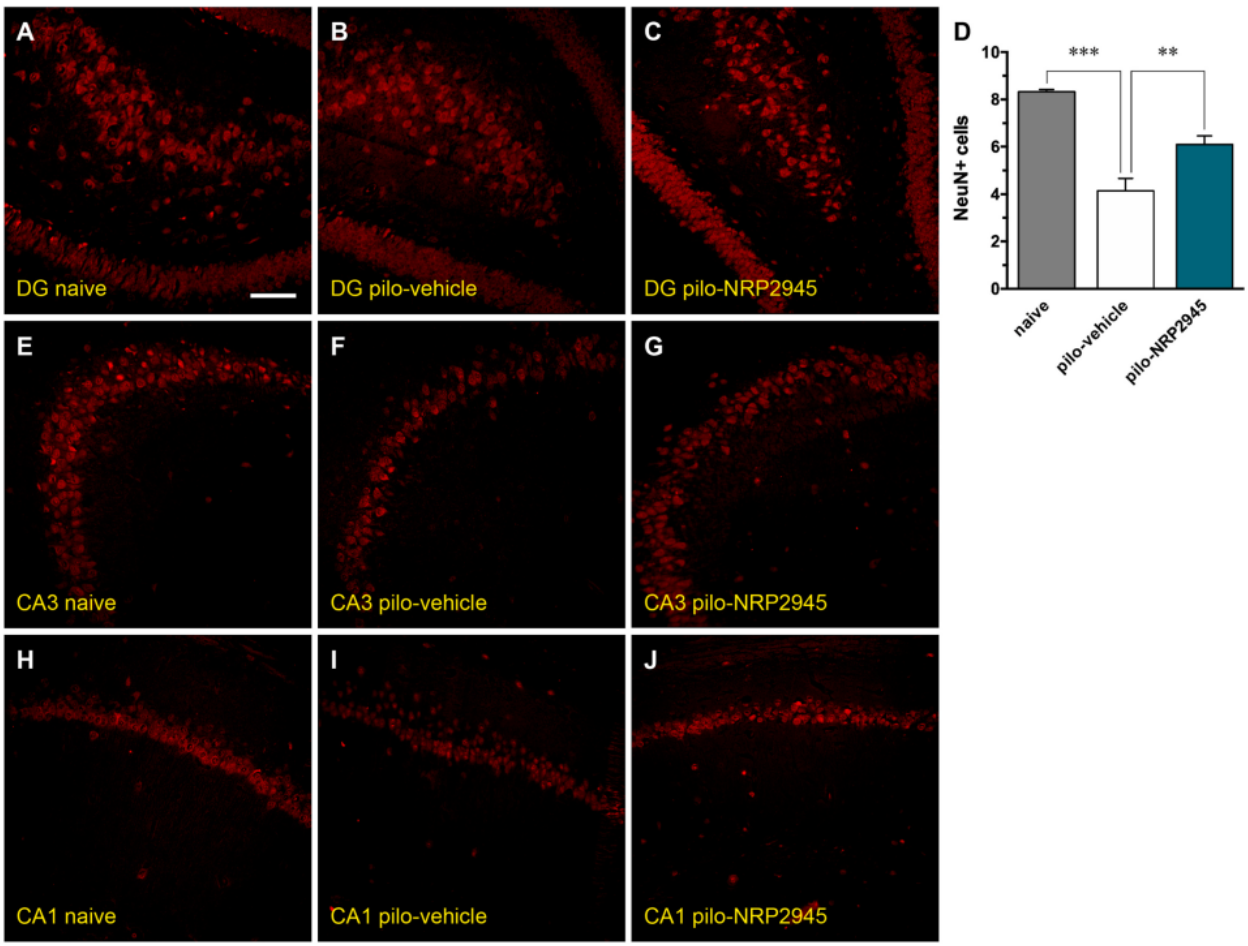


Fig. 5. NeuN immunofluorescence. Shown are representative sections of the dentate gyrus area from naïve (A), pilocarpine animals treated with vehicle (B) and pilocarpine animals treated with NRP2945 (C). Neurons are labelled in red with NeuN. Data quantification is reported in D. Images taken at the level of CA3 are shown in E (naïve), F (pilocarpine animal treated with vehicle) and G (pilocarpine animals treated with NRP2945). Note the loss of pyramidal neurons in F, a pattern that was observed in all animals of this group, but only in 4 of 7 animals treated with NRP2945. Images taken at the level of CA1 are shown in H (naïve), I (pilocarpine animal treated with vehicle) and J (pilocarpine animals treated with NRP2945). Note the loss of pyramidal neurons in I, a pattern that was observed in 3 of 9 animals of this group, but only in 1 of 7 animals treated with NRP2945. Horizontal bar in panel A (for all panels) = 100 μm. Data in panel D are the means ± S.E. M. of 5 naïve, 9 pilocapine-vehicle (pilo-vehicle) and 7 pilo-NRP2945. ***P < 0.001; **P < 0.01; ANOVA and post-hoc Tukey test for multiple comparisons.

both objects (Falcicchia et al., 2018; Paolone et al., 2019). In contrast, rats treated with NRP2945 during latency did not display measurable impairment in this test. These data should be considered with caution until confirmed in a proper battery of cognitive tests. Nonetheless, this effect of NRP2945 is in line with previous data showing that the specific CXCR4 inhibitor AMD3100 prevents the recovery of memory deficits induced by intranasal administration of a nerve growth factor analogue (hNGFp) in the 5xFAD mouse model of Alzheimer disease (Capsoni et al., 2017). BDNF release may also contribute to this effect, because direct intra-hippocampal BDNF administration was found to attenuate cognitive impairment in the pilocarpine model (Falcicchia et al., 2018).

The aim of this study was to explore the effects of NRP2945 on epilepsy development and on chronic, refractory epilepsy. The specific effects we observed on the former suggest a preventive potential for this compound, that may form the basis for the development of treatments for individuals that are at-risk of developing epilepsy after exposure to an epileptogenic insult (head trauma, stroke, encephalitis, status epilepticus). Further studies will be needed to refine and strengthen the evidence (by performing EEG monitoring and investigating other preclinical models of epileptogenesis) and to clarify the mechanism(s) of these promising effects.

Credit author statement

Francesca Lovisari: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. **Annunziata Guarino**: Methodology, Formal analysis, Investigation. **Marie Soukupova**: Methodology, Investigation. **Chiara Falcicchia**: Methodology, Investigation. **Selene Ingusci**: Methodology, Investigation. **Pietro Marino**: Methodology, Investigation, Visualization. **Mark Thomas**: Conceptualization, Funding acquisition. **Frank Sieg**: Conceptualization, Funding acquisition. **Michele Simonato**: Conceptualization, Writing - Review & Editing, Visualization, Supervision.

Funding

The compound NRP2945 and placebo ingredients were a gift from CuroNZ. CuroNZ sponsored the whole study, contributed to the study design and approved the decision to submit the article for publication, but was not involved in the collection, analysis and interpretation of

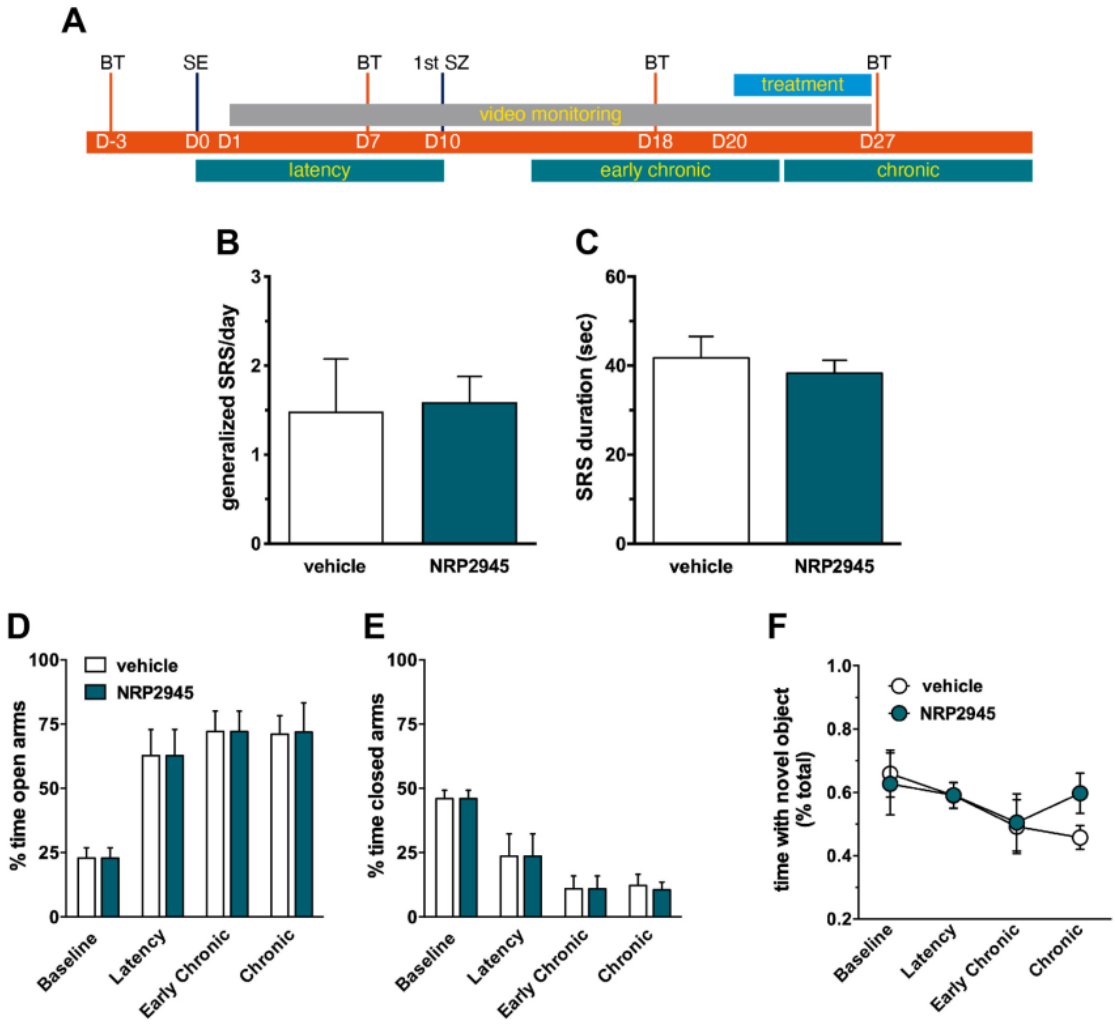


Fig. 6. (A) Timeline of the anti-seizure protocol. BT = behavioral tests; SZ = seizure; D = day. (B) and (C) Spontaneous generalized spontaneous recurrent seizures (SRS). Frequency (B) and duration (C) of generalized SRS in the anti-seizure protocol. Data are the means ± S.E.M. of 7 animals per group. (D) and (E) EPM. Time (% total) spent in the open arms (D) and in the closed arms (E) of the EPM apparatus in the different time points. (F) NOR. Data are expressed as the time spent exploring the novel object divided by the total exploration time. All data are the means ± S.E.M. of 7 animals per group.

data, nor in the writing of the report.

Declaration of competing interest

F. Sieg and M. Thomas are employees of CuroNZ Ltd.

Acknowledgements

None.

References

Bialer, M., White, H.S., 2010. Key factors in the discovery and development of new antiepileptic drugs. Nat. Rev. Drug Discov. 9, 68–82.

Bonora, M., De Marchi, E., Patergnani, S., Suski, J.M., Celsi, F., Bononi, A., Giorgi, C., Marchi, S., Rimessi, A., Duszynski, J., Pozzan, T., Wieckowski, M.R., Pinton, P., 2014. Tumor necrosis factor-alpha impairs oligodendroglial differentiation through a mitochondria-dependent process. Cell Death Differ. 21, 1198–1208.

Boylan, L.S., Flint, L.A., Labovitz, D.L., Jackson, S.C., Starner, K., Devinsky, O., 2004. Depression but not seizure frequency predicts quality of life in treatment-resistant epilepsy. Neurology 62, 258–261.

Capsoni, S., Malerba, F., Carucci, N.M., Rizzi, C., Criscuolo, C., Origlia, N., Calvello, M., Viegi, A., Meli, G., Cattaneo, A., 2017. The chemokine CXCL12 mediates the anti-amyloidogenic action of painless human nerve growth factor. Brain 140, 201–217.

Danzer, S.C., 2018. Contributions of adult-generated granule cells to hippocampal pathology in temporal lobe epilepsy: a neuronal bestiary. Brain Plast. 3, 169–181.

Dezsi, G., Sieg, F., Thomas, M., O'Brien, T.J., van der Hart, M., Jones, N.C., 2017. Disease-modifying effects of neural regeneration peptide 2945 in the GAERS model of absence epilepsy. Neurochem. Res. 42, 2055–2064.

Falcicchia, C., Paolone, G., Emerich, D.F., Lovisari, F., Bell, W.J., Fradet, T., Wahlberg, L. U., Simonato, M., 2018. Seizure-suppressant and neuroprotective effects of encapsulated BDNF-producing cells in a rat model of temporal lobe epilepsy. Mol. Ther. Methods Clin. Dev. 9, 211–224.

Fisher, R.S., van Emde Boas, W., 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.

Gorba, T., Bradoo, P., Antonic, A., Marvin, K., Liu, D.X., Lobie, P.E., Reymann, K.G., Gluckman, P.D., Sieg, F., 2006. Neural regeneration protein is a novel chemoattractive and neuronal survival-promoting factor. Exp. Cell Res. 312, 3060–3074.

Gu, B., Huang, Y.Z., He, X.P., Joshi, R.B., Jang, W., McNamara, J.O., 2015. A peptide uncoupling BDNF receptor TrkB from phospholipase Cgamma1 prevents epilepsy induced by status epilepticus. Neuron 88, 484–491.

Huang, Y.Z., He, X.P., Krishnamurthy, K., McNamara, J.O., 2019. TrkB-shc signaling protects against hippocampal injury following status epilepticus. J. Neurosci. 39, 4624–4630.

Jessberger, S., Parent, J.M., 2015. Epilepsy and adult neurogenesis. Cold Spring Harb. Perspect. Biol. 7.
Johnson, E.K., Jones, J.E., Seidenberg, M., Hermann, B.P., 2004. The relative impact of anxiety, depression, and clinical seizure features on health-related quality of life in epilepsy. Epilepsia 45, 544–550.
Klein, P., Tyrlikova, I., 2020. No prevention or cure of epilepsy as yet. Neuropharmacology 168, 107762.
Landgraf, P., Sieg, F., Wahle, P., Meyer, G., Kreutz, M.R., Pape, H.C., 2005. A maternal blood-borne factor promotes survival of the developing thalamus. Faseb. J. 19, 225–227.
Lidster, K., Jefferys, J.G., Blumcke, I., Crunelli, V., Flecknell, P., Frenguelli, B.G., Gray, W.P., Kaminski, R., Pitkanen, A., Ragan, I., Shah, M., Simonato, M., Trevelyan, A., Volk, H., Walker, M., Yates, N., Prescott, M.J., 2016. Opportunities for improving animal welfare in rodent models of epilepsy and seizures. J. Neurosci. Methods 260, 2–25.
Lin, T.W., Harward, S.C., Huang, Y.Z., McNamara, J.O., 2020. Targeting BDNF/TrkB pathways for preventing or suppressing epilepsy. Neuropharmacology 167, 107734.
Lovisari, F., Roncon, P., Soukoupova, M., Paolone, G., Labasque, M., Ingusci, S., Falcicchia, C., Marino, P., Johnson, M., Rossetti, T., Petretto, E., Leclercq, K., Kaminski, R.M., Moyon, B., Webster, Z., Simonato, M., Zucchini, S., 2020. Implication of Sestrin3 in epilepsy and its comorbidities. Brain Commun. 3, 1–14 fcaa130.
Paolone, G., Falcicchia, C., Lovisari, F., Kokaia, M., Bell, W.J., Fradet, T., Barbieri, M., Wahlberg, L.U., Emerich, D.F., Simonato, M., 2019. Long-term, targeted delivery of GDNF from encapsulated cells is neuroprotective and reduces seizures in the pilocarpine model of epilepsy. J. Neurosci. 39, 2144–2156.
Paradiso, B., Marconi, P., Zucchini, S., Berto, E., Binaschi, A., Bozac, A., Buzzi, A., Mazzuferi, M., Magri, E., Navarro Mora, G., Rodi, D., Su, T., Volpi, I., Zanetti, L., Marzola, A., Manservigi, R., Fabene, P.F., Simonato, M., 2009. Localized delivery of fibroblast growth factor-2 and brain-derived neurotrophic factor reduces spontaneous seizures in an epilepsy model. Proc. Natl. Acad. Sci. U. S. A. 106, 7191–7196.
Patra, P.H., Barker-Haliski, M., White, H.S., Whalley, B.J., Glyn, S., Sandhu, H., Jones, N., Bazelot, M., Williams, C.M., McNeish, A.J., 2019. Cannabidiol reduces seizures and associated behavioral comorbidities in a range of animal seizure and epilepsy models. Epilepsia 60, 303–314.
Paxinos, G., Watson, C., 1982. The Rat Brain in Stereotaxic Coordinates. Academic Press, New York.
Pitkanen, A., Lukasiuk, K., 2011. Mechanisms of epileptogenesis and potential treatment targets. Lancet Neurol. 10, 173–186.
Racine, R.J., Gartner, J.G., Burnham, W.M., 1972. Epileptiform activity and neural plasticity in limbic structures. Brain Res. 47, 262–268.
Sadakata, T., Mizoguchi, A., Sato, Y., Katoh-Semba, R., Fukuda, M., Mikoshiba, K., Furuichi, T., 2004. The secretory granule-associated protein CAPS2 regulates neurotrophin release and cell survival. J. Neurosci. 24, 43–52.
Sajadian, A., Esteghamat, S., Karimzadeh, F., Eshaghabadi, A., Sieg, F., Speckmann, E.J., Meuth, S., Seidenbecher, T., Budde, T., Gorji, A., 2015. Anticonvulsant effect of neural regeneration peptide 2945 on pentylenetetrazol-induced seizures in rats. Neuropeptides 49, 15–23.
Sakai, A., Matsuda, T., Doi, H., Nagaishi, Y., Kato, K., Nakashima, K., 2018. Ectopic neurogenesis induced by prenatal antiepileptic drug exposure augments seizure susceptibility in adult mice. Proc. Natl. Acad. Sci. U. S. A. 115, 4270–4275.
Scharfman, H.E., Myers, C.E., 2012. Hilar mossy cells of the dentate gyrus: a historical perspective. Front. Neural Circ. 6, 106.
Sieg, F., 2016. Mini-review of neural regeneration peptides in brain development. J. Stem Cell Res. Ther. 1, 147–148.
Temkin, N.R., 2001. Antiepileptogenesis and seizure prevention trials with antiepileptic drugs: meta-analysis of controlled trials. Epilepsia 42, 515–524.

GENERAL CONCLUSIONS

The work presented in this thesis supports the notion that the identification of pharmacological treatments capable of preventing the development of epilepsy in at-risk individuals is an achievable goal. A common trait in both the studies I conducted is that the (direct or indirect) modulation of BDNF signalling represents a mechanism by which antiepileptogenic effects can be obtained. At the same time, however, our data strongly indicate that this modulation should be highly precise, focusing on specific TrkB-activated signalling pathways and not on others, that could instead prove deleterious. Further research is therefore needed to develop new drugs (better: highly refined pharmacological tools) that would prove not only effective, but also completely safe, and therefore ready for clinical testing.

OTHER PROJECTS

In addition to my primary project, described above, during the 2020 lockdown I also contributed the part on epilepsy in a review on neuroinflammation in brain diseases.

REVIEW:

THE DICHOTOMOUS ROLE OF INFLAMMATION IN THE CNS: A MITOCHONDRIAL POINT OF VIEW

biomolecules

MDPI

Review

The Dichotomous Role of Inflammation in the CNS: A Mitochondrial Point of View

Bianca Vezzani [1,2], Marianna Carinci [1,2], Simone Patergnani [1,2], Matteo P. Pasquin [1,2], Annunziata Guarino [2,3], Nimra Aziz [2,3], Paolo Pinton [1,2,4], Michele Simonato [2,3,5] and Carlotta Giorgi [1,2,*]

1 Department of Medical Sciences, University of Ferrara, 44121 Ferrara, Italy; vzzbnc@unife.it (B.V.); crnmnn@unife.it (M.C.); simone.patergnani@unife.it (S.P.); psqmtp@unife.it (M.P.P.); paolo.pinton@unife.it (P.P.)
2 Laboratory of Technologies for Advanced Therapy (LTTA), Technopole of Ferrara, 44121 Ferrara, Italy; grnnnz@unife.it (A.G.); nimra.aziz@unife.it (N.A.); michele.simonato@unife.it (M.S.)
3 Department of BioMedical and Specialist Surgical Sciences, University of Ferrara, 44121 Ferrara, Italy
4 Maria Cecilia Hospital, GVM Care & Research, 48033 Cotignola (RA), Italy
5 School of Medicine, University Vita-Salute San Raffaele, 20132 Milan, Italy
* Correspondence: carlotta.giorgi@unife.it; Tel.: +39-0532-455803

Received: 28 August 2020; Accepted: 10 October 2020; Published: 13 October 2020

Abstract: Innate immune response is one of our primary defenses against pathogens infection, although, if dysregulated, it represents the leading cause of chronic tissue inflammation. This dualism is even more present in the central nervous system, where neuroinflammation is both important for the activation of reparatory mechanisms and, at the same time, leads to the release of detrimental factors that induce neurons loss. Key players in modulating the neuroinflammatory response are mitochondria. Indeed, they are responsible for a variety of cell mechanisms that control tissue homeostasis, such as autophagy, apoptosis, energy production, and also inflammation. Accordingly, it is widely recognized that mitochondria exert a pivotal role in the development of neurodegenerative diseases, such as multiple sclerosis, Parkinson's and Alzheimer's diseases, as well as in acute brain damage, such in ischemic stroke and epileptic seizures. In this review, we will describe the role of mitochondria molecular signaling in regulating neuroinflammation in central nervous system (CNS) diseases, by focusing on pattern recognition receptors (PRRs) signaling, reactive oxygen species (ROS) production, and mitophagy, giving a hint on the possible therapeutic approaches targeting mitochondrial pathways involved in inflammation.

Keywords: neuroinflammation; mitochondria; neurodegeneration; multiple sclerosis; Parkinson's disease; Alzheimer's disease; ischemic stroke; epilepsy

1. Introduction: The Cellular Players of Neuroinflammation

The new century, together with technological innovations, brought new insight into the intrinsic communication between the central nervous system (CNS) and the innate immune response. It was a common thought that the brain was a privileged tissue of our body, due to the presence of the blood–brain barrier (BBB) that would have avoided the access of immune cells [1,2]. This hypothesis has been challenged by an increasing number of studies, becoming nowadays an obsolete consideration, even though the CNS still conserves some unique immunological features [3]. Specifically, immune cells reside at the meninges granting surveillance to the brain, and meninges are provided of lymphatic vessels, able to drain large particles and immunomodulatory cytokines directly to the peripheral immune system through lymph nodes connections [4,5]. Nevertheless, the resident

key players of the neuroimmune system are glial cells. These CNS immune cells are classified as macroglia (oligodendrocytes and astrocytes) and microglia, they regulate several physiological processes required for neuronal survival and brain function. As far as we are now aware, besides being part of glial cells, oligodendrocytes do not have a major role in the physiological neuroinflammation, since they mainly provide physical and metabolic support to neurons promoting the myelinating process [6]. Noticeably, oligodendrocyte gap junctions' deficiency due to genetic defects has been associated with increased neuroinflammation in mouse models, indicating that the altered expression of connexins in oligodendrocytes, besides being a consequence of inflammation, can also promote a proinflammatory environment [7]. Astrocytes are the most numerous glial cells of the CNS, exerting diverse roles, such as the regulation of synaptic plasticity and, more broadly, the control of brain homeostasis, also by coordinating local energy metabolism. Importantly, they also play a role in neuroprotection by maintaining the BBB intact, due to their tight interactions with the cerebrovascular endothelium [8,9]. Furthermore, astrocytes release proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), which besides boosting the local inflammatory response by acting on microglia and neurons, is important in facilitating lymphocytes crossing the BBB into CNS parenchyma [10]. Accordingly, abnormal astrocytes activation, mainly characterized by hypertrophy of soma and processes, plays a key role in the neuroinflammatory process, also owed to the strict communication with microglia [11]. Being firstly described a century ago by Pio del Rio Hortega as the 'third element' of the CNS [12], microglia cells are now defined as the innate immune cells of the CNS characterized by the expression of CX3CR1, CD11b, Iba1, and F4/80 markers, by their myeloid origin, and by their phagocytic ability [13]. Microglia exert different functions in the CNS: they are responsible for sensing changes in the surrounding microenvironment, including both physiological changes and pathogens invasion, thus activating either their housekeeping or defense function [14].

Neuroinflammation is a natural process of defense, precisely and timely regulated, which includes a proinflammatory phase aimed to neutralize the danger, and an anti-inflammatory phase that restores the tissue homeostasis by activating the regenerative processes. While an acute neuroinflammatory response reduces injury by contributing to the repair of damaged tissue, chronic glial activation, which results from persistent stimuli, is a fundamental component of neurodegenerative diseases, and contributes to neuronal dysfunction, and therefore to CNS diseases progression [15]. As a consequence, the neuroimmune response performed by activated glial cells has a dichotomous role in the CNS. On one side, it induces the activation of repairing and regenerating mechanisms (i.e., remyelination), while on the other, the uncontrolled release of inflammatory mediators as proinflammatory cytokines, reactive oxygen species (ROS), and nitric oxide (NO) boost a chronic neuroinflammatory state, and is potentially dangerous for the neighboring cells. The aberrant release of these inflammatory molecules, together with the consequent upregulation of immune receptors on the other CNS cells, lead to tissue damage and the consequent activation of peripheral B- and T-cell responses due to the meningeal lymphatic system drainage [16]. This cascade of events enhances the inflammatory process owing to the synergistic action of microglia and lymphocytes against the antigen presenting cells [17,18]. Acute neuroinflammation usually takes place during infectious disease or during chronic autoimmune disorders such as multiple sclerosis (MS), but recent evidence has suggested how prolonged neuroinflammation is a ubiquitous pathological sign of several neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (PD and AD) [19–21]. Accordingly, the close link between neuroinflammatory state and neurodegeneration suggests that neuroimmune mechanisms might trigger neuronal degeneration, resulting in neurotoxicity and neuronal cell loss [22,23]. Interestingly, the presence of mitochondrial dysfunctions both in neurodegenerative and neuroinflammatory CNS pathologies might represent the key connection between chronic immune activation and neuronal degeneration [24–26]. Mitochondria are organelles of endosymbiotic bacterial origin involved in various cellular functions, from the regulation of energy production and metabolism to the control of cell proliferation and programmed cell death [27]. Noticeably, mitochondria are also endowed with the ability to sense and react to cellular damage and to promote efficient host immune response by

producing secondary messengers fundamental in the activation of immune cells and by contributing to the activation of inflammasomes, i.e., of the intracellular protein complexes that detect and respond to danger stimuli. Therefore, it is not surprising that increasing literature is supporting the central role of these organelles in the pathogenesis of both inflammatory and neurodegenerative CNS disorders.

In this review we are going to discuss the central role of mitochondria in driving and maintaining the neuroinflammatory process present either in chronic primarily inflammatory CNS diseases such as MS, chronic non-inflammatory neurodegenerative diseases such as PD and AD, and also in non-primarily inflammatory CNS pathologies such as epilepsy and ischemic stroke. We are going to focus on the mitochondrial pathways regulating inflammation in microglia and astrocytes because, to the best of our knowledge, these two cell types are the most involved in triggering and sustaining the neuroinflammatory process. Finally, we are going to discuss the current therapies aimed to reduce neuroinflammation in the cited pathologies.

2. Role of Mitochondria in Neuroinflammation

Neuroinflammation is an innate inflammatory response within the CNS against harmful and toxic stimuli, mediated by the activation of resident immune cells, by the recruitment of peripheral lymphocytes and, lastly but most importantly, by the production of cytokines, chemokines, ROS, and other proinflammatory secondary messengers. The main cellular players involved in the neuroinflammatory process are glial cells, such as astrocytes and microglia. For a long time, glial cells residing in a healthy brain were defined as inactive. Following damage or infection, glial cells become "activated", even though the terms "resting" and "activated" are vague and obsolete due to the high plasticity of these cells, which have shown to be able to dynamically shift between a spectrum of different phenotypes [28] (Figure 1). In fact, the advent of in vivo techniques, such as 2-photon microscopy, allowed the discovery that in their "resting" state, microglial cells are instead highly active, by surveying their microenvironment with extremely motile processes and protrusions [29]. Additionally, astrocytes, the most abundant glial cell population, participate in the immune and inflammatory responses of the CNS by sensing both exogenous and endogenous material through the expression of specific receptors. Indeed, even if mainly expressed by microglial cells, pattern recognition receptors (PRRs) that are fundamental for the primary recognition of infectious agents and of endogenous danger signals, are also expressed by astrocytes [30]. PRRs, localized on the cell surface, in the endosomes and also in the cytoplasm, upon the recognition of a specific antigen lead to intracellular signaling cascade ending with the release of proinflammatory mediators [31]. It is important to underline that astrocytes mainly rely on microglia for their activation. In fact, microglia control the surrounding microenvironment by using their dynamic ramifications to sense and detect any occurring alteration in brain homeostasis: once in contact with dangerous molecular factors, microglia acquire a less ramified phenotype, starting their immunomodulatory activity either by phagocytosis or by proinflammatory factors secretion [32]. Several molecular pathways are involved in activating and maintaining the inflammatory state within the CNS: PRRs signaling, cytokine receptor signaling, triggering receptor expressed on myeloid cells-2 (TREM2) signaling, ROS production, and secretion [32,33]. Interestingly, an increasing number of studies demonstrate the direct involvement of mitochondria in the modulation of the innate immune response by their participation in PRRs signaling, ROS production, and thus inflammasome assembly [34–36], as shown in Figure 1. Particularly, mitochondrial damage and/or dysfunction such as mitochondrial depolarization or excessive ROS production promote a selective autophagic process called mitophagy. Due to the importance of mitochondria in regulating neuroinflammation, mitophagy represents a key factor in modulating damage-associated molecular patterns (DAMPs) response, by preventing their release both in the cytoplasm and in the extracellular space. Therefore, its alteration has a fundamental role in the establishment of a proinflammatory environment in the development of CNS disorders. We are going to focus our attention on these three latter mechanisms, PRRs signaling, ROS production, and

mitophagy, briefly describing their involvement in the neuroinflammatory process and then describing their participation in MS, PD, AD, ischemic stroke, and epilepsy.

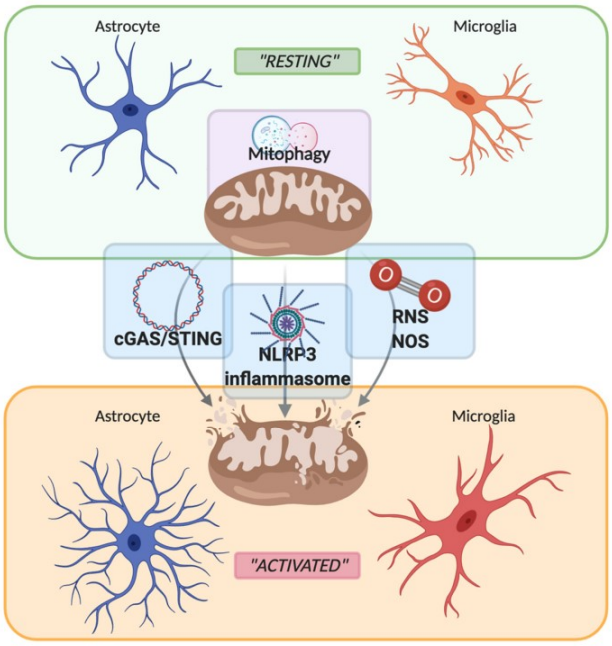


Figure 1. Schematic representation of the switch between "resting" and "activated" state of astrocyte and microglia mediated by mitochondria. Mitochondria maintain their healthy and physiological state by mitophagy. Upon stressful condition, such as inflammatory stimuli, mitochondria are disrupted with the consequent release of mtDNA, damage-associated molecular patterns (DAMPs), reactive oxygen/nitrogen species (ROS and RNS), leading to the activation of the sentinel of the central nervous system such as astrocytes and microglia. Created with BioRender.com.

2.1. PRRs Signaling: Focus on cGAS-STING Pathway

As mentioned above, the innate immune system is able to recognize pathogens through the presence of the receptor families of PRRs [37]. These receptors are present on inflammatory cells like macrophages, neutrophils, dendritic cells, microglia, and astrocytes. PRRs can be on the cell membrane, such as the Toll-like receptors (TLR), or can be present in the intracellular compartments as for the nucleotide-binding oligomerization domain-like receptors (NLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs) [38]. Physiologically, PRRs play a protective role in host defense against damaging signals, but their abnormal activation leads to chronic inflammation. As a part of the innate immune system, inflammation is initiated when PRRs detect pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins, and carbohydrates. On the other hand, in 2002 Matzinger developed the "danger theory", stating that the human body, in absence of infection, uses the same system to signal tissue damage (sterile inflammation), and activate repairing mechanisms [39]. In fact, PRRs are also able to detect DAMPs that are commonly released from injured cells following stress conditions [40]. Due to their bacterial origin [41], mitochondria represent an important source of DAMPs, thus playing an important role in immune system activation and induction of sterile inflammation. Under stress conditions, the outer mitochondrial membrane (OMM), can be damaged triggering the subsequent disruption of the inner mitochondrial membrane and the release

of mitochondrial components, such as mitochondrial DNA (mtDNA), N-formylated proteins, and cardiolipin in the cytoplasm [42,43]. The release of mitochondrial components triggers the activation of different PRRs, such as inflammasomes, cyclic GMP-AMP synthase (cGAS), and TLRs [44]. Notably, the expression of most PRRs in the CNS is not restricted to microglia and astrocytes, but it also occurs in neurons indicating that they not only suffer neuroinflammation, but they might be involved in its regulation [45–47].

mtDNA is a circular molecule of double-stranded (ds)DNA enriched in bacterial hypomethylated CpG island therefore highly capable of eliciting the PRRs response by binding to the TLR9 [48]. Recently it has been identified, through a strategy that combined quantitative mass spectrometry with conventional protein purification, a novel sensor of cytosolic dsDNA able to trigger the type-I interferon (IFN) pathway: the cGAS [49]. Briefly, by binding to two molecules of cytosolic dsDNA, cGAS converts adenosine triphosphate (ATP) and guanosine triphosphate (GTP) into the second messenger 2′,3′-cyclic GMP-AMP (cGAMP) [50], which binds and activates the ER-resident protein stimulator of interferon genes (STING) [51]. This bond causes conformational reorganization of STING, which allows its phosphorylation by the TANK-binding kinase 1 (TBK1) in the endoplasmic reticulum (ER)-Golgi intermediate compartment. After its activation, STING phosphorylates the interferon regulatory factor 3 (IRF3) which dimerizes, translocates to the nucleus and induces expression of type I IFNs. Moreover, STING activates the IkB kinase complex, which phosphorylates IkB, an inhibitor of nuclear factor-kB (NF-kB). IkB degradation allows the translocation of NF-kB into the nucleus and the consequent induction of inflammatory cytokines [52,53]. This pathway is physiologically activated during pathogen infections, and its activation is important for the correct pathogen response. However, in case of sustained dsDNA presence in the cytoplasm, its continued activation might result in abnormal neuroinflammation. Interestingly, mitochondria disruption represents a great source of cytoplasmic dsDNA. Accordingly, mitochondrial damage is not only a cause, but is also a consequence of neuroinflammation, resulting in the release of mtDNA in the cytosol and also in the extracellular space. It is therefore not surprising how the cGAS-STING pathway has recently assumed considerable importance for the understanding the molecular bases beyond various neuroinflammatory and neurodegenerative diseases (NDDs) [54].

2.2. PRRs Signaling: Focus on NLRP3 Inflammasome

Another important family of PRRs responsible for the early recognition of PAMPs and DAMPs expressed by microglia and astrocytes are the NLRs. The NLR family is characterized by the presence of a central nucleotide and oligomerization domain (NACHT), which is common to all members of the NLR family, flanked by C-terminal leucine-rich repeats (LRR) and N-terminal caspase (CARD) or pyrine (PYD) recruitment domains. LRR regions are responsible for ligand detection, while the CARD and PYD domains mediate the protein–protein interactions for the activation of downstream signaling [55]. Noticeably, some NLRs, once activated following the detection of PAMPs or DAMPs, can lead to the formation of a multiprotein complex called "inflammasome" [56]. Outstandingly, besides the inflammasomes derived from the NLR family, such as NLRP1, NLRP3, and NLRC4, also non-NLR proteins such as ALRs and pyrin can lead to inflammasomes assembly [57]. Among the cited ones, the NLRP3 (nucleotide-binding domain and leucine-rich repeat containing protein 3, also known as NALP3) inflammasome is the most studied and it is present both in microglia and astrocytes, even though a later study reported that NLRP3 was predominantly active in microglia [58,59]. The NLR domain represents the sensory component of the inflammasome, which binds to the amino-terminal domain of the adaptor apoptosis-associated speck-like protein containing CARD (ASC), once dangerous molecules are detected. ASC forms a bridge with the CARD domain, which contains the pro caspase-1, which subsequently self-catalyzes to its active form caspase-1 leading to the production of the proinflammatory cytokines IL-1β and IL-18 [60]. Moreover, caspase-1 is accountable for the cleavage of gasdermin D, which promotes inflammasome-associated pyroptotic cell death by producing pores in the cell membrane allowing also IL-1 β and IL-18 secretion [60]. Notably,

in the resting state, NLRP3 is localized in the cytosol and upon activation it relocates in mitochondria and at the mitochondria associated membranes (MAMs) together with its partner ASC [61]. The triggering signals for inflammasome assembly and delocalization are a variety of exogenous and endogenous stimuli such as microbial infections, extracellular ATP, ROS, and mtDNA. As for the cGAS-STING pathway, also the NLRP3 inflammasomes, and more broadly all the inflammasomes, have a protective role against pathogen infections and also against metabolic toxic waste accumulation, by sustaining the innate immune response in order to defeat the harmful stimuli. On the other hand, sustained NLRP3 activation due to abnormal amounts of misfolded protein or metabolic by-products, leads to chronic neuroinflammation, an ideal environment for the development of CNS disorders [62]. In this latter context, the ability of mitochondria components and products to activate the NLRP3 inflammasome indicates that it is responsible for sensing mitochondrial dysfunction, thus explaining the frequent association of mitochondrial damage with inflammatory diseases [61]. In particular, different studies reported that microglial NLRP3 inflammasome activation is a key contributor to the development of the neuroinflammatory process during neurodegeneration. Indeed, microglial NLRP3 activation has been shown to be triggered by pathogenic protein aggregates such as β-amyloid protein (Aβ) and α-synuclein (α-Syn), related to the development of amyotrophic lateral sclerosis (ALS), AD and PD [63], but also by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) commonly used to model PD in mice [64]. Moreover, it has been reported that the microglial activation of the NLRP3 inflammasome drives tau pathology in a mouse model of frontotemporal dementia, shedding a light on the role of microglia in the development of AD [65]. Lastly, also ceramide, the sphingosine-based lipid-signaling molecule linked to the development of numerous pathophysiological processes in the CNS including AD, has been reported as a modulator of NLRP3 inflammasome assembly [66]. Interestingly, STING has been proposed to activate the NLRP3 inflammasome at least by two distinct mechanisms upon cytosolic DNA stimulation, indicating a connection between cGAS-STING and NLRP3 pathways. On one hand STING interacts and recruits NLRP3 allowing its localization in the ER and facilitating the inflammasome formation, on the other hand the interaction reduces K48- and K63-linked polyubiquitination of NLRP3 favoring the inflammasome activation [67]. In conclusion, besides being fundamental in the physiological process of pathogen-driven immune response, abnormal NLRP3 inflammasome activation exerts a fundamental role in the progression of neuroinflammation and, consequently, in the development of a variety of neurodegenerative diseases, representing a promising target for therapies.

2.3. Reactive Oxygen Species

For several years now, it has been widely known that cytokine-activated microglia produce ROS and that, as stated before, ROS are responsible for activating microglia [68]. Accordingly, oxidative damage is both a cause and a result of the neuroinflammatory process leading to the neurotoxic effects observed in different NDDs. Reactive species, also called free radicals, include reactive oxygen and nitrogen species. ROS are a physiological by-product of oxygen metabolism and exert significant roles in cell signaling. They are mainly generated by mitochondria and include oxygen radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl (•OH), peroxyl ($RO_2^{\bullet}$), and alkoxyl (RO^-), and also non-radical oxidizing agents easily convertible into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen, and hydrogen peroxide (H_2O_2) [69]. In addition, reactive nitrogen species (RNS), such as nitric oxide (NO), are produced at low levels during the mitochondrial oxidative phosphorylation (OXPHOS) [70]. When the redox state is balanced, ROS act as second messengers in different signaling pathways, contributing to the conservation of cellular functionality [71]. However, when oxygen homeostasis is not maintained, the redox balance is compromised thus leading to ROS accumulation, with the consequent assembly of NLRP3 inflammasome and the disruption of the OMM, which, in the CNS, results in the induction of the neuroinflammatory state. The exacerbated production of ROS leads to the activation of glial cells resulting in proinflammatory cytokines release, which in turn stimulates the apoptosis of pericytes, important regulators of the BBB, via ROS augmentation [72]. Furthermore,

in damaged mitochondria, the rate of $O_2^{\bullet-}$ formation is increased by the loss of electrons, leading to the formation of H_2O_2. $O_2^{\bullet-}$ can therefore react with NO, which is produced by cellular NO synthase, with the consequent formation of peroxynitrite, leading to increased cell damage [73,74]. Since the brain is one of the highest ATP-demanding organs, OXPHOS is highly active in CNS cells, and is responsible for the maintenance of neuronal function like synaptic transmission and preservation of neuronal potential [75–77]. Therefore, prolonged mitochondrial dysfunction leading to the failure of ATP production and to increased ROS and RNS production is considered at the base of neuronal cell loss in neuroinflammation [76,78–80]. Nevertheless, it is important to recall the fundamental role of ROS in the maintenance of tissue homeostasis when redox balance is preserved, implying that too aggressive antioxidant therapies might compromise also the physiological role of ROS and thereby, CNS functions. Overall, a comprehensive understanding of the fine redox tuning and ROS production in neuroinflammation and during NDDs progression may help to develop new, antioxidant-based adjuvant therapies.

2.4. Mitophagy

Mitochondria, as all the other cellular organelles, experience a continuous turnover through the coordinated degradation, recycling, and new synthesis of their constituent elements [81]. In the CNS, neuronal cell functions highly depend on the efficiency of mitochondria, either in their ability to produce energy and balance inflammatory response or in their capacity of undergoing selective degradation. This latter function is known as mitophagy, a physiological process aimed to specifically eliminate damaged mitochondria, or to remove all mitochondria in specific developmental phases, in order to preserve tissue homeostasis [82]. During the neuroinflammatory process, a lot of mitochondria by-products are generated inducing mtDNA mutations and the alteration of the mitochondrial membrane potential (Ψm), exacerbating the inflammatory state [83]. In this scenario, mitophagy plays a pivotal role, by removing damaged mitochondria thus reducing the cellular damage and avoiding neuronal cell loss, preserving CNS function. This mechanism is based on ubiquitin-dependent and receptor-dependent signals released from damaged mitochondria [84].

The best-characterized mitophagic pathway in mammalian cells is the PTEN-induced putative kinase 1 (PINK1)/Parkin pathway [85], which relies on ubiquitin mediated degradation. This process is triggered by a decrease of the Ψm, due to mitochondrial permeabilization, which leads to the recruitment of PINK1 at the OMM. At this point, PINK1 enrolls the E3 ubiquitin ligase Parkin, leading to the ubiquitination of different OMM mitochondrial proteins [86]. Polyubiquitinated mitochondrial proteins are then associated with the ubiquitin-binding domains of autophagy receptors inducing the formation of the autophagosome that will be subsequently degraded by its fusion with the lysosome [87]. At least five specific ubiquitin-binding autophagy receptors were identified to connect ubiquitinated mitochondria to the phagosomes. However, it remains to be clarified if one among p62/sequestosome 1 (p62/SQSTM1), nuclear dot protein 52 (NDP52), neighbor of Brca1 (NBR1), tax 1 binding protein 1 (TAX1BP1), and optineurin (OPTN) is effectively essential for mitophagy [88,89].

The other pathway that regulates mitophagy is dependent on proteins localized on the OMM that act as receptors, such as B-cell lymphoma 2 nineteen kilodalton interacting protein 3 (BNIP3), Nix, Bcl-2-like protein 13, and FUN1. These proteins all contain the LC3-interacting region (LIR) motif, which is responsible for the recruitment of the autophagosomal machinery by the direct interaction of the mitochondria with LC3/GABARAP family members [81]. Being strictly correlated with the inflammatory process, due to its scavenger activity, the mitophagic pathway is commonly altered in CNS disorder, representing an appealing target for therapies [82].

3. From Chronic Neuroinflammation to Neurodegeneration: Multiple Sclerosis, Parkinson's, and Alzheimer's Disease

All neurodegenerative disease, including MS, PD, and AD share a common feature: chronic aberrant inflammation (Figure 2). This condition starts with a systemic inflammation that activates

the immune response in the CNS, particularly throughout the priming of brain resident microglia. This leads to the subsequent release of inflammatory mediators and the consequent upregulation of the immune response. As described before, mitochondria take part in this unfavorable condition. Particularly, activated microglia increase the production of mitochondrial oxidative species, such as ROS and RNS, which can oxidize and damage lipids, nucleic acids, proteins, and polysaccharides leading to further mitochondrial damage. All these hostile conditions act as a feedback sufficient to sustain a stressful condition that promotes tissue damage and chronic inflammation, leading to nervous tissue degeneration. Accordingly, several studies have demonstrated that inflammation and a perturbed mitochondrial population exacerbate the outcome of neurodegenerative diseases.

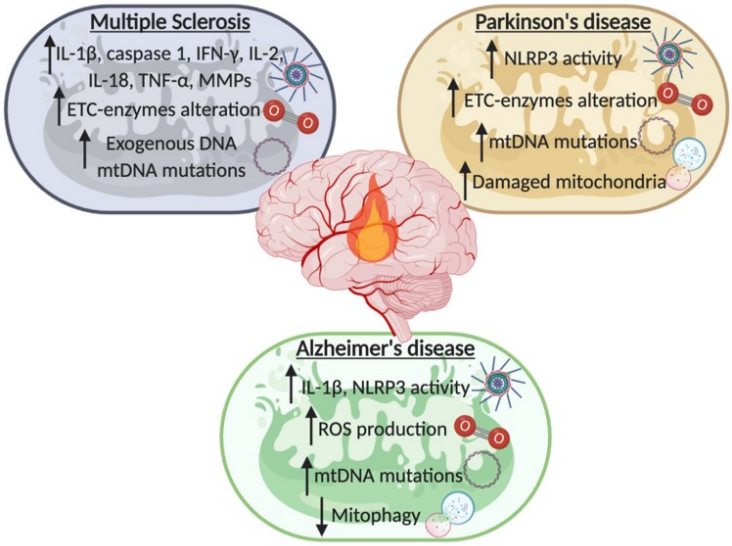


Figure 2. Schematic list of mitochondrial contribution to the recurrence of neuroinflammation during the development of neurodegenerative diseases (NDDs). In multiple sclerosis, Parkinson's disease, and Alzheimer's disease mitochondria have a fundamental role in the induction of a neuroinflammatory state by the activation of inflammasomes, variation of the electron transport chain (ETC) enzymes, modulation of reactive oxygen species (ROS) production, alteration of the mtDNA and of the mitophagic process. Created with BioRender.com.

3.1. Multiple Sclerosis

MS is the commonest primary demyelinating disease of the brain. MS displays a great inter-individual variability in disease course and severity [90]. About 1–3% of the affected people have a benign form of MS, in which any severe disability occurs after several years [91]. About 10–15% of MS patients present a progressive primary form, where symptoms and disabilities gradually get worse over time. Lastly, the majority (80–85%) present a relapsing-remitting form, where an attack is followed by a time of recovery with few or no symptoms, called remission.

MS is a T-cell–mediated autoimmune disease characterized by demyelination, gliosis, and neuronal cell loss [92]. The association of MS with a strong inflammatory process has been challenged over the years, but it is now evident that cortical demyelination occurs in association with neuroinflammation [93]. Inflammatory pathogenic T cells enter the CNS to initiate the immunological cascade leading to the activation of residing microglia and astrocytes, which, together with the further participation of B cells and dendritic cells, finally trigger the chronic CNS inflammation [94]. Indeed, increased levels of proinflammatory cytokines like IFN-γ, IL-2, IL-18, and TNF-α, have been found in human samples

obtained from MS patients [95,96]. To become biologically active TNF-α has to be cleaved by a disintegrin and metalloproteinase (ADAM-17) called TNF-α-converting enzyme (TACE). Interestingly, elevated levels of TNF-α and TACE mRNA were found in peripheral blood mononuclear cells (PBMCs) of MS patients, without an ex vivo stimulation [96]. During their migration into CNS, activated T cells express matrix metalloproteinases (MPPs) that drive the lysis of the dense subendothelial basal lamina, resulting in progressive tissue damage. Remarkably, increased levels of MMPs were observed in cerebrospinal fluid (CSF) of MS patients [97]. Notably, MPPs not only mediate tissue damage, but also regulate the inflammatory reaction through TNF-α processing [98]. Consistent with this, the most frequently used treatment for MS, namely IFN-β, acts by reducing MPPs expression and therefore by interfering with the passage of activated T cells into CNS [97].

The neuroinflammatory process can be also triggered by pathogen infection that causes the release of proinflammatory mediators within the CNS. It is therefore not surprising that different pathogens such as *Mycoplasma pneumoniae*, *Staphylococcus aureus*, *Chlamydia pneumoniae*, Epstein Barr, and Herpes viruses are associated to the development or exacerbation of MS, due to their ability to affect the cGAS-STING pathway [99]. Interestingly, it has recently been shown that the antiviral drug ganciclovir inhibits the proliferation of microglia in experimental autoimmune encephalomyelitis (EAE), the most commonly used experimental model for the human inflammatory demyelinating disease, by modulating the cGAS-STING signaling pathway [100]. Accordingly, the inhibition of component of this pathway, such as STING, IRF3, TBK1, resulted in reduced activity of ganciclovir [101].

The correlation between MS and neuroinflammation has been further supported by the involvement of NLRP3 inflammasome in the development of the disease. In fact, it has been reported that activated caspase-1 and IL-1β levels are significantly increased in MS patients and in EAE animal models [102–105]. Accordingly, mice lacking the expression of inflammasome-involved proteins, such as NLRP3, ASC, and caspase-1, resulted protected from the progression of EAE [106,107]. Moreover, administration of IFN-β weakened the progression of MS by reducing the activity of NLRP3 inflammasome [108]. As reported above, a primary cause of NLRP3 priming is mitochondrial dysfunction. Thus, it is not surprising that mitochondria play a key role in modulating MS progression, as supported by a wide number of studies describing mitochondrial impairments in both MS patients and MS mouse models. Just to cite a few, in MS lesion it has been observed an impaired activity of the electron transport chain (ETC)-enzymes of the complex IV [109], and changes in the aerobic metabolism, mainly due to alteration of mitochondrial superoxide dismutases 1 and cytochrome c levels, were found in platelets of affected patients [110]. Interestingly, these modifications on the ETC are also reflected by alteration of the ROS production, which is increased both in cellular and in animal MS models [111–113]. The excessive ROS production exacerbates the oxidative stress resulting in increased mitochondrial lipid peroxidation that leads to the final impairment of mitochondrial activity. The modification in the oxidative process is further boosted by the deregulation of the antioxidant defense mechanism, which has been found altered in MS patients' body fluids [114,115]. Since the excessive ROS production is also accountable for the induction of DNA mutation, mtDNA sequence variations were found associated with MS [116–118]. Lastly, it has been shown that also mitophagy and mitochondrial failure markers are augmented in serum and in CSF samples of MS patients, with a direct correlation with the active phase of the disease [95,119]. All these findings support the hypothesis that the neuroinflammatory process sustains the development of MS, and further highlight the central role of the mitochondria in the progression of the disease.

3.2. Parkinson's Disease

PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta, which is associated to a widespread aggregation of α-Syn forming the Lewy bodies. Accordingly, autosomal dominant mutation of the gene encoding the α-Syn protein (SNCA) determines familial PD [120]. Interestingly, α-Syn, even if predominately localized in neuron terminals, can be found at the mitochondrial surface, where it influences mitochondrial structure and functions [121–123]. SNCA

is not the only gene responsible for familial PD that directly affects mitochondrial behaviors: to date different biochemical and genetic studies revealed that the production of the PARK genes (indicated in brackets) parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7), LRRK2 (PARK8), and ATP13A2 (PARK9) are mutated in autosomal recessive Parkinsonism. All these genes work to govern mitochondrial functioning, thus strengthening the evidence that mitochondrial dysfunction is strongly involved in PD development [124].

The idea that mitochondria might be involved in PD arose in the late 1980s, when it was found that the oxidized form of the PD-inducer compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine provoked the inhibition of the complex-I of the ETC in neurons. Accordingly, compromised levels of complex-I were also found in human samples of PD patients [125,126]. Interestingly, neurons from autopsies of PD patients harbored high levels of mutations in mtDNA that correlate with mitochondrial dysfunction [127,128]. The accumulation of mtDNA mutations impairs ETC functioning, leading to compromised Ψm, reduced synthesis of ATP, and increased ROS production. Taken together these results prove the association between pathogenic mtDNA mutations and PD development.

As mentioned above, the NLRP3 inflammasome is the best characterized among the inflammasomes and it has been reported to drive the neuroinflammatory process in PD. In fact, increased expression of inflammasome components and inflammation-related factors have been found in human blood samples of PD patients [129,130]. In addition, the mitochondrial impairment observed in microglia induces an increased ROS production, thus amplifying the NLRP3 inflammasome proinflammatory signaling both in in vitro and in vivo models of PD [131]. Accordingly, it has been shown that the administration of tenuigenin, an anti-inflammatory plant extract, to PD mice models reduces the NLRP3 activation directly acting on ROS production [132]. Moreover, Pink1$^{-/-}$ or Parkin$^{-/-}$ microglia cells have been shown to have an increased NLRP3 activity. This tendency was abolished by the administration of inflammasome inhibitor, both in Pink1$^{-/-}$ or Parkin$^{-/-}$ microglia cells and in patient derived macrophages carrying the PARK2 mutations [133]. The possibility to arrest PD advancement by inhibiting NLRP3 induced neuroinflammation has been confirmed by the administration of MCC950 in rodent PD models, which resulted in a mitigation of motor deficits and reduced accumulation of α-Syn aggregates [134]. Lastly, also carbenoxolone, a heat shock protein inducer, was found to exert beneficial effects in a rat model of PD by inhibiting neuroinflammation and mitochondrial dysfunctions [135].

Ablation of PINK1-parkin pathway, associated with reduced mitophagic process, results in the accumulation of defective mitochondria, damaged mitochondrial proteins and ROS, which leads to NLRP3 inflammasome stimulation. Although this altered pathway was found in PD patient-derived cells and brains the in vivo role of mitophagy in PD remains unclear [136–139]. Indeed, mice lacking either PINK or parkin do not display PD-relevant phenotypes [140], although in these models a reduced mitophagy pathway was observed [141–143]. A recent study has tried to shed light on this aspect by using mitophagy-deficient mouse models with also an increased accumulation of mtDNA mutations, namely Pink1$^{-/-}$*/mutator* and Parkin$^{-/-}$/mutator mice. The research performed demonstrates that in these models, acute and chronic stress activate the proinflammatory cGAS-STING pathway leading to the manifestation of dopaminergic neuron loss and movement disorders [144]. Therefore, this work further supports the important connection between mitochondrial stress and inflammation in PD progression demonstrating that mitophagy exerts a crucial role in preventing neuroinflammation in this pathological context.

Taken together all the reported data suggest a tight connection between mitophagy dysfunction, ROS overproduction, and NLRP3 activation, observed in patients affected by Parkinsonism, confirming the fundamental role of mitochondrial driven neuroinflammation in the development of PD.

3.3. Alzheimer's Disease

AD is a NDD with a slow onset that gradually gets worse over time. The main symptom of AD is dementia, which causes problems with memory, thinking, and behaviors, caused by deposition of

intracellular neurofibrillary masses of pathologic forms of tau protein and extracellular plaque of Aβ. As described for the other NDDs, mitochondria play a key role also in the pathophysiology of AD. Interestingly, Aβ accumulation was found both in mitochondria of human AD patients' brains [145] and of transgenic AD mouse models [146]. In detail, it has been shown that Aβ interacts with different mitochondrial components, such as elements of the ETC, diverse mitochondrial matrix proteins, and putative component of the PTP [145–148]. In this latter scenario, the interaction between Aβ and the PTP component cyclophillin D, induces the pore opening with the consequent alteration of mitochondrial dynamics and functioning (Ca^{2+} homeostasis, ATP levels, ROS) leading to apoptotic neuronal cell death. Notably, PTP is also involved in the regulation of autophagy in AD progression.

The excessive ROS production and the consequent increased oxidative stress is another mitochondrial parameter frequently found dysregulated in AD. Indeed, increased oxidative damage correlates with the brain regions most affected in AD [149,150]. One of the primary targets of oxidative damage is mtDNA, therefore it is not surprising that in AD patient specimens mtDNA mutations are widely present [149,151,152]. As described before, increased ROS production is responsible for inflammasome recruitment, and AD is no exception. Moreover, in AD context, Aβ was found sufficient to activate NLRP3 inflammasome. Accordingly, NLRP3 knockout ameliorated Aβ-related pathology and the development of cognitive decline [153]. Interestingly, mice expressing human tau mutations as well as patients affected by primary tauopathies, such as frontotemporal dementia, exhibited increased NLRP3 levels. Additionally, in this scenario, knockdown of NLRP3 decreased tau aggregation and hyperphosphorylation levels ameliorating the clinical outcome [65]. Similar effects were also obtained by using the specific NLRP3 inhibitor MCC950 in vivo [154]. At demonstration of the determinant role of NLRP3 in AD, elevated levels of its effector molecule IL-1β were found in serum, CSF, and brain of patients with AD as well as other types of dementia [155]. Once secreted, IL-1β enhances the production of Aβ by neurons and induces the phosphorylation of the tau protein [156,157]. Accordingly, IL-1β brain injection upregulates amyloid deposits levels and provokes amyloidogenesis, while IL-1β blockade reduces neuroinflammation by decreasing fibrillar Aβ level and tau activation [158,159]. Polymorphisms of IL-18 promoter, another proinflammatory cytokine released upon NLRP3 inflammasome activation, has been shown to be associated to the risk of developing sporadic late onset AD [160]. Interestingly, IL-18 levels were elevated in body fluids of mild cognitively impaired and AD patients and its production was found elevated in mononuclear cells and macrophages of peripheral blood [161,162]. Furthermore, IL-18 increases the expression of the glycogen synthase kinase 3β and the cyclin dependent kinase 5, which are the mediators of the hyperphosphorylation of tau protein [163].

Proinflammatory cytokines production, including IL-1β, is also enhanced by saturated fatty acid metabolism. Intriguingly, this alternative pathway is the elective way to supply energy in AD brains to overcome the impaired glucose metabolism [164]. Lastly, activation of mitophagy results in diminished Aβ levels and reduced tau hyperphosphorylation leading to a regression of the cognitive impairments in AD-mouse models [165,166]. Accordingly, reduced levels of autophagic and mitophagic markers and an impaired energetic metabolism were observed in human samples obtained by AD-affected patients [167]. The reduced energy supply found in AD patients, due to altered brain glucose metabolism, is compensated by using amino acids and fatty acids as alternative energetic source [168,169]. In conclusion, also for AD development the neuroinflammatory process exerts a pivotal role and represents a powerful therapeutic target.

4. Ischemic Stroke and Mitochondrial Induced Neuroinflammation

Ischemic stroke (IS) is a pathophysiological event occurring when the occlusion of cerebral arteries leads to a transient or permanent block of blood supply to a part of the brain [170]. Among the cerebral arteries accounted for in the development of IS episodes, the middle cerebral artery is the most involved. This artery supplies blood to an extended area of the lateral surface of the brain, part of the basal ganglia and the internal capsule, areas that contain motor, sensory functions and emotions [171].

Therefore, depending on the extent of injury, people affected by an IS injury will likely go through a long-term disability or even death [172,173]. Immediately after stroke onset neurons fail to sustain cellular homoeostasis, resulting in a sequence of harmful events strictly connected to mitochondria functions [174]. Mitochondrial failure, triggered by oxygen and glucose deprivation (OGD), leads to neuronal cells damage and ultimately neuron loss. The high energy demand accompanied by limited energy reserves, make neurons the most OGD sensitive brain residing cells [175]. Depending on several factors, including duration of ischemia and circulation in collateral vessels, the failure of blood supply correlates with different outcomes [176–178]. The failure of blood supply differentially affects the infarcted brain zone: the infarct core has a low level of reperfusion and is characterized by irreversible damages, while in the penumbra, defined as the damaged but metabolically active neuronal area surrounding the ischemic core, the neuronal structure is still preserved and potentially restorable [176,179]. Notably, even though reperfusion is a mandatory step to recover ischemic damage, ischemic reperfusion (IR) is a double-edged sword. If on the one hand IR is a key factor in safeguarding the lesioned brain tissue, on the other hand it establishes the IR-injury exacerbating brain damage [177,180,181].

Recently, a central role of cytosolic dsDNA-sensing cGAS in sterile inflammation and following ischemic injury has been reported [182]. Briefly, in the middle cerebral artery occlusion in vivo model of IS, it has been found that pharmacological inhibition of dsDNA cGAS by A151, a selective antagonist, reduced microglia activation within the ischemic penumbra, inhibited the release of proinflammatory cytokines and reduced the migration of periphery neutrophils injury improving ischemic outcome. Furthermore, AIM2 inflammasome implicated in the brain damage and neuroinflammation after IS [183,184] is also inhibited by A151 [182]. In line with this evidence, CX3CR1CreER mice carrying the selective deletion of cGAS in microglia were protected from ischemic injury [182], suggesting that inhibition of dsDNA sensing cGAS could represent a promising target against IS injury.

A large number of works aimed to define the crucial targetable pathways involved in IS, especially to overcome the detrimental effects of reperfusion, identified NLRP3 mediated neuroinflammation as an eligible target [185–188]. Although the expression of different inflammasome components including NLRP1, NLRP3, NLRC4, ASC, caspase-1, and the proinflammatory cytokines IL-1β and IL-18 increases in the initial hours and early days after ischemia in the brain of rodents [183,189], mitochondrial destabilization or dysfunctions are tightly associated with only NLRP3 inflammasome activation [61,190,191].

The main causes of NLRP3 inflammasome activation in ischemic conditions are ascribable to mitochondria dysfunction, as abnormal Ca^{2+} influx, ROS production, mitochondrial membrane permeabilization with the consequent release of DAMPs and mtDNA, all conditions that are tightly linked one to the other (Figure 3). Within minutes, OGD affects mitochondria functions leading to a strong reduction of OXPHOS and therefore of ATP synthesis. Neurons in the infarct core fail to compensate the ATP cell request leading to a bust of the Na^+/K^+ ATPase pump [185,192]. This results in neuronal membrane depolarization accompanied by an extreme release of glutamate in the extracellular space finally leading to neurons loss [193]. Glutamate is an important excitatory neurotransmitter, which binds several types of receptors such as N-methyl-D-aspartate (NMDA) receptor, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor and kainate receptor [194]. Although these receptors originally were thought to be exclusive to neurons, several studies revealed their functional expression also on glial cells [195,196]. The excessive amount of glutamate after stroke leads to a hyperactivation of these receptors promoting a strong influx of Ca^{2+} into the cells [197–199]. Mitochondria are the crucial players in regulating cytosolic Ca^{2+} levels [200], thus the massive accumulation of cytosolic Ca^{2+} results into the activation of the mitochondria calcium uniporter (MCU) leading to mitochondrial depolarization, which in turn drives NLRP3 inflammasome activation and the consequently IL-1β release [201]. Consistently, it has been found that in response to the activation of NMDA receptors, MCU overexpression increases mitochondrial Ca^{2+} levels and provokes mitochondrial membrane depolarization. Inversely, genetic knockdown of MCU

reduces the NMDA-induced increase in mitochondrial Ca^{2+} followed by lower levels of mitochondrial depolarization [202]. In line with these findings, in focal cerebral ischemia rat models, the early stages of cerebral ischemia are characterized by an upregulation of the mitochondrial calcium uptake 1 (MICU1) a crucial regulator of MCU [203]. Interestingly this occurs in the acute phase of IS right when the inflammatory response takes place [204–206]. To corroborate the role of Ca^{2+} in mitochondria dysfunction-induced inflammation, it has been shown that NLRP3 inflammasome activation is reduced following the inhibition of extracellular Ca^{2+} entry or the depletion of Ca^{2+} stores in the ER [207]. Albeit K^+ efflux, a common NLRP3 inducer [208], has been proposed to be upstream of the Ca^{2+}-induced NLRP3 inflammasome activation, thus indicating that high levels of extracellular K^+ abolish NLRP3 activation [209], the crucial contribution of mitochondrial Ca^{2+} overload in sustaining inflammasome activation following IS and IR is not excluded.

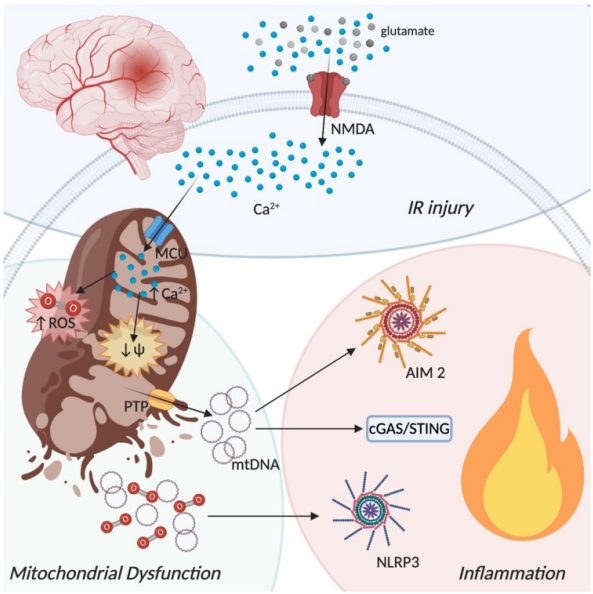


Figure 3. Ischemic-reperfusion injury from a mitochondrial perspective. Oxygen depletion occurring during an ischemic event lead to neuronal membrane depolarization with glutamate release in the extracellular space. Glutamate binds to N-methyl-D-aspartate (NMDA) receptors promoting a strong influx of Ca^{2+}. The increase of cytosolic Ca^{2+} activates the mitochondria calcium uniporter (MCU) leading to mitochondrial depolarization and increased production of reactive oxygen species (ROS) with the consequent opening of the permeability transition pore (PTP) and the final disruption of the mitochondrial membranes. mtDNA and ROS release in the cytoplasm drives the inflammatory process by the activation different pathways, such as AIM2 and the NLRP3 inflammasomes and the cGAS-STING dsDNA sensing machinery. Created with BioRender.com.

Additionally, in brain ischemic damage, mitochondria and ROS have a crucial role [210,211]. Following cerebral ischemia, the balance between ROS production and clearance is compromised, resulting in a pathogenic oxidative-stress-induced inflammation signaling. After IR, the spreading of mitochondrial activity results in a burst of ROS levels [212], worsening the inflammatory response and then the ischemic outcome. Mitochondrial ROS are predominantly generated by complexes I (NADH dehydrogenase) and III (cytocrome bc) of the ETC. Indeed, free electrons in the mitochondrial ETC leaking out and reacting with molecular oxygen, generate $O_2^{\bullet-}$ as a metabolic by-product of

respiration [213–215]. Recently, complex I has been distinguished as a major source of ROS upon IR. Briefly, following IR the succinate, which is markedly increased during ischemia, becomes oxidized. By reverse electron transport, the oxidized metabolite promotes ROS formation at the complex I, providing the initiating burst of $O_2^{\bullet-}$ that leads to IR injury [216]. In agreement, the treatment with rotenone, a mitochondrial complex I inhibitor, causes the loss of Ψm and thus increases ROS production, enhancing NLRP3-dependent IL-1β secretion [61,217]. Ca^{2+} accumulation and ROS production, during IR, lead to the mitochondrial PTP induction. The opening of PTP allows the release of mitochondrial material to the cytoplasm including DAMPs, such as cardiolipin and mtDNA [218]. The ability of cyclosporin and other PTP inhibitors to attenuate NLRP3 inflammasome activation provides a link between PTP and inflammation [219,220]. Several works reported that the inhibition of PTP by genetic or pharmacological approaches confers protection against ischemic damage [221–223]. Accordingly, it has been observed that ROS generation induced by rotenone injection, are mitigated by the inhibition of PTP or mitochondria ROS scavenger [224], indicating the important participation of PTP in the activation and sustainment of IS-induced inflammation.

Mitophagy exerts a fundamental role in cerebral ischemia by preventing all the described mitochondrial-dependent proinflammatory processes, such as extreme ROS production, loss of mitochondrial membrane polarization, and PTP opening [225–227]. Indeed, it has been reported that melatonin administration promotes inhibition of both ROS generation and NLRP3 inflammasome activation by increasing mitophagy [228]. Moreover, both methylene blue administration and rapamycin treatment have been found to enhance mitophagy, reducing ROS accumulation and mitochondrial dysfunction following cerebral ischemia [229,230]. Lastly, the overexpression of the activating transcription factor 4 has been found to ameliorate cerebral IR by suppressing NLRP3 inflammasome activation through parkin-dependent mitophagy [231]. By contrast, knockout of BNIP3-like (BNIP3L), an important player in cerebral IR-induced mitophagy, worsen cerebral IR injury in mice causing an impairment in mitophagy, condition that could be rescued by BNIP3L overexpression [232]. However, it has also been reported how excessive mitophagy could be detrimental for both the ischemic and the reperfusion state, even though the precise molecular pathway has still to be defined [233,234]. Given the importance of mitophagy in maintaining mitochondrial homeostasis, and therefore inhibiting also NLRP3- and ROS-induced neuroinflammation, the modulation of this catabolic process in IS represents an important therapeutic target.

5. The Neuroinflammatory Process in Epilepsy: The Involvement of Mitochondria

Epilepsy is a progressive neurological disorder affecting almost the 1% of the global population, characterized by recurrent seizures and by other complex features, including psychiatric and cognitive comorbidities [235,236]. More than 30% of epileptic patients are drug resistant, becoming thus affected by refractory epilepsy, which can be progressive. Epilepsy and its comorbidities can lead to a profound deterioration in the patient's quality of life [237]. Recent studies conducted on animal models have highlighted that neuroinflammation plays a crucial role in precipitating and/or sustaining seizures recurrence, ultimately facilitating neural cell loss [238]. Indeed, intracerebral application of interleukin IL-1β has been shown to increase seizure activity in experimental models [239]. The oxidative stress generated by RNS and ROS imbalance, due to mitochondria dysfunction, can lead to alterations of cellular macromolecules, such as lipids, proteins, and DNA, [240] with the consequent generation of "oxidation specific epitopes" that induces neuroinflammation [241,242] (Figure 4). Mitochondrial dysfunction is one of the prominent pathological hallmarks that aggravates the inflammation process during epilepsy. Approximately 40% of the epileptic patients have been reported to be affected by a mitochondrial disease [243].

Temporal lobe epilepsy (TLE) is an acquired epilepsy, usually triggered by an insult (such as brain injury) that leads to the development of spontaneous, recurrent seizures after a latency period of months to years [244]. This latency period corresponds to an "epileptogenesis" process, in which a normal brain is transformed to one capable of generating spontaneous seizures [245].

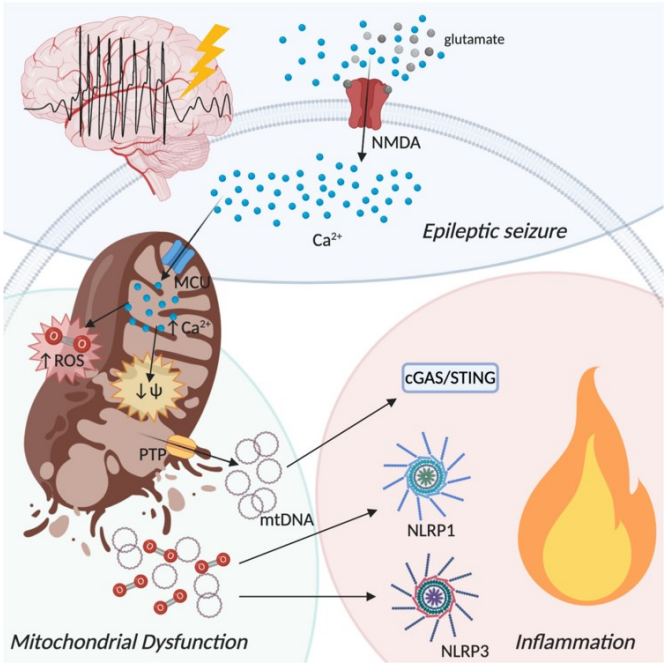


Figure 4. Epileptic seizure from a mitochondrial perspective. Repeated epileptic seizures activate NMDA receptors with the consequent influx of Ca^{2+} into the cytoplasm. Additionally, in this scenario, increased cytoplasmic Ca^{2+} concentration activates MCU. Augmented mitochondrial Ca^{2+} level triggers the activation of different enzymes responsible for reactive oxygen species production (ROS), such as nitric-oxide synthase and NADPH oxidase, and leads to membrane depolarization with the opening of the permeability transition pore (PTP) and mitochondrial membrane disruption. Release of mitochondrial components in the cytoplasm activates proinflammatory pathways such as NLRP1 and NLRP3 inflammasome assembly and the cGAS-STING dsDNA sensing machinery, leading to progressive inflammation. Created with BioRender.com.

It has been reported that inflammasomes activation contributes to the development and progression of epilepsy through the release of inflammatory mediators [246]. Remarkably, in vivo injection of NLRP3 small interfering RNAs displayed neuroprotective effects in rats following amygdala kindling-induced epilepsy [247]. Moreover, it has been speculated that amentoflavone, a natural biflavone compound with anti-inflammatory and antioxidative properties, has the ability to affect epileptogenesis and exerts neuroprotective effects through the inhibition of the NLRP3 inflammasome [248]. Interestingly, IL-10 administration in the picrotoxin seizure model results in a decreased activation of the NLRP3 inflammasome, thus of IL-1β release, suggesting a protective role in status epilepticus (SE) [249]. The role of NLRP3 inflammasome in the development of epilepsy was confirmed by a recent study showing that children with febrile seizures have higher serum levels of IL-1β, correlated to NLRP3 upregulation in PBMCs, as compared to healthy controls [250]. Recent studies have demonstrated that, like NLRP3, also the NLRP1 inflammasome is involved in SE. In fact, a NLPR1 polymorphism was reported in a Chinese Han population affected by partial seizures, suggesting a broader role of inflammasomes in inducing vulnerability to seizures [251]. Moreover, it has been demonstrated that the expression of NLRP1 and caspase 1 were increased in the hippocampus of individuals with pharmacoresistant mesial TLE, compared with the control group [252]. Accordingly, knocking down NLRP1 expression in TLE rats

led to decreased hippocampal neuronal loss and reduced seizure frequency and severity [252]. Finally, analysis of the hippocampal tissue transcriptome of patients affected by mesial TLE demonstrated an upregulation of NLRP1 compared to non-epileptic controls [253].

Besides the important role of inflammasomes activation in epileptogenesis, several studies in both humans and rodent models of TLE and SE suggest a close association between mitochondrial dysfunction and oxidative stress [254–256]. Mitochondrial respiratory deficit occurs during experimental TLE, and ROS production contributes to this event [257]. In the kainic acid model, a commonly used model of epilepsy associated with neuronal death, an increase in mitochondrial $O_2^{\bullet-}$ and of 8-hydroxy-2-deoxyguanosine levels, an indicator of oxidative DNA damage, have been observed. The intracerebroventricular infusion of the catalytic antioxidant MnTBAP 48 h before kainic acid injection has been reported to significantly reduce neuronal cell death [258]. These results confirm the strict association between mitochondria-mediated inflammation and seizure induced neuronal loss. The role of ROS in modulating seizure-induced neuroinflammation was also investigated in the pilocarpine model of TLE [240] showing that the injection of MnIIITDE-2-ImP5+, a catalytic scavenger of $O_2^{\bullet-}$, attenuated SE-induced microglial activation, mitochondrial dysfunction, and hippocampal neuronal loss. Moreover, MnIIITDE-2-ImP5+ improved short- and long-term recognition memory as well as spatial memory in epileptic rats even after treatment discontinuation. Moreover, there was no positive effect in terms of spontaneous seizures, suggesting that learning and memory improvements were not due to a reduction of the overall seizure burden [259]. The occurrence of repeated seizures leads to the activation of NMDA receptors that, as described above, induces strong influx of Ca^{2+} into the cytoplasm. Increased cytoplasmic Ca^{2+} concentration results in the activation of MCU, thus increasing mitochondrial Ca^{2+} levels that trigger the activation of various enzymes, such as nitric-oxide synthase, calpains, and NADPH oxidase, leading to the progressive inflammation [260]. Indeed, a prolonged seizure-like activity increases ROS production in an NMDA receptor-dependent manner in glioneuronal cultures, and this activity can be reduced with the inhibition of NADPH oxidase or xanthine oxidase [261]. Accordingly, the administration of a NMDA receptor antagonist after in vivo SE provided significant neuronal protection [262]. In a clinical study of parahippocampal and hippocampal tissue samples from 74 mesial TLE patients, mitochondrial dysfunction due to ROS-mediated mtDNA mutagenesis has been shown to promote neuronal cell death and epileptogenesis [263]. Accordingly, inducible NO synthase inhibition, and therefore the reduction of peroxynitrite production, may alleviate neuroinflammation and represent a neuroprotective strategy against SE [260]. Increased ROS lead to the accumulation of damaged mitochondria in the brain, which are normally removed by mitophagy [264]. Interestingly, it has been found that mitophagy is highly active in samples from hippocampi and temporal lobe cortices obtained from patients with refractory TLE, but it is unable to remove damaged mitochondria completely, thereby favoring neuronal death [265]. In this scenario, incomplete mitophagy correlates with TLE pathology. On the other hand, the treatment with DA3-CH, a glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide receptor agonist, has a neuroprotective effect in the pilocarpine model of epilepsy, because of its ability to attenuate mitophagy and, consequently, neuronal death [266]. Furthermore, the inhibition of succinate dehydrogenase in kainic acid or pilocarpine-induced SE results in a decrease of succinate levels, oxidative stress, and mitophagy, preventing neuronal damage and reducing severity of epileptic seizures [267]. Therefore, mitophagy appears to play a double faceted role in epilepsy, and the conditions in which it has a protective or pathogenic role are still controversial. In conclusion, some controversies notwithstanding, the contribution of mitochondria in epilepsy is likely important, and may lead to identification of conceptually new therapeutic approaches.

6. Current Therapies Targeting Neuroinflammation

As largely described above, mitochondria have a great impact on the neuroinflammatory process that is beyond the development of different brain disorders, such as NDDs, epilepsy, and IS, but, from a therapeutic point of view, directly targeting mitochondria is still a complicated route. In fact, current

therapies aim to treat neuroinflammation with a wider approach. One of the most characterized proinflammatory molecules is the IL-1β, and its impact on seizures occurrence was described more than 20 years ago when Redman and collaborators registered neurotoxicity after daily administration of 50 ng/kg i.v. IL-1β in patients with metastatic renal cells carcinoma [268]. Since then, many steps forward were made to understand the inflammation process and its neurotoxic contribution.

6.1. Targeting Neuroinflammation in Multiple Sclerosis

MS is characterized by a demyelinating autoimmune nature suggesting the promising results of therapies aimed to reduce inflammation. Patients affected by MS lack the ability to complete a successful remyelination process after the progression of demyelination [269]. In particular, inefficient clearance of myelin debris seems to play a crucial role in preventing a proper remyelination [270]. In this context, the lack of CX3C chemokine receptor 1 (CX3CR1), was described to compromise remyelination in mouse models [270]. In CX3CR1-deficient mice, the clearance of myelin debris was blocked, obstructing the correct remyelination. These data highlighted the crucial role of microglia in the clearance of myelin debris after a primary demyelinating insult. Additionally, IFN-β secreted by microglia enhanced the removal of myelin debris in the MS model of experimental autoimmune encephalomyelitis (EAE) [271]. These data indicate that remyelination cannot be successful if myelin debris are still present, thus pointing to the myelin clearance process as a promising target. Besides the great importance of proinflammatory cytokines, such as IFN-β, in modulating myelin debris clearance, excessive inflammatory response is detrimental in MS development, therefore different therapeutic approaches aim to decrease the neuroinflammation in MS. In particular, an interesting strategy to counteract immune system response is provided by alemtuzumab. Patients treated with this compound showed a reduction of proinflammatory cytokines. Furthermore, alemtuzumab showed a long-lasting therapeutic benefit with the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β) [272]. However, the presence of secondary autoimmunities caused by alemtuzumab has limited its clinical employment [273]. Among the drugs with neuroinflammatory effects, FDA has recently approved ocrelizumab which targets CD20-positive B-cells and prevents damage to nerve cells. There are many ongoing phase III/IV clinical trials evaluating the dose efficacy of ocrelizumab by recording the improvement of ambulatory functions (NCT04544436; NCT04387734). Hoffmann-La Roche has also started a phase III trial of fenebrutinib, a dual inhibitor of both B-cells and myeloid lineage-cells activation, in patients with primary progressive MS (NCT04544449). Similarly, ofatumumab, a human anti-CD20 antibody responsible for specific B-cells lysis and depletion at the lymph nodes, is undergoing phase III clinical trial (NCT04486716). Moreover, the Food and Drug Administration has approved the use of fingolimod to treat relapsing-remitting MS. Fingolimod is a sphingosine-1-phosphate-receptor modulator that blocks the recirculation of autoaggressive lymphocytes without suppressing the immune response [274]. Various clinical trials have confirmed positive effects conferred by fingolimod treatment with lower relapse rate and better magnetic resonance imaging outcome [275,276].

6.2. Targeting Neuroinflammation in Parkinson's Disease

High levels of IL-1β were reported in Parkinsonian patients [277], thus many studies have investigated the contribution of inflammation to the onset of PD. In particular, the focus has pointed to the IL-1 receptor/Toll-like receptor 4 axis, as the trigger of the neuroinflammatory process [278]. However, therapies specifically targeting this pathway are still missing and are mainly focused on PD symptoms. Among these therapies, L-dopa still represents the most effective symptomatic pharmacological treatment for PD [279]. Despite this, several strategies have been recently studied to modulate the inflammatory response. For instance, minocycline, a widely used antibiotic, seems to protect nigral dopaminergic neurons and to decrease glial cells activation [280], warranting its neuroprotective role in PD. Unfortunately, this treatment is still controversial because, despite its compelling features, it has also been reported to cause severe neural cell loss in animal models [281]. Besides minocycline, other compounds have been described to have a neuroprotective effect in the

treatment of Parkinsonism. For instance, the synthetic steroid dexamathasone prevents dopaminergic neurons degeneration in a mouse PD model [282], and the synthetic compound naloxone blocks microglia activation thus reducing the inflammatory damage [283]. Aside from its cardiovascular application, nimodipine, a calcium channel blocker, exhibits a neuroprotective effect on dopaminergic neurons by downregulating microglial activation, thus decreasing TNF-α and IL-1β production [284]. Another compound with promising effect in targeting neuroinflammation in PD is semaglutide, a synthetic analogue of glucagon-like peptide 1 (GLP1), which stimulates GLP1R, and it is already used to treat type 2 diabetes. GLP1R activation inhibits the production of proinflammatory cytokines and slows down the neurodegenerative process [285]. An ongoing phase II clinical trial will assess the effects of semaglutide on both motor and non-motor symptoms of PD (NCT03659682). Another GLP1 agonist, exenatide, is undergoing a phase III clinical trial to verify its neuroprotective effect in Parkinsonian patients (NCT04232969). As previously described, oxidative stress has a crucial role in triggering the degeneration of dopaminergic neurons. Accordingly, studies on different antioxidant therapies, such as idebenone a quinone analogue, and tocotrienols, primary form of vitamin E, are undergoing, respectively, phase II and phase IV clinical trial for their effect on motor and non-motor symptoms in patients with early PD (NCT03727295, NCT04491383).

6.3. Targeting Neuroinflammation in Alzheimer's Disease

Nimodipine together with the non-steroidal anti-inflammatory drugs (NSAIDs) are the elective drugs used to treat neuroinflammation in AD. Nimodipine prevents Aβ-dependent injury, and its administration both in in vitro and in vivo models resulted in a strong inhibition of IL-1β release and accumulation [286]. Ibuprofen, which is one of the most prominent NSAIDs, is involved in many ongoing clinical studies for AD treatment, but its action is still controversial. In fact, a clinical study reported that long NSAID treatment reduced the probability of developing AD [287], while on the other hand another one reported that AD patients treated with NSAIDs have a worse outcome [288]. Recently, fenamate NSAIDs were reported to have beneficial effects in a mouse model of AD [289], while a pioglitazone phase III clinical trial was terminated because of lack of efficacy [290]. Chronic treatment with AF710B (ANAVEX 3-71), a selective allosteric M1 muscarinic and sigma-1 receptor agonist, showed anti-amyloid and anti-neuroinflammatory effects suggesting its ability in damping inflammation in animal models of AD [291]. Following the encouraging preclinical results, a phase I clinical trial is assessing tolerability and safety of ANAVEX3-71 compound in healthy volunteers (NCT04442945). The same pharmaceutical company developed another similar compound, ANAVEX2-73, which is now ongoing phase III clinical trial to evaluate its anti-amyloid and anti-inflammatory effects on AD patients (NCT03790709). Primary outcome measures will be available in the next few years. Nilvadipine, a calcium channel blocker, due to its anti-inflammatory potential shown in preclinical studies, underwent a phase III clinical trial to treat AD. Unfortunately, the trial reported no benefits of nilvadipine in the treatment of mild to moderate AD (NCT02017340). Lastly, epidemiological studies proposed the treatment of AD with NSAIDs, but the encouraging results recorded in animal models have not yet been confirmed in human patients [292].

6.4. Targeting Neuroinflammation in Ischemic Stroke

As observed in MS, the oral administration of fingolimod displayed promising results in ameliorating acute IS outcome [293]. Treated patients exhibited a reduction of lesions and a better clinical recovery after 3 months. As well as for PD, minocycline showed controversial results in stroke patients: while a phase II trial reported a good outcome, a pilot study has shown that minocycline treatment was safe but not efficacious [294,295], indicating the need of further investigation. The recombinant human IL-1 receptor antagonist, namely anakinra, showed encouraging results in a phase II clinical trial by decreasing the neuroinflammatory process after 3 months from the administration, even though this promising result was not reflected by the clinical outcome [296,297]. A novel combined therapy of molecular hydrogen H_2, an antioxidant, plus minocycline, named H_2M is undergoing a

pilot randomized control trial to test its efficacy in preventing brain tissue damage (NCT03320018). Lastly, the efficacy of the neuroprotectant nerinetide is being investigated in patients with acute IS after the promising results obtained in vitro showing a reduction of the hypoxic damage in cultured neurons mimicking IR injuries [298] (NCT04462536).

6.5. Targeting Neuroinflammation in Epilepsy

As well as for PD, toll-like receptor 4 has been described to significantly affect the neuroinflammatory process and the pathogenesis of epilepsy [299,300]. Therefore, IL-1R has become a front-runner among the possible therapeutic targets. Accordingly, promising results, such as the reduction of total number of seizures, have emerged from the use of anakinra to treat epilepsy and related syndromes [301]. Interestingly, levetiracetam, one of the most commonly used anti-epilepsy drugs, reported anti-inflammatory effects in vitro [302]. Cannabidiol has also been investigated for both its anti-inflammatory and antioxidant properties [303]. Even though different phase III clinical trials are currently assessing the efficacy of cannabidiol in decreasing the number of seizures, FDA has recently approved epidiolex, a pharmaceutical compound containing cannabidiol, for the treatment of seizures associated with two severe forms of epilepsy (NCT02224690) (see Table 1).

Table 1. List of recent studies and drug therapies targeting neuroinflammation in Parkinson's disease, Alzheimer's disease, multiple sclerosis, ischemic stroke, and epilepsy. The clinically approved column refers to the approval of the drug for the treatment of the specific CNS disease. Refer to the text for the explanation of controversial effects.

Therapy	Disease	Effects	Clinically Approved	Reference
Alemtuzumab	MS	Controversial	No	[272,273]
ANAVEX2-73	AD	Anti-inflammatory; Antioxidant	No	NCT03790709
ANAVEX3-71	AD	Anti-inflammatory	No	[291]; NCT04442945
Cannabidiol	Epilepsy	Anti-inflammatory; Antioxidant	Yes	[303]; NCT02224690
Dexamethasone	PD	Neuroprotectant	No	[282]
Exenatide	PD	Neuroprotectant	No	NCT04232969
Fenamate NSAIDs	AD	Anti-inflammatory	No	[289]
Fenebrutinib	MS	Anti-inflammatory	No	NCT04544449
Fingolimod	IS	Neuroprotectant	No	[293]
Fingolimod	MS	Anti-inflammatory	No	[274–276]
H2M	IS	Antioxidant; neuroprotectant.	No	[287,288]
Ibuprofen	AD	Controversial	No	[287,288]
Idebenone	PD	Antioxidant	No	[299,300]
IL-1Ra	Epilepsy	Anticonvulsant	No	[299,300]
IL-1Ra	IS	Controversial	No	[296,297]
Levetiracetam	Epilepsy	Anticonvulsant; anti-inflammatory	Yes	[302]
Minocycline	IS	Controversial	No	[294,295]
Minocycline	PD	Controversial	No	[280,281]
Naloxone	PD	Neuroprotectant	No	[283]
Nerinetide	IS	Neuroprotectant	No	[298]; NCT04462536
Nilvadipine	AD	Controversial	No	NCT02017340
Nimodipine	AD	Anti-inflammatory	No	[286]
Nimodipine	PD	Neuroprotectant	No	[284]
Ocrelizumab	MS	Neuroprotectant	Yes	NCT04544436; NCT04387734
Ofatumumab	MS	Anti-inflammatory	No	NCT04486716
Pioglitazone	AD	Controversial	No	[290]
Semaglutide	PD	Anti-inflammatory	No	[285];NCT03659682
TLR4 deletion	PD	Neuroprotectant; Anti-inflammatory	No	[278]
Tocotrienols	PD	Antioxidant	No	NCT04491383

7. Conclusions

Neuroinflammation has been shown to play a pivotal role in CNS disorders, being mainly responsible for the neuronal cell loss and the exacerbation of the pathology. As widely described,

mitochondria have a prominent part in this process: they are responsible for inflammasome assembly and ROS production, and they enclose a large amount of DAMPs, accountable for sustaining the inflammatory process. Interestingly, all these proinflammatory roles are balanced by mitophagy, which is responsible for eliminating abnormal mitochondria in order to interrupt the inflammatory sprouts. These aspects are finely balanced in CNS physiological homeostasis, since acute inflammation cover also a protective role in case of pathogens infection, but the minimal perturbation of this fine regulation triggers the neurodegenerative process. As neurodegeneration involves many altered pathways, researchers have focused the attention on many possible targets including abnormal oxidative stress and uncontrolled neuroinflammation. Researchers are investigating why pathways that are crucial for cell survival are dysregulated in neurodegenerative diseases. For instance, neuroinflammation is vital in activating the regenerative process in IS and epilepsy, but its atypical stimulation leads to opposite results. The actual used therapies act mainly on symptomatic relief, and different pharmacological mechanisms of used drugs are yet to be fully elucidated. At the same time, even if many preclinical studies reported encouraging results, various clinical trials have shown controversial outcome or poor efficacy. This scenario could be ascribed to one of the biggest challenges of clinical trials, that is patients' heterogeneity and the presence of comorbidities. For these reasons, as far as now, no therapies have shown efficacy in preventing neurodegenerative diseases or at least in significantly reducing their progression. However, an increasing number of studies are focusing on deregulated neuroinflammatory pathways as a common feature in NDDS. In particular, in MS, due to its autoimmune nature, drugs targeting neuroinflammation have already been approved and have displayed the most prominent results.

In conclusion, even if many aspects of inflammation-driven neurodegeneration are still unclear and further studies are needed to exploit all the pathways beyond this phenomenon, dysregulated inflammatory responses appear as a common feature for progression of brain diseases. Therefore, targeting neuroinflammation, also by acting on mitochondria, in NDDs, IS, and epilepsy represents a promising complementary therapy to obtain better clinical outcomes.

Author Contributions: Conceptualization, writing—original draft, writing—review and editing, B.V.; writing—original draft, writing—review and editing M.C., M.P.P.; writing—original draft S.P., A.G., N.A.; funding acquisition, review and editing, P.P.; review and editing, M.S.; conceptualization, funding acquisition, review and editing, C.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Italian Association for Cancer Research (AIRC), the Italian Ministry of Health and the ERC-STG (Project: 853057—InflaPML) to C.G. S.P. was supported by Fondazione Umberto Veronesi.

Acknowledgments: P.P. thanks Camilla degli Scrovegni for continuous support. The Signal Transduction Laboratory of the University of Ferrara (P.P., C.G., B.V., M.C., S.P. and M.P.P.) thanks Associazione Ricerca Oncologica Sperimentale Estense (A-Rose) for the ongoing support to their researches.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AD	Alzheimer's disease
AIM2	absent in melanoma 2
ALRs	AIM2-like receptors
ALS	amyotrophic lateral sclerosis
ASC	apoptosis-associated speck-like protein containing CARD
ATP	adenosine triphosphate
Aβ	β-amyloid protein
BBB	blood–brain barrier
BNIP3	B-cell lymphoma 2 nineteen kilodalton interacting protein 3
BNIP3L	BNIP3-like
cGAMP	2′,3′-cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthetase
CNS	central nervous system
CSF	cerebrospinal fluid
CXC3CR1	CX3C chemokine receptor 1
DAMPs	damage associated molecular patterns

dsDNA	double strand DNA
EAE	experimental autoimmune encephalomyelitis
ER	endoplasmic reticulum
ETC	electron transport chain
GTP	guanosine triphosphate
GLP1	glucagon-like peptide 1
IFN	interferon
IL-10	interleukin-10
IL-18	interleukin-18
IL-1β	interleukin-1β
IL-6	interleukin-6
IR	ischemic reperfusion
IRF3	interferon regulatory factor 3
IS	ischemic stroke
LIR	LC3-interacting region
LRR	leucine-rich repeats
MAMs	mitochondria associated membranes
MCU	mitochondria calcium uniporter
MICU1	mitochondrial calcium uptake 1
MPPs	matrix metalloproteinases
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	multiple sclerosis
mtDNA	mitochondrial DNA
NACHT	nucleotide and oligomerization domain
NBR1	neighbor of Brca1
NDDs	neurodegenerative diseases
NDP52	nuclear dot protein 52
NF-kB	nuclear factor-kB
NLRP3	nucleotide-binding domain and leucine-rich repeat containing protein 3
NLRs	nucleotide-binding oligomerization domain-like (NOD) receptors
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSAIDs	non steroidal anti-inflammatory drugs
OGD	oxygen and glucose deprivation
OMM	outer mitochondrial membrane
OPTN	optineurin
OXPHOS	oxidative phosphorylation
p62/SQSTM1	p62/sequestosome 1
PAMPs	pathogen-associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PD	Parkinson's disease
PINK1	PTEN-induced putative kinase 1
PRRs	pattern recognition receptors
PTP	permeability transition pore
PYD	pyrine
RNS	reactive nitrogen species
ROS	reactive oxygen species
SE	status epilepticus
STING	stimulator of interferon genes
TACE	TNF-a-converting enzyme
TAX1BP1	tax 1 binding protein 1
TBK1	TANK-binding kinase 1
TGF-β	transforming growth factor β
TLE	temporal lobe epilepsy
TLR	Toll-like receptor
TNF-α	tumor necrosis factor α
TREM2	triggering receptor expressed on myeloid cells-2
α-Syn	α-synuclein
Ψm	mitochondrial membrane potential

References

1. Barker, C.F.; Billingham, R.E. Immunologically privileged sites. *Adv. Immunol.* **1977**, *25*, 1–54. [PubMed]

2. Medawar, P.B. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br. J. Exp. Pathol.* **1948**, *29*, 58–69. [PubMed]
3. Louveau, A.; Harris, T.H.; Kipnis, J. Revisiting the Mechanisms of CNS Immune Privilege. *Trends Immunol.* **2015**, *36*, 569–577. [CrossRef] [PubMed]
4. Louveau, A.; Smirnov, I.; Keyes, T.J.; Eccles, J.D.; Rouhani, S.J.; Peske, J.D.; Derecki, N.C.; Castle, D.; Mandell, J.W.; Lee, K.S.; et al. Structural and functional features of central nervous system lymphatic vessels. *Nature* **2015**, *523*, 337–341. [CrossRef] [PubMed]
5. Aspelund, A.; Antila, S.; Proulx, S.T.; Karlsen, T.V.; Karaman, S.; Detmar, M.; Wiig, H.; Alitalo, K. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med.* **2015**, *212*, 991–999. [CrossRef]
6. Bradl, M.; Lassmann, H. Oligodendrocytes: Biology and pathology. *Acta Neuropathol.* **2010**, *119*, 37–53. [CrossRef] [PubMed]
7. Papaneophytou, C.; Georgiou, E.; Kleopa, K.A. The role of oligodendrocyte gap junctions in neuroinflammation. *Channels* **2019**, *13*, 247–263. [CrossRef] [PubMed]
8. Prat, A.; Biernacki, K.; Wosik, K.; Antel, J.P. Glial cell influence on the human blood-brain barrier. *Glia* **2001**, *36*, 145–155. [CrossRef]
9. Koehler, R.C.; Gebremedhin, D.; Harder, D.R. Role of astrocytes in cerebrovascular regulation. *J. Appl. Physiol.* **2006**, *100*, 307–317. [CrossRef]
10. Gimenez, M.A.; Sim, J.; Archambault, A.S.; Klein, R.S.; Russell, J.H. A tumor necrosis factor receptor 1-dependent conversation between central nervous system-specific T cells and the central nervous system is required for inflammatory infiltration of the spinal cord. *Am. J. Pathol.* **2006**, *168*, 1200–1209. [CrossRef]
11. Carson, M.J.; Thrash, J.C.; Walter, B. The cellular response in neuroinflammation: The role of leukocytes, microglia and astrocytes in neuronal death and survival. *Clin. Neurosci. Res.* **2006**, *6*, 237–245. [CrossRef] [PubMed]
12. Del Río Hortega, P. Noticia de un nuevo y fácil método para la coloración de la neuroglia y el tejido conjuntivo. *Trab. Lab. Investig. Biol.* **1918**, *15*, 367–378.
13. Ransohoff, R.M.; El Khoury, J. Microglia in Health and Disease. *Cold Spring Harb. Perspect. Biol.* **2015**, *8*, a020560. [CrossRef] [PubMed]
14. Hickman, S.; Izzy, S.; Sen, P.; Morsett, L.; El Khoury, J. Microglia in neurodegeneration. *Nat. Neurosci.* **2018**, *21*, 1359–1369. [CrossRef] [PubMed]
15. Streit, W.J.; Mrak, R.E.; Griffin, W.S.T. Microglia and neuroinflammation: A pathological perspective. *J. Neuroinflamm.* **2004**, *1*, 14. [CrossRef] [PubMed]
16. Da Mesquita, S.; Fu, Z.; Kipnis, J. The Meningeal Lymphatic System: A New Player in Neurophysiology. *Neuron* **2018**, *100*, 375–388. [CrossRef]
17. McGavern, D.B.; Homann, D.; Oldstone, M.B.A. T cells in the central nervous system: The delicate balance between viral clearance and disease. *J. Infect. Dis.* **2002**, *186* (Suppl. S2), S145–S151. [CrossRef]
18. Sabatino, J.J.; Pröbstel, A.-K.; Zamvil, S.S. B cells in autoimmune and neurodegenerative central nervous system diseases. *Nat. Rev. Neurosci.* **2019**, *20*, 728–745. [CrossRef]
19. Heneka, M.T.; O'Banion, M.K. Inflammatory processes in Alzheimer's disease. *J. Neuroimmunol.* **2007**, *184*, 69–91. [CrossRef]
20. Hirsch, E.C.; Hunot, S. Neuroinflammation in Parkinson's disease: A target for neuroprotection? *Lancet Neurol.* **2009**, *8*, 382–397. [CrossRef]
21. Björkqvist, M.; Wild, E.J.; Tabrizi, S.J. Harnessing immune alterations in neurodegenerative diseases. *Neuron* **2009**, *64*, 21–24. [CrossRef] [PubMed]
22. Di Filippo, M.; Chiasserini, D.; Tozzi, A.; Picconi, B.; Calabresi, P. Mitochondria and the link between neuroinflammation and neurodegeneration. *J. Alzheimer's Dis.* **2010**, *20* (Suppl. S2), S369–S379. [CrossRef] [PubMed]
23. Aktas, O.; Ullrich, O.; Infante-Duarte, C.; Nitsch, R.; Zipp, F. Neuronal damage in brain inflammation. *Arch. Neurol.* **2007**, *64*, 185–189. [CrossRef] [PubMed]
24. de Barcelos, I.P.; Troxell, R.M.; Graves, J.S. Mitochondrial Dysfunction and Multiple Sclerosis. *Biology* **2019**, *8*, 37. [CrossRef]
25. Abou-Sleiman, P.M.; Muqit, M.M.K.; Wood, N.W. Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nat. Rev. Neurosci.* **2006**, *7*, 207–219. [CrossRef]

26. Silva, D.F.; Selfridge, J.E.; Lu, J.; Lezi, E.; Cardoso, S.M.; Swerdlow, R.H. Mitochondrial abnormalities in Alzheimer's disease: Possible targets for therapeutic intervention. *Adv. Pharmacol.* **2012**, *64*, 83–126. [CrossRef]
27. Antico Arciuch, V.G.; Elguero, M.E.; Poderoso, J.J.; Carreras, M.C. Mitochondrial regulation of cell cycle and proliferation. *Antioxid. Redox Signal.* **2012**, *16*, 1150–1180. [CrossRef]
28. Cherry, J.D.; Olschowka, J.A.; O'Banion, M.K. Are "resting" microglia more "m2"? *Front. Immunol.* **2014**, *5*, 594. [CrossRef]
29. Nimmerjahn, A.; Kirchhoff, F.; Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **2005**, *308*, 1314–1318. [CrossRef]
30. Farina, C.; Aloisi, F.; Meinl, E. Astrocytes are active players in cerebral innate immunity. *Trends Immunol.* **2007**, *28*, 138–145. [CrossRef]
31. Craft, J.M.; Watterson, D.M.; Van Eldik, L.J. Neuroinflammation: A potential therapeutic target. *Expert Opin. Ther. Targets* **2005**, *9*, 887–900. [CrossRef] [PubMed]
32. ElAli, A.; Rivest, S. Microglia Ontology and Signaling. *Front. Cell Dev. Biol.* **2016**, *4*. [CrossRef] [PubMed]
33. Chitnis, T.; Weiner, H.L. CNS inflammation and neurodegeneration. *J. Clin. Investig.* **2017**, *127*, 3577–3587. [CrossRef] [PubMed]
34. Seth, R.B.; Sun, L.; Ea, C.-K.; Chen, Z.J. Identification and Characterization of MAVS, a Mitochondrial Antiviral Signaling Protein that Activates NF-κB and IRF3. *Cell* **2005**, *122*, 669–682. [CrossRef] [PubMed]
35. Liu, S.; Feng, M.; Guan, W. Mitochondrial DNA sensing by STING signaling participates in inflammation, cancer and beyond. *Int. J. Cancer* **2016**, *139*, 736–741. [CrossRef]
36. Liu, Q.; Zhang, D.; Hu, D.; Zhou, X.; Zhou, Y. The role of mitochondria in NLRP3 inflammasome activation. *Mol. Immunol.* **2018**, *103*, 115–124. [CrossRef]
37. Janeway, C.A. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today* **1992**, *13*, 11–16. [CrossRef]
38. Voet, S.; Srinivasan, S.; Lamkanfi, M.; van Loo, G. Inflammasomes in neuroinflammatory and neurodegenerative diseases. *EMBO Mol. Med.* **2019**, *11*. [CrossRef]
39. Matzinger, P. The danger model: A renewed sense of self. *Science* **2002**, *296*, 301–305. [CrossRef]
40. Newton, K.; Dixit, V.M. Signaling in innate immunity and inflammation. *Cold Spring Harb. Perspect. Biol.* **2012**, *4*. [CrossRef]
41. Sagan, L. On the origin of mitosing cells. *J. Theor. Biol.* **1967**, *14*, 255–274. [CrossRef]
42. Grazioli, S.; Pugin, J. Mitochondrial Damage-Associated Molecular Patterns: From Inflammatory Signaling to Human Diseases. *Front. Immunol.* **2018**, *9*. [CrossRef] [PubMed]
43. Patrushev, M.; Kasymov, V.; Patrusheva, V.; Ushakova, T.; Gogvadze, V.; Gaziev, A. Mitochondrial permeability transition triggers the release of mtDNA fragments. *Cell. Mol. Life Sci.* **2004**, *61*, 3100–3103. [CrossRef] [PubMed]
44. Bader, V.; Winklhofer, K. Mitochondria at the interface between neurodegeneration and neuroinflammation. *Semin. Cell Dev. Biol.* **2020**, 163–171. [CrossRef] [PubMed]
45. Kigerl, K.A.; de Rivero Vaccari, J.P.; Dietrich, W.D.; Popovich, P.G.; Keane, R.W. Pattern recognition receptors and central nervous system repair. *Exp. Neurol.* **2014**, *258*, 5–16. [CrossRef]
46. Adamczak, S.E.; De Rivero Vaccari, J.P.; Dale, G.; Brand, F.J.; Nonner, D.; Bullock, M.; Dahl, G.P.; Dietrich, W.D.; Keane, R.W. Pyroptotic neuronal cell death mediated by the AIM2 inflammasome. *J. Cereb. Blood Flow Metab.* **2014**, *34*, 621–629. [CrossRef] [PubMed]
47. Cameron, J.S.; Alexopoulou, L.; Sloane, J.A.; DiBernardo, A.B.; Ma, Y.; Kosaras, B.; Flavell, R.; Strittmatter, S.M.; Volpe, J.; Sidman, R.; et al. Toll-like receptor 3 is a potent negative regulator of axonal growth in mammals. *J. Neurosci.* **2007**, *27*, 13033–13041. [CrossRef]
48. Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; et al. A Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408*, 740–745. [CrossRef]
49. Sun, L.; Wu, J.; Du, F.; Chen, X.; Chen, Z.J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **2013**, *339*, 786–791. [CrossRef]
50. Ablasser, A.; Goldeck, M.; Cavlar, T.; Deimling, T.; Witte, G.; Röhl, I.; Hopfner, K.-P.; Ludwig, J.; Hornung, V. cGAS produces a 2′-5′-linked cyclic dinucleotide second messenger that activates STING. *Nature* **2013**, *498*, 380–384. [CrossRef]

51. Shang, G.; Zhang, C.; Chen, Z.J.; Bai, X.-C.; Zhang, X. Cryo-EM structures of STING reveal its mechanism of activation by cyclic GMP-AMP. *Nature* **2019**, *567*, 389–393. [CrossRef] [PubMed]
52. Liu, S.; Cai, X.; Wu, J.; Cong, Q.; Chen, X.; Li, T.; Du, F.; Ren, J.; Wu, Y.-T.; Grishin, N.V.; et al. Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* **2015**, *347*, aaa2630. [CrossRef] [PubMed]
53. Gui, X.; Yang, H.; Li, T.; Tan, X.; Shi, P.; Li, M.; Du, F.; Chen, Z.J. Autophagy induction via STING trafficking is a primordial function of the cGAS pathway. *Nature* **2019**, *567*, 262–266. [CrossRef] [PubMed]
54. Chin, A.C. Neuroinflammation and the cGAS-STING pathway. *J. Neurophysiol.* **2019**, *121*, 1087–1091. [CrossRef]
55. Srinivasula, S.M.; Poyet, J.-L.; Razmara, M.; Datta, P.; Zhang, Z.; Alnemri, E.S. The PYRIN-CARD Protein ASC Is an Activating Adaptor for Caspase-1. *J. Biol. Chem.* **2002**, *277*, 21119–21122. [CrossRef]
56. Man, S.M.; Kanneganti, T.-D. Regulation of inflammasome activation. *Immunol. Rev.* **2015**, *265*, 6–21. [CrossRef]
57. Guo, H.; Callaway, J.B.; Ting, J.P.-Y. Inflammasomes: Mechanism of action, role in disease, and therapeutics. *Nat. Med.* **2015**, *21*, 677–687. [CrossRef]
58. Lu, M.; Sun, X.-L.; Qiao, C.; Liu, Y.; Ding, J.-H.; Hu, G. Uncoupling protein 2 deficiency aggravates astrocytic endoplasmic reticulum stress and nod-like receptor protein 3 inflammasome activation. *Neurobiol. Aging* **2014**, *35*, 421–430. [CrossRef]
59. Gustin, A.; Kirchmeyer, M.; Koncina, E.; Felten, P.; Losciuto, S.; Heurtaux, T.; Tardivel, A.; Heuschling, P.; Dostert, C. NLRP3 Inflammasome Is Expressed and Functional in Mouse Brain Microglia but Not in Astrocytes. *PLoS ONE* **2015**, *10*, e0130624. [CrossRef]
60. Shi, J.; Zhao, Y.; Wang, K.; Shi, X.; Wang, Y.; Huang, H.; Zhuang, Y.; Cai, T.; Wang, F.; Shao, F. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* **2015**, *526*, 660–665. [CrossRef]
61. Zhou, R.; Yazdi, A.S.; Menu, P.; Tschopp, J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* **2011**, *469*, 221–225. [CrossRef] [PubMed]
62. Singhal, G.; Jaehne, E.J.; Corrigan, F.; Toben, C.; Baune, B.T. Inflammasomes in neuroinflammation and changes in brain function: A focused review. *Front. Neurosci.* **2014**, *8*. [CrossRef] [PubMed]
63. Deora, V.; Lee, J.D.; Albornoz, E.A.; McAlary, L.; Jagaraj, C.J.; Robertson, A.A.B.; Atkin, J.D.; Cooper, M.A.; Schroder, K.; Yerbury, J.J.; et al. The microglial NLRP3 inflammasome is activated by amyotrophic lateral sclerosis proteins. *Glia* **2020**, *68*, 407–421. [CrossRef] [PubMed]
64. Lee, E.; Hwang, I.; Park, S.; Hong, S.; Hwang, B.; Cho, Y.; Son, J.; Yu, J.-W. MPTP-driven NLRP3 inflammasome activation in microglia plays a central role in dopaminergic neurodegeneration. *Cell Death Differ.* **2019**, *26*, 213–228. [CrossRef] [PubMed]
65. Ising, C.; Venegas, C.; Zhang, S.; Scheiblich, H.; Schmidt, S.V.; Vieira-Saecker, A.; Schwartz, S.; Albasset, S.; McManus, R.M.; Tejera, D.; et al. NLRP3 inflammasome activation drives tau pathology. *Nature* **2019**, *575*, 669–673. [CrossRef]
66. Scheiblich, H.; Schlütter, A.; Golenbock, D.T.; Latz, E.; Martinez-Martinez, P.; Heneka, M.T. Activation of the NLRP3 inflammasome in microglia: The role of ceramide. *J. Neurochem.* **2017**, *143*, 534–550. [CrossRef] [PubMed]
67. Wang, W.; Hu, D.; Wu, C.; Feng, Y.; Li, A.; Liu, W.; Wang, Y.; Chen, K.; Tian, M.; Xiao, F.; et al. STING promotes NLRP3 localization in ER and facilitates NLRP3 deubiquitination to activate the inflammasome upon HSV-1 infection. *PLoS Pathog.* **2020**, *16*, e1008335. [CrossRef]
68. Colton, C.A.; Gilbert, D.L. Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett.* **1987**, *223*, 284–288. [CrossRef]
69. Handy, D.E.; Loscalzo, J. Redox regulation of mitochondrial function. *Antioxid. Redox Signal.* **2012**, *16*, 1323–1367. [CrossRef]
70. Bedard, K.; Krause, K.-H. The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. *Physiol. Rev.* **2007**, *87*, 245–313. [CrossRef]
71. Ray, P.D.; Huang, B.-W.; Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **2012**, *24*, 981–990. [CrossRef] [PubMed]
72. Kobylarek, D.; Iwanowski, P.; Lewandowska, Z.; Limphaibool, N.; Szafranek, S.; Labrzycka, A.; Kozubski, W. Advances in the Potential Biomarkers of Epilepsy. *Front. Neurol.* **2019**, *10*. [CrossRef] [PubMed]

73. Barker, J.E.; Bolaños, J.P.; Land, J.M.; Clark, J.B.; Heales, S.J. Glutathione protects astrocytes from peroxynitrite-mediated mitochondrial damage: Implications for neuronal/astrocytic trafficking and neurodegeneration. *Dev. Neurosci.* **1996**, *18*, 391–396. [CrossRef] [PubMed]
74. Eliasson, M.J.; Huang, Z.; Ferrante, R.J.; Sasamata, M.; Molliver, M.E.; Snyder, S.H.; Moskowitz, M.A. Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J. Neurosci.* **1999**, *19*, 5910–5918. [CrossRef] [PubMed]
75. Bélanger, M.; Allaman, I.; Magistretti, P.J. Brain energy metabolism: Focus on astrocyte-neuron metabolic cooperation. *Cell Metab.* **2011**, *14*, 724–738. [CrossRef] [PubMed]
76. Rose, J.; Brian, C.; Woods, J.; Pappa, A.; Panayiotidis, M.I.; Powers, R.; Franco, R. Mitochondrial dysfunction in glial cells: Implications for neuronal homeostasis and survival. *Toxicology* **2017**, *391*, 109–115. [CrossRef]
77. Yin, F.; Sancheti, H.; Patil, I.; Cadenas, E. Energy metabolism and inflammation in brain aging and Alzheimer's disease. *Free Radic. Biol. Med.* **2016**, *100*, 108–122. [CrossRef]
78. Fernandez-Fernandez, S.; Almeida, A.; Bolaños, J.P. Antioxidant and bioenergetic coupling between neurons and astrocytes. *Biochem. J.* **2012**, *443*, 3–11. [CrossRef]
79. Herrero-Mendez, A.; Almeida, A.; Fernández, E.; Maestre, C.; Moncada, S.; Bolaños, J.P. The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat. Cell Biol.* **2009**, *11*, 747–752. [CrossRef]
80. van Horssen, J.; van Schaik, P.; Witte, M. Inflammation and mitochondrial dysfunction: A vicious circle in neurodegenerative disorders? *Neurosci. Lett.* **2019**, *710*, 132931. [CrossRef]
81. Martinez-Vicente, M. Neuronal Mitophagy in Neurodegenerative Diseases. *Front. Mol. Neurosci.* **2017**, *10*, 64. [CrossRef] [PubMed]
82. Palikaras, K.; Tavernarakis, N. Mitophagy in neurodegeneration and aging. *Front. Genet.* **2012**, *3*, 297. [CrossRef] [PubMed]
83. Patergnani, S.; Pinton, P. Mitophagy and Mitochondrial Balance. *Methods Mol Biol.* **2015**, *1241*, 181–194. [CrossRef] [PubMed]
84. Wang, Y.; Liu, N.; Lu, B. Mechanisms and roles of mitophagy in neurodegenerative diseases. *CNS Neurosci. Ther.* **2019**, *25*, 859–875. [CrossRef] [PubMed]
85. Youle, R.J.; Narendra, D.P. Mechanisms of mitophagy. *Nat. Rev. Mol. Cell Biol.* **2011**, *12*, 9–14. [CrossRef] [PubMed]
86. Jin, S.M.; Lazarou, M.; Wang, C.; Kane, L.A.; Narendra, D.P.; Youle, R.J. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J. Cell Biol.* **2010**, *191*, 933–942. [CrossRef] [PubMed]
87. Jin, S.M.; Youle, R.J. PINK1- and Parkin-mediated mitophagy at a glance. *J. Cell Sci.* **2012**, *125*, 795–799. [CrossRef] [PubMed]
88. Geisler, S.; Holmström, K.M.; Skujat, D.; Fiesel, F.C.; Rothfuss, O.C.; Kahle, P.J.; Springer, W. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat. Cell Biol.* **2010**, *12*, 119–131. [CrossRef] [PubMed]
89. Pickles, S.; Vigié, P.; Youle, R.J. Mitophagy and Quality Control Mechanisms in Mitochondrial Maintenance. *Curr. Biol.* **2018**, *28*, R170–R185. [CrossRef] [PubMed]
90. Lublin, F.D.; Reingold, S.C.; Cohen, J.A.; Cutter, G.R.; Sorensen, P.S.; Thompson, A.J.; Wolinsky, J.S.; Balcer, L.J.; Banwell, B.; Barkhof, F.; et al. Defining the clinical course of multiple sclerosis: The 2013 revisions. *Neurology* **2014**, *83*, 278–286. [CrossRef]
91. Lublin, F.D. New multiple sclerosis phenotypic classification. *Eur. Neurol.* **2014**, *72* (Suppl. S1), 1–5. [CrossRef] [PubMed]
92. Patergnani, S.; Fossati, V.; Bonora, M.; Giorgi, C.; Marchi, S.; Missiroli, S.; Rusielewicz, T.; Wieckowski, M.R.; Pinton, P. Mitochondria in Multiple Sclerosis: Molecular Mechanisms of Pathogenesis. *Int Rev Cell Mol Biol.* **2017**, *382*, 49–103. [CrossRef]
93. Ciccarelli, O. Multiple sclerosis in 2018: New therapies and biomarkers. *Lancet Neurol.* **2019**, *18*, 10–12. [CrossRef]
94. Khaibullin, T.; Ivanova, V.; Martynova, E.; Cherepnev, G.; Khabirov, F.; Granatov, E.; Rizvanov, A.; Khaiboullina, S. Elevated Levels of Proinflammatory Cytokines in Cerebrospinal Fluid of Multiple Sclerosis Patients. *Front. Immunol.* **2017**, *8*. [CrossRef]

95. Patergnani, S.; Castellazzi, M.; Bonora, M.; Marchi, S.; Casetta, I.; Pugliatti, M.; Giorgi, C.; Granieri, E.; Pinton, P. Autophagy and mitophagy elements are increased in body fluids of multiple sclerosis-affected individuals. *J. Neurol. Neurosurg. Psychiatry* **2018**, *89*, 439–441. [CrossRef]
96. Seifert, T.; Kieseier, B.C.; Ropele, S.; Strasser-Fuchs, S.; Quehenberger, F.; Fazekas, F.; Hartung, H.-P. TACE mRNA expression in peripheral mononuclear cells precedes new lesions on MRI in multiple sclerosis. *Mult. Scler. J.* **2002**, *8*, 447–451. [CrossRef]
97. Leppert, D.; Ford, J.; Stabler, G.; Grygar, C.; Lienert, C.; Huber, S.; Miller, K.M.; Hauser, S.L.; Kappos, L. Matrix metalloproteinase-9 (gelatinase B) is selectively elevated in CSF during relapses and stable phases of multiple sclerosis. *Brain* **1998**, *121 Pt 1*, 2327–2334. [CrossRef]
98. Leppert, D.; Waubant, E.; Bürk, M.R.; Oksenberg, J.R.; Hauser, S.L. Interferon beta-1b inhibits gelatinase secretion and in vitro migration of human T cells: A possible mechanism for treatment efficacy in multiple sclerosis. *Ann. Neurol.* **1996**, *40*, 846–852. [CrossRef]
99. Libbey, J.E.; Cusick, M.F.; Fujinami, R.S. Role of pathogens in multiple sclerosis. *Int. Rev. Immunol.* **2014**, *33*, 266–283. [CrossRef]
100. Ding, Z.; Mathur, V.; Ho, P.P.; James, M.L.; Lucin, K.M.; Hoehne, A.; Alabsi, H.; Gambhir, S.S.; Steinman, L.; Luo, J.; et al. Antiviral drug ganciclovir is a potent inhibitor of microglial proliferation and neuroinflammation. *J. Exp. Med.* **2014**, *211*, 189–198. [CrossRef]
101. Mathur, V.; Burai, R.; Vest, R.T.; Bonanno, L.N.; Lehallier, B.; Zardeneta, M.E.; Mistry, K.N.; Do, D.; Marsh, S.E.; Abud, E.M.; et al. Activation of the STING-Dependent Type I Interferon Response Reduces Microglial Reactivity and Neuroinflammation. *Neuron* **2017**, *96*, 1290–1302.e6. [CrossRef] [PubMed]
102. Ming, X.; Li, W.; Maeda, Y.; Blumberg, B.; Raval, S.; Cook, S.D.; Dowling, P.C. Caspase-1 expression in multiple sclerosis plaques and cultured glial cells. *J. Neurol. Sci.* **2002**, *197*, 9–18. [CrossRef]
103. Cao, Y.; Goods, B.A.; Raddassi, K.; Nepom, G.T.; Kwok, W.W.; Love, J.C.; Hafler, D.A. Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Sci. Transl. Med.* **2015**, *7*, 287ra74. [CrossRef]
104. Huang, W.-X.; Huang, P.; Hillert, J. Increased expression of caspase-1 and interleukin-18 in peripheral blood mononuclear cells in patients with multiple sclerosis. *Mult. Scler. J.* **2004**, *10*, 482–487. [CrossRef]
105. Barclay, W.; Shinohara, M.L. Inflammasome activation in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). *Brain Pathol.* **2017**, *27*, 213–219. [CrossRef]
106. Gris, D.; Ye, Z.; Iocca, H.A.; Wen, H.; Craven, R.R.; Gris, P.; Huang, M.; Schneider, M.; Miller, S.D.; Ting, J.P.-Y. NLRP3 Plays a Critical Role in the Development of Experimental Autoimmune Encephalomyelitis by Mediating Th1 and Th17 Responses. *J. Immunol.* **2010**, *185*, 974–981. [CrossRef]
107. Shaw, P.J.; Lukens, J.R.; Burns, S.; Chi, H.; McGargill, M.A.; Kanneganti, T.-D. Cutting edge: Critical role for PYCARD/ASC in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* **2010**, *184*, 4610–4614. [CrossRef] [PubMed]
108. Malhotra, S.; Río, J.; Urcelay, E.; Nurtdinov, R.; Bustamante, M.F.; Fernández, O.; Oliver, B.; Zettl, U.; Brassat, D.; Killestein, J.; et al. NLRP3 inflammasome is associated with the response to IFN-β in patients with multiple sclerosis. *Brain* **2015**, *138*, 644–652. [CrossRef]
109. Mahad, D.; Ziabreva, I.; Lassmann, H.; Turnbull, D. Mitochondrial defects in acute multiple sclerosis lesions. *Brain* **2008**, *131*, 1722–1735. [CrossRef]
110. Iñarrea, P.; Alarcia, R.; Alava, M.A.; Capablo, J.L.; Casanova, A.; Iñiguez, C.; Iturralde, M.; Larrodé, P.; Martín, J.; Mostacero, E.; et al. Mitochondrial complex enzyme activities and cytochrome C expression changes in multiple sclerosis. *Mol. Neurobiol.* **2014**, *49*, 1–9. [CrossRef]
111. Bonora, M.; De Marchi, E.; Patergnani, S.; Suski, J.M.; Celsi, F.; Bononi, A.; Giorgi, C.; Marchi, S.; Rimessi, A.; Duszyński, J.; et al. Tumor necrosis factor-α impairs oligodendroglial differentiation through a mitochondria-dependent process. *Cell Death Differ.* **2014**, *21*, 1198–1208. [CrossRef] [PubMed]
112. Bros, H.; Millward, J.M.; Paul, F.; Niesner, R.; Infante-Duarte, C. Oxidative damage to mitochondria at the nodes of Ranvier precedes axon degeneration in ex vivo transected axons. *Exp. Neurol.* **2014**, *261*, 127–135. [CrossRef] [PubMed]
113. Ravelli, K.G.; Santos, G.D.; dos Santos, N.B.; Munhoz, C.D.; Azzi-Nogueira, D.; Campos, A.C.; Pagano, R.L.; Britto, L.R.; Hernandes, M.S. Nox2-dependent neuroinflammation in an EAE model of multiple sclerosis. *Transl. Neurosci.* **2019**, *10*, 1–9. [CrossRef] [PubMed]

114. Jansen, E.H.; Ruskovska, T. Comparative Analysis of Serum (Anti)oxidative Status Parameters in Healthy Persons. *Int. J. Mol. Sci.* **2013**, *14*, 6106–6115. [CrossRef] [PubMed]
115. Adamczyk, B.; Adamczyk-Sowa, M. New Insights into the Role of Oxidative Stress Mechanisms in the Pathophysiology and Treatment of Multiple Sclerosis. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 1973834. [CrossRef]
116. Tranah, G.J.; Santaniello, A.; Caillier, S.J.; D'Alfonso, S.; Martinelli Boneschi, F.; Hauser, S.L.; Oksenberg, J.R. Mitochondrial DNA sequence variation in multiple sclerosis. *Neurology* **2015**, *85*, 325–330. [CrossRef]
117. Andalib, S.; Talebi, M.; Sakhinia, E.; Farhoudi, M.; Sadeghi-Bazargani, H.; Motavallian, A.; Pilehvar-Soltanahmadi, Y. Multiple sclerosis and mitochondrial gene variations: A review. *J. Neurol. Sci.* **2013**, *330*, 10–15. [CrossRef]
118. Campbell, G.R.; Ziabreva, I.; Reeve, A.K.; Krishnan, K.J.; Reynolds, R.; Howell, O.; Lassmann, H.; Turnbull, D.M.; Mahad, D.J. Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis. *Ann. Neurol.* **2011**, *69*, 481–492. [CrossRef]
119. Castellazzi, M.; Patergnani, S.; Donadio, M.; Giorgi, C.; Bonora, M.; Fainardi, E.; Casetta, I.; Granieri, E.; Pugliatti, M.; Pinton, P. Correlation between auto/mitophagic processes and magnetic resonance imaging activity in multiple sclerosis patients. *J. Neuroinflamm.* **2019**, *16*, 131. [CrossRef]
120. Nussbaum, R.L. The Identification of Alpha-Synuclein as the First Parkinson Disease Gene. *J. Parkinsons Dis.* **2017**, *7*, S43–S49. [CrossRef]
121. Liu, G.; Zhang, C.; Yin, J.; Li, X.; Cheng, F.; Li, Y.; Yang, H.; Uéda, K.; Chan, P.; Yu, S. α-Synuclein is differentially expressed in mitochondria from different rat brain regions and dose-dependently down-regulates complex I activity. *Neurosci. Lett.* **2009**, *454*, 187–192. [CrossRef] [PubMed]
122. Devi, L.; Raghavendran, V.; Prabhu, B.M.; Avadhani, N.G.; Anandatheerthavarada, H.K. Mitochondrial Import and Accumulation of α-Synuclein Impair Complex I in Human Dopaminergic Neuronal Cultures and Parkinson Disease Brain. *J. Biol. Chem.* **2008**, *283*, 9089–9100. [CrossRef] [PubMed]
123. Zhu, Y.; Duan, C.; Lü, L.; Gao, H.; Zhao, C.; Yu, S.; Uéda, K.; Chan, P.; Yang, H. α-Synuclein overexpression impairs mitochondrial function by associating with adenylate translocator. *Int. J. Biochem. Cell Biol.* **2011**, *43*, 732–741. [CrossRef]
124. Dawson, T.M.; Dawson, V.L. The role of parkin in familial and sporadic Parkinson's disease. *Mov. Disord.* **2010**, *25*, S32–S39. [CrossRef] [PubMed]
125. Schapira, A.H.; Cooper, J.M.; Dexter, D.; Jenner, P.; Clark, J.B.; Marsden, C.D. Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem.* **1990**, *54*, 823–827. [CrossRef]
126. Thomas, R.R.; Keeney, P.M.; Bennett, J.P. Impaired complex-I mitochondrial biogenesis in Parkinson disease frontal cortex. *J. Parkinsons Dis.* **2012**, *2*, 67–76. [CrossRef]
127. Bender, A.; Krishnan, K.J.; Morris, C.M.; Taylor, G.A.; Reeve, A.K.; Perry, R.H.; Jaros, E.; Hersheson, J.S.; Betts, J.; Klopstock, T.; et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat. Genet.* **2006**, *38*, 515–517. [CrossRef]
128. Kraytsberg, Y.; Kudryavtseva, E.; McKee, A.C.; Geula, C.; Kowall, N.W.; Khrapko, K. Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. *Nat. Genet.* **2006**, *38*, 518–520. [CrossRef]
129. Fan, Z.; Pan, Y.-T.; Zhang, Z.-Y.; Yang, H.; Yu, S.-Y.; Zheng, Y.; Ma, J.-H.; Wang, X.-M. Systemic activation of NLRP3 inflammasome and plasma α-synuclein levels are correlated with motor severity and progression in Parkinson's disease. *J. Neuroinflamm.* **2020**, *17*, 11. [CrossRef]
130. Wang, X.; Chi, J.; Huang, D.; Ding, L.; Zhao, X.; Jiang, L.; Yu, Y.; Gao, F. α-synuclein promotes progression of Parkinson's disease by upregulating autophagy signaling pathway to activate NLRP3 inflammasome. *Exp. Ther. Med.* **2019**, *9*, 931–938. [CrossRef]
131. Sarkar, S.; Malovic, E.; Harishchandra, D.S.; Ghaisas, S.; Panicker, N.; Charli, A.; Palanisamy, B.N.; Rokad, D.; Jin, H.; Anantharam, V.; et al. Mitochondrial impairment in microglia amplifies NLRP3 inflammasome proinflammatory signaling in cell culture and animal models of Parkinson's disease. *NPJ Parkinson's Dis.* **2017**, *3*, 30. [CrossRef]
132. Fan, Z.; Liang, Z.; Yang, H.; Pan, Y.; Zheng, Y.; Wang, X. Tenuigenin protects dopaminergic neurons from inflammation via suppressing NLRP3 inflammasome activation in microglia. *J. Neuroinflamm.* **2017**, *14*, 256. [CrossRef]

133. Mouton-Liger, F.; Rosazza, T.; Sepulveda-Diaz, J.; Ieang, A.; Hassoun, S.-M.; Claire, E.; Mangone, G.; Brice, A.; Michel, P.P.; Corvol, J.-C.; et al. Parkin deficiency modulates NLRP3 inflammasome activation by attenuating an A20-dependent negative feedback loop. *Glia* **2018**, *66*, 1736–1751. [CrossRef] [PubMed]
134. Gordon, R.; Albornoz, E.A.; Christie, D.C.; Langley, M.R.; Kumar, V.; Mantovani, S.; Robertson, A.A.B.; Butler, M.S.; Rowe, D.B.; O'Neill, L.A.; et al. Inflammasome inhibition prevents α-synuclein pathology and dopaminergic neurodegeneration in mice. *Sci. Transl. Med.* **2018**, *10*, eaah4066. [CrossRef] [PubMed]
135. Thakur, P.; Nehru, B. Inhibition of Neuroinflammation and Mitochondrial Dysfunctions by Carbenoxolone in the Rotenone Model of Parkinson's Disease. *Mol. Neurobiol.* **2015**, *51*, 209–219. [CrossRef] [PubMed]
136. Rakovic, A.; Grünewald, A.; Voges, L.; Hofmann, S.; Orolicki, S.; Lohmann, K.; Klein, C. PINK1-Interacting Proteins: Proteomic Analysis of Overexpressed PINK1. *Parkinsons Dis.* **2011**, *2011*, 1–9. [CrossRef]
137. Rakovic, A.; Grunewald, A.; Seibler, P.; Ramirez, A.; Kock, N.; Orolicki, S.; Lohmann, K.; Klein, C. Effect of endogenous mutant and wild-type PINK1 on Parkin in fibroblasts from Parkinson disease patients. *Hum. Mol. Genet.* **2010**, *19*, 3124–3137. [CrossRef] [PubMed]
138. Tanaka, A.; Cleland, M.M.; Xu, S.; Narendra, D.P.; Suen, D.-F.; Karbowski, M.; Youle, R.J. Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J. Cell Biol.* **2010**, *191*, 1367–1380. [CrossRef] [PubMed]
139. Gandhi, S. PINK1 protein in normal human brain and Parkinson's disease. *Brain* **2006**, *129*, 1720–1731. [CrossRef]
140. Jagmag, S.A.; Tripathi, N.; Shukla, S.D.; Maiti, S.; Khurana, S. Evaluation of Models of Parkinson's Disease. *Front. Neurosci.* **2015**, *9*, 503. [CrossRef] [PubMed]
141. Giaime, E.; Shen, J. Regulation of mitochondrial function by PINK1, Parkin and DJ-1. *Mol. Neurodegener.* **2013**, *8*, O13. [CrossRef]
142. Giaime, E.; Yamaguchi, H.; Gautier, C.A.; Kitada, T.; Shen, J. Loss of DJ-1 Does Not Affect Mitochondrial Respiration but Increases ROS Production and Mitochondrial Permeability Transition Pore Opening. *PLoS ONE* **2012**, *7*, e40501. [CrossRef]
143. Gandhi, S.; Wood-Kaczmar, A.; Yao, Z.; Plun-Favreau, H.; Deas, E.; Klupsch, K.; Downward, J.; Latchman, D.S.; Tabrizi, S.J.; Wood, N.W.; et al. PINK1-Associated Parkinson's Disease Is Caused by Neuronal Vulnerability to Calcium-Induced Cell Death. *Mol. Cell* **2009**, *33*, 627–638. [CrossRef] [PubMed]
144. Sliter, D.A.; Martinez, J.; Hao, L.; Chen, X.; Sun, N.; Fischer, T.D.; Burman, J.L.; Li, Y.; Zhang, Z.; Narendra, D.P.; et al. Parkin and PINK1 mitigate STING-induced inflammation. *Nature* **2018**, *561*, 258–262. [CrossRef] [PubMed]
145. Du, H.; Guo, L.; Fang, F.; Chen, D.; Sosunov, A.A.; McKhann, G.M.; Yan, Y.; Wang, C.; Zhang, H.; Molkentin, J.D.; et al. Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease. *Nat. Med.* **2008**, *14*, 1097–1105. [CrossRef] [PubMed]
146. Yao, J.; Du, H.; Yan, S.S.; Fang, F.; Wang, C.; Lue, L.-F.; Guo, L.; Chen, D.; Stern, D.M.; Gunn Moore, F.J.; et al. Inhibition of Amyloid-(A) Peptide-Binding Alcohol Dehydrogenase-A Interaction Reduces A Accumulation and Improves Mitochondrial Function in a Mouse Model of Alzheimer's Disease. *J. Neurosci.* **2011**, *31*, 2313–2320. [CrossRef] [PubMed]
147. Tillement, L.; Lecanu, L.; Yao, W.; Greeson, J.; Papadopoulos, V. The spirostenol (22R, 25R)-20α-spirost-5-en-3β-yl hexanoate blocks mitochondrial uptake of Aβ in neuronal cells and prevents Aβ-induced impairment of mitochondrial function. *Steroids* **2006**, *71*, 725–735. [CrossRef]
148. Walls, K.C.; Coskun, P.; Gallegos-Perez, J.L.; Zadourian, N.; Freude, K.; Rasool, S.; Blurton-Jones, M.; Green, K.N.; LaFerla, F.M. Swedish Alzheimer mutation induces mitochondrial dysfunction mediated by HSP60 mislocalization of amyloid precursor protein (APP) and beta-amyloid. *J. Biol. Chem.* **2012**, *287*, 30317–30327. [CrossRef]
149. Wang, J.; Markesbery, W.R.; Lovell, M.A. Increased oxidative damage in nuclear and mitochondrial DNA in mild cognitive impairment. *J. Neurochem.* **2006**, *96*, 825–832. [CrossRef]
150. Bonda, D.J.; Wang, X.; Lee, H.-G.; Smith, M.A.; Perry, G.; Zhu, X. Neuronal failure in Alzheimer's disease: A view through the oxidative stress looking-glass. *Neurosci. Bull.* **2014**, *30*, 243–252. [CrossRef]
151. Coskun, P.E.; Beal, M.F.; Wallace, D.C. Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 10726–10731. [CrossRef] [PubMed]

152. Keogh, M.J.; Chinnery, P.F. Mitochondrial DNA mutations in neurodegeneration. *Biochim. Biophys. Acta Bioenerg.* **2015**, *1847*, 1401–1411. [CrossRef] [PubMed]
153. Heneka, M.T.; Kummer, M.P.; Stutz, A.; Delekate, A.; Schwartz, S.; Vieira-Saecker, A.; Griep, A.; Axt, D.; Remus, A.; Tzeng, T.-C.; et al. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* **2013**, *493*, 674–678. [CrossRef] [PubMed]
154. Stancu, I.-C.; Cremers, N.; Vanrusselt, H.; Couturier, J.; Vanoosthuyse, A.; Kessels, S.; Lodder, C.; Brône, B.; Huaux, F.; Octave, J.-N.; et al. Aggregated Tau activates NLRP3–ASC inflammasome exacerbating exogenously seeded and non-exogenously seeded Tau pathology in vivo. *Acta Neuropathol.* **2019**, *137*, 599–617. [CrossRef]
155. Shaftel, S.S.; Griffin, W.S.T.; O'Banion, M.K. The role of interleukin-1 in neuroinflammation and Alzheimer disease: An evolving perspective. *J. Neuroinflamm.* **2008**, *5*, 7. [CrossRef] [PubMed]
156. González-Reyes, R.E.; Nava-Mesa, M.O.; Vargas-Sánchez, K.; Ariza-Salamanca, D.; Mora-Muñoz, L. Involvement of Astrocytes in Alzheimer's Disease from a Neuroinflammatory and Oxidative Stress Perspective. *Front. Mol. Neurosci.* **2017**, *10*. [CrossRef]
157. Griffin, W.; Liu, L.; Li, Y.; Mrak, R.; Barger, S. Interleukin-1 mediates Alzheimer and Lewy body pathologies. *J Neuroinflamm.* **2006**, *3*, 1–9. [CrossRef]
158. Sheng, J.G.; Ito, K.; Skinner, R.D.; Mrak, R.E.; Rovnaghi, C.R.; Van Eldik, L.J.; Griffin, W.S. In vivo and in vitro evidence supporting a role for the inflammatory cytokine interleukin-1 as a driving force in Alzheimer pathogenesis. *Neurobiol. Aging* **1996**, *17*, 761–766. [CrossRef]
159. Kitazawa, M.; Cheng, D.; Tsukamoto, M.R.; Koike, M.A.; Wes, P.D.; Vasilevko, V.; Cribbs, D.H.; LaFerla, F.M. Blocking IL-1 Signaling Rescues Cognition, Attenuates Tau Pathology, and Restores Neuronal β-Catenin Pathway Function in an Alzheimer's Disease Model. *J. Immunol.* **2011**, *187*, 6539–6549. [CrossRef]
160. Bossu, P.; Ciaramella, A.; Moro, M.L.; Bellincampi, L.; Bernardini, S.; Federici, G.; Trequattrini, A.; Macciardi, F.; Spoletini, I.; Di Iulio, F.; et al. Interleukin 18 gene polymorphisms predict risk and outcome of Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatry* **2007**, *78*, 807–811. [CrossRef]
161. Bossù, P.; Ciaramella, A.; Salani, F.; Bizzoni, F.; Varsi, E.; Di Iulio, F.; Giubilei, F.; Gianni, W.; Trequattrini, A.; Moro, M.L.; et al. Interleukin-18 produced by peripheral blood cells is increased in Alzheimer's disease and correlates with cognitive impairment. *Brain Behav. Immun.* **2008**, *22*, 487–492. [CrossRef] [PubMed]
162. Di Rosa, M.; Dell'Ombra, N.; Zambito, A.M.; Malaguarnera, M.; Nicoletti, F.; Malaguarnera, L. Chitotriosidase and inflammatory mediator levels in Alzheimer's disease and cerebrovascular dementia. *Eur. J. Neurosci.* **2006**, *23*, 2648–2656. [CrossRef] [PubMed]
163. Ojala, J.O.; Sutinen, E.M.; Salminen, A.; Pirttilä, T. Interleukin-18 increases expression of kinases involved in tau phosphorylation in SH-SY5Y neuroblastoma cells. *J. Neuroimmunol.* **2008**, *205*, 86–93. [CrossRef] [PubMed]
164. Gupta, S.; Knight, A.G.; Gupta, S.; Keller, J.N.; Bruce-Keller, A.J. Saturated long-chain fatty acids activate inflammatory signaling in astrocytes. *J. Neurochem.* **2012**, *120*, 1060–1071. [CrossRef]
165. Du, F.; Yu, Q.; Yan, S.; Hu, G.; Lue, L.-F.; Walker, D.G.; Wu, L.; Yan, S.F.; Tieu, K.; Yan, S.S. PINK1 signalling rescues amyloid pathology and mitochondrial dysfunction in Alzheimer's disease. *Brain* **2017**, *140*, 3233–3251. [CrossRef] [PubMed]
166. Fang, E.F.; Hou, Y.; Palikaras, K.; Adriaanse, B.A.; Kerr, J.S.; Yang, B.; Lautrup, S.; Hasan-Olive, M.M.; Caponio, D.; Dan, X.; et al. Mitophagy inhibits amyloid-β and tau pathology and reverses cognitive deficits in models of Alzheimer's disease. *Nat. Neurosci.* **2019**, *22*, 401–412. [CrossRef]
167. Castellazzi, M.; Patergnani, S.; Donadio, M.; Giorgi, C.; Bonora, M.; Bosi, C.; Brombo, G.; Pugliatti, M.; Seripa, D.; Zuliani, G.; et al. Autophagy and mitophagy biomarkers are reduced in sera of patients with Alzheimer's disease and mild cognitive impairment. *Sci. Rep.* **2019**, *9*, 20009. [CrossRef]
168. Veitch, D.P.; Weiner, M.W.; Aisen, P.S.; Beckett, L.A.; Cairns, N.J.; Green, R.C.; Harvey, D.; Jack, C.R.; Jagust, W.; Morris, J.C.; et al. Understanding disease progression and improving Alzheimer's disease clinical trials: Recent highlights from the Alzheimer's Disease Neuroimaging Initiative. *Alzheimer's Dement.* **2019**, *15*, 106–152. [CrossRef]
169. Toledo, J.B.; Arnold, M.; Kastenmüller, G.; Chang, R.; Baillie, R.A.; Han, X.; Thambisetty, M.; Tenenbaum, J.D.; Suhre, K.; Thompson, J.W.; et al. Metabolic network failures in Alzheimer's disease: A biochemical road map. *Alzheimer's Dement.* **2017**, *13*, 965–984. [CrossRef]

170. Hatano, S. Experience from a multicentre stroke register: A preliminary report. *Bull. World Health Organ.* **1976**, *54*, 541–553.
171. Hui, C.; Tadi, P.; Patti, L. *Ischemic Stroke*; StatPearls Publishing: Treasure Island, FL, USA, 2020.
172. Katan, M.; Luft, A. Global Burden of Stroke. *Semin. Neurol.* **2018**, *38*, 208–211. [CrossRef] [PubMed]
173. Hossmann, K.-A. Pathophysiology and Therapy of Experimental Stroke. *Cell. Mol. Neurobiol.* **2006**, *26*, 1055–1081. [CrossRef] [PubMed]
174. Khoshnam, S.E.; Winlow, W.; Farzaneh, M.; Farbood, Y.; Moghaddam, H.F. Pathogenic mechanisms following ischemic stroke. *Neurol. Sci.* **2017**, *38*, 1167–1186. [CrossRef] [PubMed]
175. Ames, A. CNS energy metabolism as related to function. *Brain Res. Rev.* **2000**, *34*, 42–68. [CrossRef]
176. Bandera, E.; Botteri, M.; Minelli, C.; Sutton, A.; Abrams, K.R.; Latronico, N. Cerebral Blood Flow Threshold of Ischemic Penumbra and Infarct Core in Acute Ischemic Stroke. *Stroke* **2006**, *37*, 1334–1339. [CrossRef] [PubMed]
177. Siket, M.S. Treatment of Acute Ischemic Stroke. *Emerg. Med. Clin. N. Am.* **2016**, *34*, 861–882. [CrossRef]
178. Jung, S.; Gilgen, M.; Slotboom, J.; El-Koussy, M.; Zubler, C.; Kiefer, C.; Luedi, R.; Mono, M.-L.; Heldner, M.R.; Weck, A.; et al. Factors that determine penumbral tissue loss in acute ischaemic stroke. *Brain* **2013**, *136*, 3554–3560. [CrossRef]
179. Murphy, B.D.; Fox, A.J.; Lee, D.H.; Sahlas, D.J.; Black, S.E.; Hogan, M.J.; Coutts, S.B.; Demchuk, A.M.; Goyal, M.; Aviv, R.I.; et al. White matter thresholds for ischemic penumbra and infarct core in patients with acute stroke: CT perfusion study. *Radiology* **2008**, *247*, 818–825. [CrossRef]
180. Eltzschig, H.K.; Eckle, T. Ischemia and reperfusion—From mechanism to translation. *Nat. Med.* **2011**, *17*, 1391–1401. [CrossRef]
181. Kalogeris, T.; Baines, C.P.; Krenz, M.; Korthuis, R.J. Cell Biology of Ischemia/Reperfusion Injury. *Int. Rev. Cell Mol. Biol.* **2012**, *298*, 229–317. [CrossRef]
182. Li, Q.; Cao, Y.; Dang, C.; Han, B.; Han, R.; Ma, H.; Hao, J.; Wang, L. Inhibition of double-strand DNA-sensing cGAS ameliorates brain injury after ischemic stroke. *EMBO Mol. Med.* **2020**, *12*. [CrossRef] [PubMed]
183. Lammerding, L.; Slowik, A.; Johann, S.; Beyer, C.; Zendedel, A. Poststroke Inflammasome Expression and Regulation in the Peri-Infarct Area by Gonadal Steroids after Transient Focal Ischemia in the Rat Brain. *Neuroendocrinology* **2016**, *103*, 460–475. [CrossRef]
184. Kim, H.; Seo, J.S.; Lee, S.-Y.; Ha, K.-T.; Choi, B.T.; Shin, Y.-I.; Ju Yun, Y.; Shin, H.K. AIM2 inflammasome contributes to brain injury and chronic post-stroke cognitive impairment in mice. *Brain Behav. Immun.* **2020**. [CrossRef] [PubMed]
185. Fann, D.Y.-W.; Lee, S.-Y.; Manzanero, S.; Chunduri, P.; Sobey, C.G.; Arumugam, T.V. Pathogenesis of acute stroke and the role of inflammasomes. *Ageing Res. Rev.* **2013**, *12*, 941–966. [CrossRef] [PubMed]
186. Ismael, S.; Zhao, L.; Nasoohi, S.; Ishrat, T. Inhibition of the NLRP3-inflammasome as a potential approach for neuroprotection after stroke. *Sci. Rep.* **2018**, *8*, 5971. [CrossRef] [PubMed]
187. Su, S.-H.; Wu, Y.-F.; Lin, Q.; Wang, D.-P.; Hai, J. URB597 protects against NLRP3 inflammasome activation by inhibiting autophagy dysfunction in a rat model of chronic cerebral hypoperfusion. *J. Neuroinflamm.* **2019**, *16*, 260. [CrossRef] [PubMed]
188. Mo, Z.; Tang, C.; Li, H.; Lei, J.; Zhu, L.; Kou, L.; Li, H.; Luo, S.; Li, C.; Chen, W.; et al. Eicosapentaenoic acid prevents inflammation induced by acute cerebral infarction through inhibition of NLRP3 inflammasome activation. *Life Sci.* **2020**, *242*, 117133. [CrossRef] [PubMed]
189. Yang-Wei Fann, D.; Lee, S.-Y.; Manzanero, S.; Tang, S.-C.; Gelderblom, M.; Chunduri, P.; Bernreuther, C.; Glatzel, M.; Cheng, Y.-L.; Thundyil, J.; et al. Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated neuronal death in ischemic stroke. *Cell Death Dis.* **2013**, *4*, e790. [CrossRef] [PubMed]
190. Gong, Z.; Pan, J.; Shen, Q.; Li, M.; Peng, Y. Mitochondrial dysfunction induces NLRP3 inflammasome activation during cerebral ischemia/reperfusion injury. *J. Neuroinflamm.* **2018**, *15*, 242. [CrossRef]
191. Gurung, P.; Lukens, J.R.; Kanneganti, T.-D. Mitochondria: Diversity in the regulation of the NLRP3 inflammasome. *Trends Mol. Med.* **2015**, *21*, 193–201. [CrossRef]
192. Mongin, A.A. Disruption of ionic and cell volume homeostasis in cerebral ischemia: The perfect storm. *Pathophysiology* **2007**, *14*, 183–193. [CrossRef] [PubMed]
193. Lai, T.W.; Zhang, S.; Wang, Y.T. Excitotoxicity and stroke: Identifying novel targets for neuroprotection. *Prog. Neurobiol.* **2014**, *115*, 157–188. [CrossRef] [PubMed]

194. Epstein, F.H.; Lipton, S.A.; Rosenberg, P.A. Excitatory Amino Acids as a Final Common Pathway for Neurologic Disorders. *N. Engl. J. Med.* **1994**, *330*, 613–622. [CrossRef] [PubMed]
195. Schipke, C.G.; Ohlemeyer, C.; Matyash, M.; Nolte, C.; Kettenmann, H.; Kirchhoff, F. Astrocytes of the mouse neocortex express functional N-methyl-D-aspartate receptors. *FASEB J.* **2001**, *15*, 1270–1272. [CrossRef] [PubMed]
196. Kaindl, A.M.; Degos, V.; Peineau, S.; Gouadon, E.; Chhor, V.; Loron, G.; Le Charpentier, T.; Josserand, J.; Ali, C.; Vivien, D.; et al. Activation of microglial N-methyl-D-aspartate receptors triggers inflammation and neuronal cell death in the developing and mature brain. *Ann. Neurol.* **2012**, *72*, 536–549. [CrossRef] [PubMed]
197. White, R.; Reynolds, I. Mitochondrial depolarization in glutamate-stimulated neurons: An early signal specific to excitotoxin exposure. *J. Neurosci.* **1996**, *16*, 5688–5697. [CrossRef] [PubMed]
198. Mody, I. NMDA receptor-dependent excitotoxicity: The role of intracellular Ca2+ release. *Trends Pharmacol. Sci.* **1995**, *16*, 356–359. [CrossRef]
199. Smith, J.P.; Cunningham, L.A.; Partridge, L.D. Coupling of AMPA receptors with the Na+/Ca2+ exchanger in cultured rat astrocytes. *Brain Res.* **2000**, *887*, 98–109. [CrossRef]
200. Chinopoulos, C.; Adam-Vizi, V. Mitochondrial Ca2+ sequestration and precipitation revisited. *FEBS J.* **2010**, *277*, 3637–3651. [CrossRef]
201. Triantafilou, K.; Hughes, T.R.; Triantafilou, M.; Morgan, B.P. The complement membrane attack complex triggers intracellular Ca2+ fluxes leading to NLRP3 inflammasome activation. *J. Cell Sci.* **2013**, *126*, 2903–2913. [CrossRef]
202. Qiu, J.; Tan, Y.-W.; Hagenston, A.M.; Martel, M.-A.; Kneisel, N.; Skehel, P.A.; Wyllie, D.J.A.; Bading, H.; Hardingham, G.E. Mitochondrial calcium uniporter Mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. *Nat. Commun.* **2013**, *4*, 2034. [CrossRef] [PubMed]
203. Zhang, K.; Yan, J.; Wang, L.; Tian, X.; Zhang, T.; Li, B.; Wang, W.; Guo, L.; Liu, X. Expression of MICU1 after experimental focal cerebral ischemia in adult rats. *Chin. Neurosurg. J.* **2017**, *3*, 13. [CrossRef]
204. Hill, J.K.; Gunion-Rinker, L.; Kulhanek, D.; Lessov, N.; Kim, S.; Clark, W.M.; Dixon, M.P.; Nishi, R.; Stenzel-Poore, M.P.; Eckenstein, F.P. Temporal modulation of cytokine expression following focal cerebral ischemia in mice. *Brain Res.* **1999**, *820*, 45–54. [CrossRef]
205. Lambertsen, K.L.; Meldgaard, M.; Ladeby, R.; Finsen, B. A Quantitative Study of Microglial—Macrophage Synthesis of Tumor Necrosis Factor during Acute and Late Focal Cerebral Ischemia in Mice. *J. Cereb. Blood Flow Metab.* **2005**, *25*, 119–135. [CrossRef] [PubMed]
206. Clausen, B.H.; Lambertsen, K.L.; Babcock, A.A.; Holm, T.H.; Dagnaes-Hansen, F.; Finsen, B. Interleukin-1beta and tumor necrosis factor-alpha are expressed by different subsets of microglia and macrophages after ischemic stroke in mice. *J. Neuroinflamm.* **2008**, *5*, 46. [CrossRef] [PubMed]
207. Murakami, T.; Ockinger, J.; Yu, J.; Byles, V.; McColl, A.; Hofer, A.M.; Horng, T. Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 11282–11287. [CrossRef] [PubMed]
208. Pétrilli, V.; Papin, S.; Dostert, C.; Mayor, A.; Martinon, F.; Tschopp, J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ.* **2007**, *14*, 1583–1589. [CrossRef] [PubMed]
209. Muñoz-Planillo, R.; Kuffa, P.; Martínez-Colón, G.; Smith, B.L.; Rajendiran, T.M.; Núñez, G. K+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter. *Immunity* **2013**, *38*, 1142–1153. [CrossRef] [PubMed]
210. Sanderson, T.H.; Reynolds, C.A.; Kumar, R.; Przyklenk, K.; Hüttemann, M. Molecular Mechanisms of Ischemia–Reperfusion Injury in Brain: Pivotal Role of the Mitochondrial Membrane Potential in Reactive Oxygen Species Generation. *Mol. Neurobiol.* **2013**, *47*, 9–23. [CrossRef]
211. Cruz, C.M.; Rinna, A.; Forman, H.J.; Ventura, A.L.M.; Persechini, P.M.; Ojcius, D.M. ATP Activates a Reactive Oxygen Species-dependent Oxidative Stress Response and Secretion of Proinflammatory Cytokines in Macrophages. *J. Biol. Chem.* **2007**, *282*, 2871–2879. [CrossRef] [PubMed]
212. Loor, G.; Kondapalli, J.; Iwase, H.; Chandel, N.S.; Waypa, G.B.; Guzy, R.D.; Vanden Hoek, T.L.; Schumacker, P.T. Mitochondrial oxidant stress triggers cell death in simulated ischemia–reperfusion. *Biochim. Biophys. Acta Mol. Cell Res.* **2011**, *1813*, 1382–1394. [CrossRef] [PubMed]

213. Murphy, M.P. How mitochondria produce reactive oxygen species. *Biochem. J.* **2009**, *417*, 1–13. [CrossRef] [PubMed]
214. Kussmaul, L.; Hirst, J. The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 7607–7612. [CrossRef] [PubMed]
215. Bleier, L.; Dröse, S. Superoxide generation by complex III: From mechanistic rationales to functional consequences. *Biochim. Biophys. Acta Bioenerg.* **2013**, *1827*, 1320–1331. [CrossRef]
216. Chouchani, E.T.; Pell, V.R.; Gaude, E.; Aksentijević, D.; Sundier, S.Y.; Robb, E.L.; Logan, A.; Nadtochiy, S.M.; Ord, E.N.J.; Smith, A.C.; et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* **2014**, *515*, 431–435. [CrossRef]
217. Li, N.; Ragheb, K.; Lawler, G.; Sturgis, J.; Rajwa, B.; Melendez, J.A.; Robinson, J.P. Mitochondrial Complex I Inhibitor Rotenone Induces Apoptosis through Enhancing Mitochondrial Reactive Oxygen Species Production. *J. Biol. Chem.* **2003**, *278*, 8516–8525. [CrossRef]
218. Horng, T. Calcium signaling and mitochondrial destabilization in the triggering of the NLRP3 inflammasome. *Trends Immunol.* **2014**, *35*, 253–261. [CrossRef]
219. Zhuang, Y.; Yasinta, M.; Hu, C.; Zhao, M.; Ding, G.; Bai, M.; Yang, L.; Ni, J.; Wang, R.; Jia, Z.; et al. Mitochondrial dysfunction confers albumin-induced NLRP3 inflammasome activation and renal tubular injury. *Am. J. Physiol. Renal Physiol.* **2015**, *308*, F857–F866. [CrossRef]
220. Nakahira, K.; Haspel, J.A.; Rathinam, V.A.K.; Lee, S.-J.; Dolinay, T.; Lam, H.C.; Englert, J.A.; Rabinovitch, M.; Cernadas, M.; Kim, H.P.; et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat. Immunol.* **2011**, *12*, 222–230. [CrossRef]
221. Schinzel, A.C.; Takeuchi, O.; Huang, Z.; Fisher, J.K.; Zhou, Z.; Rubens, J.; Hetz, C.; Danial, N.N.; Moskowitz, M.A.; Korsmeyer, S.J. Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 12005–12010. [CrossRef]
222. Sun, J.; Li, Y.; Ding, Y.; Wang, J.; Geng, J.; Yang, H.; Ren, J.; Tang, J.; Gao, J. Neuroprotective effects of gallic acid against hypoxia/reoxygenation-induced mitochondrial dysfunctions in vitro and cerebral ischemia/reperfusion injury in vivo. *Brain Res.* **2014**, *1589*, 126–139. [CrossRef] [PubMed]
223. Matsumoto, S.; Murozono, M.; Kanazawa, M.; Nara, T.; Ozawa, T.; Watanabe, Y. Edaravone and cyclosporine A as neuroprotective agents for acute ischemic stroke. *Acute Med. Surg.* **2018**, *5*, 213–221. [CrossRef] [PubMed]
224. Ma, Q.; Chen, S.; Hu, Q.; Feng, H.; Zhang, J.H.; Tang, J. NLRP3 inflammasome contributes to inflammation after intracerebral hemorrhage. *Ann. Neurol.* **2014**, *75*, 209–219. [CrossRef] [PubMed]
225. Wang, Y.; Nartiss, Y.; Steipe, B.; McQuibban, G.A.; Kim, P.K. ROS-induced mitochondrial depolarization initiates PARK2/PARKIN-dependent mitochondrial degradation by autophagy. *Autophagy* **2012**, *8*, 1462–1476. [CrossRef] [PubMed]
226. Elmore, S.P.; Qian, T.; Grissom, S.F.; Lemasters, J.J. The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J.* **2001**, *15*, 1–17. [CrossRef]
227. Lyamzaev, K.G.; Tokarchuk, A.V.; Panteleeva, A.A.; Mulkidjanian, A.Y.; Skulachev, V.P.; Chernyak, B.V. Induction of autophagy by depolarization of mitochondria. *Autophagy* **2018**, *14*, 921–924. [CrossRef]
228. Cao, S.; Shrestha, S.; Li, J.; Yu, X.; Chen, J.; Yan, F.; Ying, G.; Gu, C.; Wang, L.; Chen, G. Melatonin-mediated mitophagy protects against early brain injury after subarachnoid hemorrhage through inhibition of NLRP3 inflammasome activation. *Sci. Rep.* **2017**, *7*, 2417. [CrossRef]
229. Di, Y.; He, Y.-L.; Zhao, T.; Huang, X.; Wu, K.-W.; Liu, S.-H.; Zhao, Y.-Q.; Fan, M.; Wu, L.-Y.; Zhu, L.-L. Methylene Blue Reduces Acute Cerebral Ischemic Injury via the Induction of Mitophagy. *Mol. Med.* **2015**, *21*, 420–429. [CrossRef]
230. Li, Q.; Zhang, T.; Wang, J.; Zhang, Z.; Zhai, Y.; Yang, G.-Y.; Sun, X. Rapamycin attenuates mitochondrial dysfunction via activation of mitophagy in experimental ischemic stroke. *Biochem. Biophys. Res. Commun.* **2014**, *444*, 182–188. [CrossRef]
231. He, Q.; Li, Z.; Meng, C.; Wu, J.; Zhao, Y.; Zhao, J. Parkin-Dependent Mitophagy is Required for the Inhibition of ATF4 on NLRP3 Inflammasome Activation in Cerebral Ischemia-Reperfusion Injury in Rats. *Cells* **2019**, *8*, 897. [CrossRef]

232. Yuan, Y.; Zheng, Y.; Zhang, X.; Chen, Y.; Wu, X.; Wu, J.; Shen, Z.; Jiang, L.; Wang, L.; Yang, W.; et al. BNIP3L/NIX-mediated mitophagy protects against ischemic brain injury independent of PARK2. *Autophagy* **2017**, *13*, 1754–1766. [CrossRef] [PubMed]
233. Shi, R.-Y.; Zhu, S.-H.; Li, V.; Gibson, S.B.; Xu, X.-S.; Kong, J.-M. BNIP3 Interacting with LC3 Triggers Excessive Mitophagy in Delayed Neuronal Death in Stroke. *CNS Neurosci. Ther.* **2014**, *20*, 1045–1055. [CrossRef] [PubMed]
234. Feng, J.; Chen, X.; Guan, B.; Li, C.; Qiu, J.; Shen, J. Inhibition of Peroxynitrite-Induced Mitophagy Activation Attenuates Cerebral Ischemia-Reperfusion Injury. *Mol. Neurobiol.* **2018**, *55*, 6369–6386. [CrossRef] [PubMed]
235. Leach, J.P. Treatment of epilepsy—Towards precision. *F1000Research* **2018**, *7*. [CrossRef]
236. Fisher, R.S.; Acevedo, C.; Arzimanoglou, A.; Bogacz, A.; Cross, J.H.; Elger, C.E.; Engel, J.; Forsgren, L.; French, J.A.; Glynn, M.; et al. ILAE official report: A practical clinical definition of epilepsy. *Epilepsia* **2014**, *55*, 475–482. [CrossRef]
237. Laxer, K.D.; Trinka, E.; Hirsch, L.J.; Cendes, F.; Langfitt, J.; Delanty, N.; Resnick, T.; Benbadis, S.R. The consequences of refractory epilepsy and its treatment. *Epilepsy Behav.* **2014**, *37*, 59–70. [CrossRef]
238. Rana, A.; Musto, A.E. The role of inflammation in the development of epilepsy. *J. Neuroinflamm.* **2018**, *15*, 144. [CrossRef]
239. Vezzani, A.; Conti, M.; De Luigi, A.; Ravizza, T.; Moneta, D.; Marchesi, F.; De Simoni, M.G. Interleukin-1beta immunoreactivity and microglia are enhanced in the rat hippocampus by focal kainate application: Functional evidence for enhancement of electrographic seizures. *J. Neurosci.* **1999**, *19*, 5054–5065. [CrossRef]
240. McElroy, P.B.; Liang, L.-P.; Day, B.J.; Patel, M. Scavenging reactive oxygen species inhibits status epilepticus-induced neuroinflammation. *Exp. Neurol.* **2017**, *298*, 13–22. [CrossRef]
241. Pauletti, A.; Terrone, G.; Shekh-Ahmad, T.; Salamone, A.; Ravizza, T.; Rizzi, M.; Pastore, A.; Pascente, R.; Liang, L.-P.; Villa, B.R.; et al. Targeting oxidative stress improves disease outcomes in a rat model of acquired epilepsy. *Brain* **2017**, *140*, 1885–1899. [CrossRef]
242. Terrone, G.; Balosso, S.; Pauletti, A.; Ravizza, T.; Vezzani, A. Inflammation and reactive oxygen species as disease modifiers in epilepsy. *Neuropharmacology* **2020**, *167*, 107742. [CrossRef] [PubMed]
243. Rahman, S. Pathophysiology of mitochondrial disease causing epilepsy and status epilepticus. *Epilepsy Behav.* **2015**, *49*, 71–75. [CrossRef] [PubMed]
244. Téllez-Zenteno, J.F.; Hernández-Ronquillo, L. A review of the epidemiology of temporal lobe epilepsy. *Epilepsy Res. Treat.* **2012**, *2012*, 630853. [CrossRef] [PubMed]
245. Pitkänen, A.; Lukasiuk, K. Mechanisms of epileptogenesis and potential treatment targets. *Lancet Neurol.* **2011**, *10*, 173–186. [CrossRef]
246. Shen, K.; Mao, Q.; Yin, X.; Zhang, C.; Jin, Y.; Deng, A.; Gu, Z.; Chen, B. NLRP3 Inflammasome Activation Leads to Epileptic Neuronal Apoptosis. *Curr. Neurovasc. Res.* **2018**, *15*, 276–281. [CrossRef]
247. Meng, X.-F.; Tan, L.; Tan, M.-S.; Jiang, T.; Tan, C.-C.; Li, M.-M.; Wang, H.-F.; Yu, J.-T. Inhibition of the NLRP3 inflammasome provides neuroprotection in rats following amygdala kindling-induced status epilepticus. *J. Neuroinflamm.* **2014**, *11*, 212. [CrossRef]
248. Rong, S.; Wan, D.; Fan, Y.; Liu, S.; Sun, K.; Huo, J.; Zhang, P.; Li, X.; Xie, X.; Wang, F.; et al. Amentoflavone Affects Epileptogenesis and Exerts Neuroprotective Effects by Inhibiting NLRP3 Inflammasome. *Front. Pharmacol.* **2019**, *10*, 856. [CrossRef]
249. Sun, Y.; Ma, J.; Li, D.; Li, P.; Zhou, X.; Li, Y.; He, Z.; Qin, L.; Liang, L.; Luo, X. Interleukin-10 inhibits interleukin-1β production and inflammasome activation of microglia in epileptic seizures. *J. Neuroinflamm.* **2019**, *16*, 66. [CrossRef]
250. Liu, Z.; Xian, H.; Ye, X.; Chen, J.; Ma, Y.; Huang, W. Increased levels of NLRP3 in children with febrile seizures. *Brain Dev.* **2020**, *42*, 336–341. [CrossRef]
251. Wang, H.; Xu, P.; Liao, D.; Dang, R.; He, X.; Guo, Y.; Jiang, P. Association between NLPR1, NLPR3, and P2X7R Gene Polymorphisms with Partial Seizures. *BioMed Res. Int.* **2017**, *2017*, 9547902. [CrossRef]
252. Tan, C.-C.; Zhang, J.-G.; Tan, M.-S.; Chen, H.; Meng, D.-W.; Jiang, T.; Meng, X.-F.; Li, Y.; Sun, Z.; Li, M.-M.; et al. NLRP1 inflammasome is activated in patients with medial temporal lobe epilepsy and contributes to neuronal pyroptosis in amygdala kindling-induced rat model. *J. Neuroinflamm.* **2015**, *12*, 18. [CrossRef] [PubMed]

253. Dixit, A.B.; Banerjee, J.; Srivastava, A.; Tripathi, M.; Sarkar, C.; Kakkar, A.; Jain, M.; Chandra, P.S. RNA-seq analysis of hippocampal tissues reveals novel candidate genes for drug refractory epilepsy in patients with MTLE-HS. *Genomics* **2016**, *107*, 178–188. [CrossRef] [PubMed]
254. Vezzani, A.; French, J.; Bartfai, T.; Baram, T.Z. The role of inflammation in epilepsy. *Nat. Rev. Neurol.* **2011**, *7*, 31–40. [CrossRef] [PubMed]
255. Ryan, K.; Backos, D.S.; Reigan, P.; Patel, M. Post-translational oxidative modification and inactivation of mitochondrial complex I in epileptogenesis. *J. Neurosci.* **2012**, *32*, 11250–11258. [CrossRef]
256. Waldbaum, S.; Liang, L.-P.; Patel, M. Persistent impairment of mitochondrial and tissue redox status during lithium-pilocarpine-induced epileptogenesis. *J. Neurochem.* **2010**, *115*, 1172–1182. [CrossRef]
257. Rowley, S.; Liang, L.-P.; Fulton, R.; Shimizu, T.; Day, B.; Patel, M. Mitochondrial respiration deficits driven by reactive oxygen species in experimental temporal lobe epilepsy. *Neurobiol. Dis.* **2015**, *75*, 151–158. [CrossRef]
258. Liang, L.P.; Ho, Y.S.; Patel, M. Mitochondrial superoxide production in kainate-induced hippocampal damage. *Neuroscience* **2000**, *101*, 563–570. [CrossRef]
259. Pearson, J.N.; Rowley, S.; Liang, L.-P.; White, A.M.; Day, B.J.; Patel, M. Reactive oxygen species mediate cognitive deficits in experimental temporal lobe epilepsy. *Neurobiol. Dis.* **2015**, *82*, 289–297. [CrossRef]
260. Walker, M.C. Pathophysiology of status epilepticus. *Neurosci. Lett.* **2018**, *667*, 84–91. [CrossRef]
261. Kovac, S.; Domijan, A.-M.; Walker, M.C.; Abramov, A.Y. Seizure activity results in calcium- and mitochondria-independent ROS production via NADPH and xanthine oxidase activation. *Cell Death Dis.* **2014**, *5*, e1442. [CrossRef]
262. Ding, S.; Fellin, T.; Zhu, Y.; Lee, S.-Y.; Auberson, Y.P.; Meaney, D.F.; Coulter, D.A.; Carmignoto, G.; Haydon, P.G. Enhanced astrocytic Ca^{2+} signals contribute to neuronal excitotoxicity after status epilepticus. *J. Neurosci.* **2007**, *27*, 10674–10684. [CrossRef] [PubMed]
263. Volmering, E.; Niehusmann, P.; Peeva, V.; Grote, A.; Zsurka, G.; Altmüller, J.; Nürnberg, P.; Becker, A.J.; Schoch, S.; Elger, C.E.; et al. Neuropathological signs of inflammation correlate with mitochondrial DNA deletions in mesial temporal lobe epilepsy. *Acta Neuropathol.* **2016**, *132*, 277–288. [CrossRef] [PubMed]
264. Shefa, U.; Jeong, N.Y.; Song, I.O.; Chung, H.-J.; Kim, D.; Jung, J.; Huh, Y. Mitophagy links oxidative stress conditions and neurodegenerative diseases. *Neural Regen. Res.* **2019**, *14*, 749–756. [CrossRef] [PubMed]
265. Wu, M.; Liu, X.; Chi, X.; Zhang, L.; Xiong, W.; Chiang, S.M.V.; Zhou, D.; Li, J. Mitophagy in Refractory Temporal Lobe Epilepsy Patients with Hippocampal Sclerosis. *Cell. Mol. Neurobiol.* **2018**, *38*, 479–486. [CrossRef] [PubMed]
266. Tian, M.-J.; Wang, R.-F.; Hölscher, C.; Mi, R.-L.; Yuan, Z.-Y.; Li, D.-F.; Xue, G.-F. The novel GLP-1/GIP dual receptor agonist DA3-CH is neuroprotective in the pilocarpine-induced epileptogenesis rat model. *Epilepsy Res.* **2019**, *154*, 97–106. [CrossRef] [PubMed]
267. Zhang, Y.; Zhang, M.; Zhu, W.; Yu, J.; Wang, Q.; Zhang, J.; Cui, Y.; Pan, X.; Gao, X.; Sun, H. Succinate accumulation induces mitochondrial reactive oxygen species generation and promotes status epilepticus in the kainic acid rat model. *Redox Biol.* **2020**, *28*, 101365. [CrossRef] [PubMed]
268. Redman, B.G.; Abubakr, Y.; Chou, T.; Esper, P.; Flaherty, L.E. Phase II trial of recombinant interleukin-1 beta in patients with metastatic renal cell carcinoma. *J. Immunother. Emphas. Tumor Immunol.* **1994**, *16*, 211–215. [CrossRef]
269. Chari, D.M. Remyelination in multiple sclerosis. *Int. Rev. Neurobiol.* **2007**, *79*, 589–620. [CrossRef]
270. Lampron, A.; Larochelle, A.; Laflamme, N.; Préfontaine, P.; Plante, M.-M.; Sánchez, M.G.; Yong, V.W.; Stys, P.K.; Tremblay, M.-È.; Rivest, S. Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *J. Exp. Med.* **2015**, *212*, 481–495. [CrossRef]
271. Kocur, M.; Schneider, R.; Pulm, A.-K.; Bauer, J.; Kropp, S.; Gliem, M.; Ingwersen, J.; Goebels, N.; Alferink, J.; Prozorovski, T.; et al. IFNβ secreted by microglia mediates clearance of myelin debris in CNS autoimmunity. *Acta Neuropathol. Commun.* **2015**, *3*, 20. [CrossRef]
272. De Mercanti, S.; Rolla, S.; Cucci, A.; Bardina, V.; Cocco, E.; Vladic, A.; Soldo-Butkovic, S.; Habek, M.; Adamec, I.; Horakova, D.; et al. Alemtuzumab long-term immunologic effect: Treg suppressor function increases up to 24 months. *Neurol. Neuroimmunol. Neuroinflamm.* **2016**, *3*, e194. [CrossRef] [PubMed]
273. Baker, D.; Herrod, S.S.; Alvarez-Gonzalez, C.; Giovannoni, G.; Schmierer, K. Interpreting Lymphocyte Reconstitution Data From the Pivotal Phase 3 Trials of Alemtuzumab. *JAMA Neurol.* **2017**, *74*, 961–969. [CrossRef] [PubMed]

274. Pinschewer, D.D.; Ochsenbein, A.F.; Odermatt, B.; Brinkmann, V.; Hengartner, H.; Zinkernagel, R.M. FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory. *J. Immunol.* **2000**, *164*, 5761–5770. [CrossRef] [PubMed]
275. Kappos, L.; Antel, J.; Comi, G.; Montalban, X.; O'Connor, P.; Polman, C.H.; Haas, T.; Korn, A.A.; Karlsson, G.; Radue, E.W.; et al. Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N. Engl. J. Med.* **2006**, *355*, 1124–1140. [CrossRef]
276. Cohen, J.A.; Barkhof, F.; Comi, G.; Hartung, H.-P.; Khatri, B.O.; Montalban, X.; Pelletier, J.; Capra, R.; Gallo, P.; Izquierdo, G.; et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N. Engl. J. Med.* **2010**, *362*, 402–415. [CrossRef]
277. Nagatsu, T.; Mogi, M.; Ichinose, H.; Togari, A. Changes in cytokines and neurotrophins in Parkinson's disease. *J. Neural Transm. Suppl.* **2000**, 277–290. [CrossRef]
278. Campolo, M.; Paterniti, I.; Siracusa, R.; Filippone, A.; Esposito, E.; Cuzzocrea, S. TLR4 absence reduces neuroinflammation and inflammasome activation in Parkinson's diseases in vivo model. *Brain Behav. Immun.* **2019**, *76*, 236–247. [CrossRef]
279. Muthuraman, M.; Koirala, N.; Ciolac, D.; Pintea, B.; Glaser, M.; Groppa, S.; Tamás, G.; Groppa, S. Deep Brain Stimulation and L-DOPA Therapy: Concepts of Action and Clinical Applications in Parkinson's Disease. *Front. Neurol.* **2018**, *9*, 711. [CrossRef] [PubMed]
280. Tomás-Camardiel, M.; Rite, I.; Herrera, A.J.; de Pablos, R.M.; Cano, J.; Machado, A.; Venero, J.L. Minocycline reduces the lipopolysaccharide-induced inflammatory reaction, peroxynitrite-mediated nitration of proteins, disruption of the blood-brain barrier, and damage in the nigral dopaminergic system. *Neurobiol. Dis.* **2004**, *16*, 190–201. [CrossRef] [PubMed]
281. Diguet, E.; Fernagut, P.-O.; Wei, X.; Du, Y.; Rouland, R.; Gross, C.; Bezard, E.; Tison, F. Deleterious effects of minocycline in animal models of Parkinson's disease and Huntington's disease. *Eur. J. Neurosci.* **2004**, *19*, 3266–3276. [CrossRef]
282. Castaño, A.; Herrera, A.J.; Cano, J.; Machado, A. The degenerative effect of a single intranigral injection of LPS on the dopaminergic system is prevented by dexamethasone, and not mimicked by rh-TNF-alpha, IL-1beta and IFN-gamma. *J. Neurochem.* **2002**, *81*, 150–157. [CrossRef]
283. Liu, B.; Du, L.; Hong, J.S. Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 607–617.
284. Li, Y.; Hu, X.; Liu, Y.; Bao, Y.; An, L. Nimodipine protects dopaminergic neurons against inflammation-mediated degeneration through inhibition of microglial activation. *Neuropharmacology* **2009**, *56*, 580–589. [CrossRef]
285. Zhang, L.; Zhang, L.; Li, L.; Hölscher, C. Semaglutide is Neuroprotective and Reduces α-Synuclein Levels in the Chronic MPTP Mouse Model of Parkinson's Disease. *J. Parkinsons Dis.* **2019**, *9*, 157–171. [CrossRef]
286. Sanz, J.M.; Chiozzi, P.; Colaianna, M.; Zotti, M.; Ferrari, D.; Trabace, L.; Zuliani, G.; Di Virgilio, F. Nimodipine inhibits IL-1β release stimulated by amyloid β from microglia. *Br. J. Pharmacol.* **2012**, *167*, 1702–1711. [CrossRef]
287. Vlad, S.C.; Miller, D.R.; Kowall, N.W.; Felson, D.T. Protective effects of NSAIDs on the development of Alzheimer disease. *Neurology* **2008**, *70*, 1672–1677. [CrossRef]
288. Jaturapatporn, D.; Isaac, M.G.E.K.N.; McCleery, J.; Tabet, N. Aspirin, steroidal and non-steroidal anti-inflammatory drugs for the treatment of Alzheimer's disease. *Cochrane Database Syst. Rev.* **2012**, CD006378. [CrossRef]
289. Daniels, M.J.D.; Rivers-Auty, J.; Schilling, T.; Spencer, N.G.; Watremez, W.; Fasolino, V.; Booth, S.J.; White, C.S.; Baldwin, A.G.; Freeman, S.; et al. Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models. *Nat. Commun.* **2016**, *7*, 12504. [CrossRef]
290. Sato, T.; Hanyu, H.; Hirao, K.; Kanetaka, H.; Sakurai, H.; Iwamoto, T. Efficacy of PPAR-γ agonist pioglitazone in mild Alzheimer disease. *Neurobiol. Aging* **2011**, *32*, 1626–1633. [CrossRef]
291. Hall, H.; Iulita, M.F.; Gubert, P.; Flores Aguilar, L.; Ducatenzeiler, A.; Fisher, A.; Cuello, A.C. AF710B, an M1/sigma-1 receptor agonist with long-lasting disease-modifying properties in a transgenic rat model of Alzheimer's disease. *Alzheimer's Dement.* **2018**, *14*, 811–823. [CrossRef]
292. Woodling, N.S.; Colas, D.; Wang, Q.; Minhas, P.; Panchal, M.; Liang, X.; Mhatre, S.D.; Brown, H.; Ko, N.; Zagol-Ikapitte, I.; et al. Cyclooxygenase inhibition targets neurons to prevent early behavioural decline in Alzheimer's disease model mice. *Brain* **2016**, *139*, 2063–2081. [CrossRef] [PubMed]

293. Fu, Y.; Zhang, N.; Ren, L.; Yan, Y.; Sun, N.; Li, Y.-J.; Han, W.; Xue, R.; Liu, Q.; Hao, J.; et al. Impact of an immune modulator fingolimod on acute ischemic stroke. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 18315–18320. [CrossRef] [PubMed]
294. Lampl, Y.; Boaz, M.; Gilad, R.; Lorberboym, M.; Dabby, R.; Rapoport, A.; Anca-Hershkowitz, M.; Sadeh, M. Minocycline treatment in acute stroke: An open-label, evaluator-blinded study. *Neurology* **2007**, *69*, 1404–1410. [CrossRef] [PubMed]
295. Kohler, E.; Prentice, D.A.; Bates, T.R.; Hankey, G.J.; Claxton, A.; van Heerden, J.; Blacker, D. Intravenous minocycline in acute stroke: A randomized, controlled pilot study and meta-analysis. *Stroke* **2013**, *44*, 2493–2499. [CrossRef] [PubMed]
296. Emsley, H.C.A.; Smith, C.J.; Georgiou, R.F.; Vail, A.; Hopkins, S.J.; Rothwell, N.J.; Tyrrell, P.J.; Acute Stroke Investigators. A randomised phase II study of interleukin-1 receptor antagonist in acute stroke patients. *J. Neurol. Neurosurg. Psychiatry* **2005**, *76*, 1366–1372. [CrossRef]
297. Smith, C.J.; Hulme, S.; Vail, A.; Heal, C.; Parry-Jones, A.R.; Scarth, S.; Hopkins, K.; Hoadley, M.; Allan, S.M.; Rothwell, N.J.; et al. SCIL-STROKE (Subcutaneous Interleukin-1 Receptor Antagonist in Ischemic Stroke): A Randomized Controlled Phase 2 Trial. *Stroke* **2018**, *49*, 1210–1216. [CrossRef]
298. Hill, M.D.; Goyal, M.; Menon, B.K.; Nogueira, R.G.; McTaggart, R.A.; Demchuk, A.M.; Poppe, A.Y.; Buck, B.H.; Field, T.S.; Dowlatshahi, D.; et al. Efficacy and safety of nerinetide for the treatment of acute ischaemic stroke (ESCAPE-NA1): A multicentre, double-blind, randomised controlled trial. *Lancet* **2020**, *395*, 878–887. [CrossRef]
299. Vezzani, A.; Moneta, D.; Conti, M.; Richichi, C.; Ravizza, T.; De Luigi, A.; De Simoni, M.G.; Sperk, G.; Andell-Jonsson, S.; Lundkvist, J.; et al. Powerful anticonvulsant action of IL-1 receptor antagonist on intracerebral injection and astrocytic overexpression in mice. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 11534–11539. [CrossRef]
300. Vezzani, A.; Maroso, M.; Balosso, S.; Sanchez, M.-A.; Bartfai, T. IL-1 receptor/Toll-like receptor signaling in infection, inflammation, stress and neurodegeneration couples hyperexcitability and seizures. *Brain Behav. Immun.* **2011**, *25*, 1281–1289. [CrossRef]
301. Kenney-Jung, D.L.; Vezzani, A.; Kahoud, R.J.; LaFrance-Corey, R.G.; Ho, M.-L.; Muskardin, T.W.; Wirrell, E.C.; Howe, C.L.; Payne, E.T. Febrile infection-related epilepsy syndrome treated with anakinra. *Ann. Neurol.* **2016**, *80*, 939–945. [CrossRef]
302. Stienen, M.N.; Haghikia, A.; Dambach, H.; Thöne, J.; Wiemann, M.; Gold, R.; Chan, A.; Dermietzel, R.; Faustmann, P.M.; Hinkerohe, D.; et al. Anti-inflammatory effects of the anticonvulsant drug levetiracetam on electrophysiological properties of astroglia are mediated via TGFβ1 regulation. *Br. J. Pharmacol.* **2011**, *162*, 491–507. [CrossRef]
303. Silvestro, S.; Mammana, S.; Cavalli, E.; Bramanti, P.; Mazzon, E. Use of Cannabidiol in the Treatment of Epilepsy: Efficacy and Security in Clinical Trials. *Molecules* **2019**, *24*, 1459. [CrossRef]

COLLABORATION NOT RELATED TO EPILEPSY

The NOP antagonist BTRX-246040 increases stress resilience in mice without affecting adult neurogenesis in the hippocampus

Flora D'Oliveira da Silva[1]*, Joaquim Azevedo Neto[2]*, Chiara Sturaro[2], Annunziata Guarino[2], Cathaline Robert[1], Elaine C. Gavioli[3], Girolamo Calo[4], Lionel Mouledous[1#], and Chiara Ruzza[2,5#]

[1]Research Center on Animal Cognition (CRCA), Center of Integrative Biology (CBI), University of Toulouse, CNRS UMR-5169, UPS, Toulouse, France.

[2]Department of Neuroscience and Rehabilitation, University of Ferrara, Ferrara, Italy

[3]Department of Biophysics and Pharmacology, Federal University of Rio Grande do Norte, Natal, Brazil

[4]Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Italy

[5]LTTA Laboratory for Advanced Therapies, Technopole of Ferrara, Ferrara, Italy

* These authors equally contributed

# These authors equally contributed

Corresponding author

Chiara Ruzza

chiara.ruzza@unife.it

Abstract

Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand of an inhibitory G protein coupled receptor named N/OFQ peptide receptor (NOP). Clinical and preclinical findings suggest that the blockade of the NOP signaling induces antidepressant-like effects. Additionally, the blockade of the NOP receptor during inescapable stress exposure prevented the acquisition of the helplessness phenotype, suggesting that NOP antagonists are able to increase stress resilience. BTRX-246040 (aka LY2940094) is a novel NOP receptor antagonist with high affinity, potency and selectivity for the NOP over classical opioid receptors. BTRX-246040 is under development for the treatment of depression, eating disorders and alcohol abuse and it already entered clinical trials.

In the present study, the antidepressant effects of BTRX-246040 were evaluated in mice subjected to the forced swimming test and to the learned helplessness model of depression. Additionally, the ability of BTRX-246040 to prevent the development of the helpless behavior and to modulate adult hippocampal neurogenesis has been investigated.

BTRX-246040 (30 mg/kg, i.p.) produced antidepressant-like effects in the forced swimming test and in the learned helplessness model. More interestingly, when given before the stress induction sessions it was able to prevent the development of the helplessness behavior. Under these experimental conditions, BTRX-246040 did not modulate adult hippocampal neurogenesis, neither in naive nor in stressed mice.

This study, performed with a clinically viable ligand, further corroborates growing evidence indicating that the blockade of the NOP signaling may provide an innovative strategy for the treatment of stress related psychopathologies.

Keywords

Nociceptin/orphanin FQ, NOP receptor, BTRX-246040, mouse, learned helplessness, adult neurogenesis, stress vulnerability.

Abbreviations

FST, forced swimming test; LA, locomotor activity; LH, learned helplessness; N/OFQ, Nociceptin/orphanin FQ; NOP, Nociceptin/orphanin FQ receptor

1. Introduction

Nociceptin/orphanin FQ (N/OFQ) is a heptadecapeptide discovered in the middle of 90´ using the reverse pharmacology approach. This peptide is the endogenous ligand of an inhibitory G protein coupled receptor named N/OFQ peptide receptor (NOP) (Meunier et al., 1995; Reinscheid et al., 1995). Clinical and preclinical findings suggest that the blockade of the NOP signaling induces antidepressant-like effects (Gavioli et al., 2019; Gavioli and Calo', 2013). Previous studies performed in our laboratories using the learned helplessness (LH) model of depression demonstrated that NOP antagonists revert the acquired helpless behavior, which is considered a depressive-like behavior. Thus, NOP antagonists evoke effects similar to those elicited by classical antidepressants (Holanda et al., 2016). Additionally, the blockade of the NOP receptor during inescapable stress exposure prevented the acquisition of helplessness, an effect not shared by classical antidepressants (Holanda et al., 2020, 2019a).

Little is known about the mechanisms by which NOP receptor antagonists promote stress resilience. The hippocampus, exerting a negative feedback control on the hypothalamic-pituitary-adrenal (HPA) axis (Jacobson and Sapolsky, 1991), is an important region for the regulation of the stress response. Adult neurogenesis in the dentate gyrus (DG) of the hippocampus is modulated by stress (Schoenfeld and Gould, 2012) and plays a role in stress resilience (Anacker et al., 2018; Snyder et al., 2011). Additionally, a link between the behavioral effects of classical antidepressants and their ability to increase adult hippocampal neurogenesis is now largely recognized (Malberg et al., 2021). Both N/OFQ and the NOP receptor are expressed in the hippocampus (Houtani et al., 2000; Manabe et al., 1998; Neal et al., 1999; Ozawa et al., 2015), N/OFQ levels are increased in the hippocampus after acute stress exposure (Nativio et al., 2012), and the bilateral infusions of a selective NOP antagonist into the dorsal hippocampus produced antidepressant-like effects in the mouse forced swim and tail suspension tests (Goeldner et al., 2010). Thus, the hypothesis that NOP antagonists produce their protective effects from stress by modulating adult neurogenesis in the hippocampus is worth to be explored.

BTRX-246040 (aka LY2940094) is a novel potent and NOP selective antagonist (Statnick et al., 2016; Toledo et al., 2014). In preclinical studies in rodents, BTRX-246040 produced robust antidepressant effects (Post et al., 2016b; Witkin et al., 2016), and displayed potential therapeutic utility in treating alcohol addiction (Rorick-Kehn et al., 2016) and disorders of appetitive behavior (Statnick et al., 2016). Moreover, in small proof of concept clinical studies BTRX-246040 was safe and well tolerated and showed efficacy in depressed (Post et al., 2016b) and alcohol dependent (Post et al., 2016a) patients. The *in vitro* pharmacological activity of BTRX-246040 has been recently

characterized in detail and compared to those of the standard NOP antagonist SB-612111. BTRX-246040 resulted a pure and selective antagonist at human recombinant and murine native NOP receptors displaying 3 - 10 fold higher potency than the standard antagonist SB-612111 (Ferrari et al., 2020).

The aim of the present study was the *in vivo* evaluation of BTRX-246040 in mice, in the forced swimming test and in the LH model, in curative and preventive protocols. Additionally, the putative association between hippocampal adult neurogenesis, stress resilience and BTRX-246040 preventive action was examined in mice exposed to the LH paradigm.

2. Material and methods

2.1. Animals - All experimental procedures adopted in this study were as humane as possible, complied with the European Communities Council directives (2010/63/E) and Italian regulations (D.Lgs, 26/2014). Protocols were approved by the Animal Welfare Body of the University of Ferrara and by the Italian Ministry of Health (License N° 302/2017). In vivo studies have been reported according to the ARRIVE guidelines (Kilkenny et al., 2010). Male CD-1 mice 12 weeks old were used in this study. Mice were bred and housed in a specific pathogen free animal facility of the University of Ferrara (LARP), in 425 × 266 × 155 mm polycarbonate cages (Tecniplast, VA, Italy), 3-4 mice/cage, under standard conditions (22 °C, 55 % humidity, 12 h light-dark cycle, lights on 7.00 am) with food (4RF, Mucedola, MI, Italy) and water ad libitum. A mouse red house (Tecniplast, VA, Italy) and nesting materials were present in each cage. The experiments were performed in the mornings between 8:30 and 13:00 h.

2.2. Drugs - BTRX-246040 was kindly provided by BlackThorn Therapeutics and solubilised firstly in 1% DMSO in saline solution containing 30% of cyclodextrin.

2.3. Forced swimming test (FST) - This assay was performed as described previously by Gavioli et al., (2003). Mice were placed individually in glass cylinders (height 18 cm; diameter 17 cm) containing 12 cm of water at 24 ± 1°C, for two swim sessions: an initial 15 min training session on day 1, and 24 h later (day 2), a 5 min test session. Results were relative to the 5 min test session. The immobility time (i.e. the time spent floating in the water without struggling) was recorded by an experienced observer. BTRX-246040 (3 - 30 mg/kg, i.p.) was administered 60 min before the test session.

2.4. Learned helplessness model (LH) - In the LH model BTRX-246040 (30 mg/kg, i.p., 60 min pretreatment) has been tested both for its ability to reverse the acquired LH phenotype (protocol 1,

described by (Holanda et al., 2016), and for its ability to prevent the acquisition of the LH phenotype (protocol 2, described by (Holanda et al., 2019b). Animals were individually placed in a Plexiglas box with a stainless steel grid floor (0.3 x 1 cm) attached to an electric shock generator (Ugo Basile, Gemonio, VA, Italy). The apparatus is divided in two compartments (47 x 18 x 25 cm) by a guillotine door (12 x 25 cm). A protocol with 2 consecutive induction sections was chosen, based on a previous pilot study, with the aim to induce the helpless phenotype in approximately 50% of the animals. Under these experimental conditions, mice were subjected to unpredictable (0.5 mA, 1-10 s automatically randomized shock duration, 1-20 s automatically randomized interval) and inescapable (guillotine door closed) electric footshocks during 2 consecutive days (induction sessions).

Protocol 1 (curative protocol) - Twenty-four hours after the last induction session mice were exposed to the screening session. During the screening session mice were exposed to 30 escapable electric footshocks (0.5 mA) with a cut off duration of 20 s. A mouse is defined 'helpless' when it fails escaping for 20 or more times. Only helpless mice were moved, twenty-four hours after the screening session, to the test session. In the test session the number of escapes was evaluated. The assessment of movement between chamber sides was recorded automatically by the instrument. 60 min before the test session mice were injected with BTRX-246040 (30 mg/kg, i.p.) or vehicle.

Protocol 2 (preventive protocol) - Twenty-four hours after the last induction session mice were exposed to the screening session. During the screening session the guillotine door that divides the two compartments is open and the mouse can terminate the electric shock moving through the door to the other side of the apparatus (escapable electric footshocks). During the screening session mice were exposed to 30 escapable electric footshocks (0.5 mA) with a cut off duration of 20 s. A mouse is defined 'helpless' when it fails escaping for 20 or more times. The administration of BTRX-246040 (30 mg/kg, i.p.) or vehicle was performed 60 min before each induction session, and mouse behavior was assessed, 24 h later, in the screening session. The % of mice developing helplessness during the screening session was reported.

2.5. Escapable electric footshock task – These series of experiments were performed in order to evaluate drug effects on mouse behavior under an aversive operant conditioning task (i.e., escapable electric footshock). In this protocol, during the induction sessions, mice learn that they can terminate the footshock by changing the side of the box. Thus, this protocol can be used to assess the effect of drugs on the cognitive performance. Electric footshocks were delivered to mice placed in the chamber with the guillotine door open during all the 4 or 3 days of experiment (i.e. protocol 1: 2 induction sessions, 1 screening session, and 1 test session; protocol 2: 2 induction sessions and 1 test session). The number of cycles, footshock intensities and duration of each experimental session was the same

as in the inescapable electric footshock protocol. To mimic LH protocol 1, mice were treated with BTRX-246040 (30 mg/kg, i.p.) or vehicle 60 min before the test session. To mimic LH protocol 2, on days 1 and 2, 60 min prior to the induction sessions, mice were treated with BTRX-246040 (30 mg/kg, i.p.) or vehicle. The number of escapes from the electrified chamber was automatically recorded.

2.6. Locomotor activity test (LA) – this experimental series investigated the effects of BTRX-246040 on spontaneous locomotion. Mice were positioned in a square plastic cage (40 x 40 cm), one mouse per cage. Four mice were monitored in parallel by a video camera. The distance moved (in meters) was recorded during 30 min. Mice were treated with BTRX-246040 30 mg/kg or vehicle 60 min before the test. Locomotor activity was measured immediately after the tail withdrawal assay. The ANY-maze video tracking system (Ugo Basile, Gemonio, VA, Italy, application version 4.52c Beta) was used for automatically registering the locomotor activity.

2.7. Tail withdrawal assay (TW) - the animals were placed in a holder and the distal half of the tail was immersed in water at 52°C. Withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut off time of 10 s was chosen to avoid tissue damage. Tail withdrawal time was determined 60 min after i.p. injection of BTRX-246040 30 mg/kg.

2.8. In vivo BrdU labelling and tissue collection – 28 days before the LH protocol 2 procedure, mice were injected i.p. with BrdU (100 mg/kg, solubilized in saline solution, Merck), 3 times over a period of 12 hours. Immediately after LH experiment mice were anesthetized with isoflurane, perfused with phosphate buffer followed by 4% paraformaldehyde in phosphate buffer. Naïve mice were processed the same way, 30 days after BrdU injection but without stress exposure. The brains were removed and post-fixed in 4% paraformaldehyde in phosphate buffer overnight and kept at 4°C. 24 h after perfusion brain samples were placed in a 30% sucrose solution containing 0.1% sodium azide. Brain sections (30 µm) were collected using a Leica SM2010R cryomicrotome (Leica biosystems, Nanterre, France) equipped with a freezing stage following a rostro-caudal axis over the whole hippocampus.

2.9. DCX / BrdU immunochemistry - one section every 12 was used for doublecortine (DCX) and BrdU co-labelling. Free floating sections were washed three times for 15 minutes in PBS + 0.25% Triton-X100 (PBST), then incubated for 15 minutes with 10% ethanol and 10% H_20_2 in PBST and washed again 3 times for 10 minutes in PBST. They were incubated in HCl 2N for one hour and then in borate buffer for 5 + 15 minutes. They were washed again before being blocked in 10% normal

goat serum for one hour and then incubated with rat anti-BrdU (1:400; ab6326, Abcam, Amsterdam, Netherlands) and rabbit anti-DCX antibody (1:500; 326003, Synaptic System, Goettingen, Germany) in PBST overnight at room temperature. Sections were rinsed 3 times for 10 minutes in PBST, then incubated for 2 hours with donkey anti-rat (Alexa 488; 1:500; A21208, Invitrogen, Eugen, Oregon, USA) and goat anti-rabbit antibody (Alexa 555; 1:500; A27039, Invitrogen, Bengaluru, India) in PBST before being washed three times for 10 minutes in PBST. Cell nuclei were stained with Hoechst (1:10000) during the first wash. Sections were mounted in Mowiol solution and coverslipped.

2.10. Ki67 immunochemistry - one section every 12 was used for Ki67 immunochemistry. After three rinses of 15 minutes with PBST, sections were incubated with 10% MeOH and 10% H_2O_2 in PBST. Sections were rinsed again and then incubated for one hour with 5% normal goat serum (NGS), 1% Bovine Serum Albumin (BSA), 0,5% Tween-20 and 0,25% sodium azide in PBST. They were then incubated overnight at room temperature with rabbit anti-Ki67 antibody (1:500; M3062, Eurobio, Les Ulis, France) in PBST + 0,25% azide. The next day, after three rinses of 10 minutes with PBST, sections were incubated for two hours at room temperature with a biotin-conjugated goat anti-rabbit antibody (1:500; BA1000, Vector Laboratories, Burlingame, California, USA) in PBST. After two 10 minute rinses, sections were incubated with an horseradish peroxidase Avidin Biotin Conjugation solution (ABC kit, 1:200; PK-6100, Vector Laboratories, Burlingame, California, USA) during one hour. Sections were finally stained in 3,3'-diaminobenzidine substrate kit (DAB kit, SK-4100, Vector Laboratories, Burlingame, California, USA) and the reaction stopped with PBST and 0,25% azide. Sections were rinsed three times in PBST and mounted on gelatin-coated slides. Counter-staining was done with Nuclear fast red followed by dehydration in ethanol, clearing in toluene and coverslipping in Eukit medium.

2.11. Quantification of BrdU+, DCX+, BrdU/DCX+ and Ki67+ cells - cell counting was performed manually over the whole DG using the Mercator software (Explora Nova, La Rochelle, France) at x40 magnification with a Leica DM6000 B fluorescence microscope (Leica, Nanterre, France). The Paxinos and Watson atlas was used to locate each section relative to the Bregma and -2.5 mm was considered to be the limit between the dorsal and ventral parts of the DG. To calculate the density of DCX and BrdU-immunoreactive cells (DCX+ and BrdU+), the total surface of the dentate gyrus granular layer was measured using Hoechst labelling with the Mercator region tool. The double immuno-labeling for DCX and BrdU was determined by colocalization of BrdU labelling of nuclei and DCX labelling of somas in the z-axis. For Ki67-immunoreactive cells (Ki67+) density, the length of the sub-granular zone was measured with the Mercator line tool using the fast-red counter-staining.

2.12. Data analysis - for this study the sample sizes were calculated a priori performing the power analysis with the G*Power software 3.1.9.4 (α 0.05, β 80%, effect size of 0.75). Data are expressed as mean ± S.E.M. of n animals. After testing data normal distribution using the Kolmogorov-Smirnov test, data were analyzed using one-way or two-way ANOVA followed by Dunnet's or Tukey's post-hoc test or Student´s t test, as specified. In the LH paradigm, protocol 2, data are presented as percentage of helpless mice, and they were analyzed using chi-square test. To test statistical correlation Pearson correlation analysis was applied. Differences were considered statistically significant when $p<0.05$.

3. Results

3.1. Behavioral effects of BTRX-246040 - In the FST mice treated with vehicle spent ~ 200 sec immobile during the 5 min test session. BTRX-246040 given i.p. 60 min before the test reduced in a dose dependent manner the immobility time of mice, being active at 10 and 30 mg/kg (one-way ANOVA followed by Dunnet's post hoc test, $F(3,20) = 17.20$; $p < 0.0001$, figure 1). Based on this result, the 30 mg/kg dose has been chosen for subsequent LH experiments.

In LH experiments BTRX-246040 has been evaluated for its ability to treat mice that already developed helpless behavior (protocol 1, figure 2A) and for its ability to prevent the development of helpless behavior when administered before the induction sessions (protocol 2, figure 2B). 30 mice started the LH experiment, protocol 1. Among these, 13 (44 %) developed helplessness and were treated with vehicle or BTRX-246040. Under these experimental conditions BTRX-246040 was effective in reverting the helpless behavior of mice by significantly increasing the number of escapes on the test session (Student t test, $t = 7.08$, $df = 11$, $p < 0.0001$). On the other hand, when tested in the LH protocol 2, BTRX-246040 significantly prevented the development of helpless behavior reducing the % of helpless mice during the screening session. Specifically, 31 mice entered the LH test, protocol 2, among these 16 were treated with vehicle and 15 with BTRX-246040 30 mg/kg. In the vehicle group 10 mice (62.5%) became helpless after the 2 induction sessions. Differently, in the BTRX-246040 group only 3 mice (20%) became helpless (Chi-square test, chi-square = 5.74, $df = 1$, $p = 0.016$).

Finally, experiments were performed in order to assess BTRX-246040 effects on cognitive performance, locomotion and nociception since these putative actions may bias the interpretation of LH results. As shown in figure 3A and 3B, in the escapable electric footshock task, both protocol 1

and 2, no differences were detected between mice treated with vehicle and BTRX-246040 30 mg/kg. Additionally, BTRX-246040 did not alter mouse locomotion and nociception (figure 3C and 3D).

3.2. Effects of LH induction and BTRX-246040 treatment on adult neurogenesis in the dentate gyrus of the hippocampus – Mice subjected to the protocol 2 were injected with BrdU 4 weeks before LH induction. In parallel, a group of mice was injected with BrdU and euthanized 4 weeks later without exposure to any stressful stimulus. Parameters reflecting various steps of the adult neurogenesis process in unstressed (naïve) animals and in mice subjected to the LH procedure with or without previous treatment with BTRX-246040 were analysed.

The proliferation of neural stem cells and neuronal progenitors was assessed using the proliferation marker Ki67 (Scholzen and Gerdes, 2000) (figure 4A). No difference was detected in the density of Ki67 positive cells along the subgranular zone of the DG between naïve and stressed mice, regardless of treatment and LH development (figure 4B and C). Similar results were obtained focusing on the survival of 4-week-old cells previously labeled with BrdU (figure 5A – 5C). The maturation of 4-week-old neurons was also assessed by quantifying the percentage of BrdU positive cells co-expressing the immature neuron marker DCX (Rao and Shetty, 2004) (figure 5D – 5F). Again, the stress procedure, the LH development and BTRX-246040 treatment did not affect this parameter.

Finally, we quantified the density of immature neurons in the granular layer of the DG in naïve and stressed mice (Figure 6A - 6C). Stress exposure induced a significant increase in the density of DCX positive cells in both vehicle and BTRX-246040 treated animals (one-way ANOVA followed by Tukey's post hoc test, $F(2, 33) = 6.043$, $p = 0.0058$; Figure 6D). This phenomenon was observed in both the dorsal and the ventral parts of the DG (dorsal, one-way ANOVA followed by Tukey's post hoc test, $F(2, 33) = 4.539$, $p = 0.0181$; figure 6E; ventral, one-way ANOVA followed by Tukey's post hoc test, $F(2, 33) = 5.283$, $p = 0.0102$; figure 6F). In a parallel experiment, BTRX-246040 did not affect the density of DCX positive cells in naïve mice (figure 6G).

In mice exposed to stress and treated with vehicle, the density of DCX positive cells was significantly higher in LH than in no LH mice (two-way ANOVA followed by Tukey's post hoc test, treatment factor: $F(1, 26) = 2.034$, $p = 0.1657$, LH factor: $F(1, 26) = 5.045$, $p = 0.0334$, interaction: $F(1, 26) = 5.361$, $p = 0.0287$; figure 7A). When the dorsal and ventral parts of the DG were analyzed separately, a similar result was found in the ventral (two-way ANOVA followed by Tukey's post hoc test, treatment factor: $F(1, 26) = 2.019$, $p = 0.1672$, LH factor: $F(1, 26) = 7.452$, $p = 0.0112$, interaction: $F(1, 26) = 6.836$, $p = 0.0147$; figure 7C) but not in the dorsal region (figure 7B). In order to better characterize the relationship between LH behavior and the density of immature neurons, the

correlation between the density of DCX positive cells and the number of escape failures during the LH model has been analyzed. When the whole DG was considered, a significant positive correlation was found for the vehicle group (Pearson's correlation coefficient r = 0.6864, p = 0.0047, figure 7D) but not for the BTRX-246040 group. When the dorsal and ventral DG where separately analyzed, a correlation was detected in the ventral DG only for vehicle mice (Pearson's correlation coefficient r = 0.7282, p = 0.0021, figure 7F) but not in the dorsal DG (figure 7E).

4. Discussion

The present study demonstrates that the clinically viable NOP antagonist BTRX-246040 behaves as an antidepressant drug in mice exposed to the FST and to the LH paradigm. More interestingly, when given before stress exposure, BTRX-246040 prevents the development of LH behavior, suggesting that NOP antagonists, differently from classical antidepressant drugs, can be useful to reduce stress vulnerability. We tested whether the mechanism by which NOP antagonists produce their protective effects is the modulation of adult neurogenesis in the DG of the hippocampus. We demonstrated that the stress exposure induces, 24 hours later, an increase of the number of immature neurons and that this increase is directly related to the intensity of the LH behavior. However, despite its protective behavioral effects, BTRX-246040 treatment did not affect the number of immature neurons in mice exposed to stress. This result suggests that, at least under the present experimental conditions, the mechanisms by which NOP antagonists exert antidepressant effects and reduce stress vulnerability are not related to adult neurogenesis in the hippocampus.

BTRX-246040 is a selective and potent NOP antagonist (Ferrari et al., 2020; Statnick et al., 2016) under development for the treatment of depression, eating disorders and alcohol abuse (Browne and Lucki, 2019; Witkin et al., 2019). In a small clinical proof of concept study performed in patients with major depressive disorders (MDD) BTRX-246040 (40 mg, p.o.) was well tolerated and decreased depression symptoms after 8 weeks (Post et al., 2016b). Similar results were recently reported by Dawson and colleagues (Dawson et al., 2021). Thus, BTRX-246040, being the first NOP antagonist useful for clinical studies, represents a research tool of paramount importance to translate pre-clinical results in findings useful to the clinical practice.

In mice subjected to the FST, BTRX-246040 produced antidepressant like effects, being active from the dose of 10 mg/kg. This result replicates findings obtained in previous studies, using the same assay in both rats and mice (Post et al., 2016b; Witkin et al., 2016). The antidepressant like effects of BTRX-246040 where then confirmed in the LH model of depression, where it reverted the LH

acquired behavior after one single administration. This effect of BTRX-246040 is similar to those reported for the classical NOP antagonists UFP-101 and SB-612111 (Holanda et al., 2016) and corroborates the widely recognized hypothesis that the blockage of the NOP receptor produces antidepressant effects in rodents subjected to different models of depression (Gavioli et al., 2019; Gavioli and Calo', 2013; Witkin et al., 2014). As far as the potency of the NOP antagonist is concerned, BTRX-246040 displayed antidepressant-like effects from the dose of 10 mg/kg and was then used at the dose of 30 mg/kg in the LH experiments. Of note, the standard NOP antagonist SB-612111 is active as antidepressant from the dose of 3 mg/kg (Holanda et al., 2016), thus being 3 fold more potent than BTRX-246040. The in vivo rank order of potency SB-612111 > BTRX-246040 is different from that reported when the two NOP antagonists were systematically compared in vitro in different assays where BTRX-246040 displayed similar or even higher potency than SB-612111 (Ferrari et al., 2020). This discrepancy between the in vitro and in vivo potency of SB-612111 and BTRX-246040 can probably be ascribed to the different pharmacokinetic features of the two molecules.

What is new in the present research is the ability of BTRX-246040 to prevent the development of the LH phenotype when administered before stress exposure. Similar results were already obtained with the NOP antagonist SB-612111 and with the genetic blockage of the NOP receptor in NOP knockout mice (Holanda et al., 2020, 2019b). As recently reviewed (Gavioli et al., 2021; Ubaldi et al., 2021), a growing body of evidence suggests that the activation of the N/OFQ – NOP system during stress exposure contributes to the development of depressive-like behaviors (Der-Avakian et al., 2017; Holanda et al., 2019b). Under this view, the blockage of the NOP receptor can be proposed as an innovative strategy to increase stress resilience and to prevent depression episodes and stress-triggered diseases in vulnerable subjects. This study, performed with a clinically viable well tolerated NOP ligand, corroborates this hypothesis and paves the way for clinical proof of concept studies in selected patients.

Finally, an effort has been done to characterize the LH model focusing on hippocampal neurogenesis, and to test whether the protective effect of BTRX-246040 is related to its ability to modulate adult neurogenesis in the DG of the hippocampus. Our hypothesis was based on the following findings: i) chronic as well as acute stress are reported to inhibit adult hippocampal neurogenesis (Llorens-Martín and Trejo, 2011; Malberg and Duman, 2003; Schoenfeld and Gould, 2012). ii) adult neurogenesis is a key element for the effectiveness of both classical (chronic administration) and rapid acting antidepressants (Autry et al., 2011; Li et al., 2010; Ly et al., 2018; Malberg and Schechter, 2005;

Malberg et al., 2000; Malberg and Duman, 2003; Santarelli et al., 2003; Surget et al., 2011; Yamada and Jinno, 2019). iii) N/OFQ and the NOP receptor are expressed in the hippocampus (Houtani et al., 2000; Manabe et al., 1998; Neal et al., 1999; Ozawa et al., 2015), N/OFQ is released in the hippocampus after acute restraint stress (Nativio et al., 2012) and facilitates despair (Goeldner et al., 2010). iv) in primary cultures of hippocampal neurons N/OFQ inhibits dendritic growth and counteracts BDNF positive effects on dendritic growth (Alder et al., 2013). v) chronic administration of the NOP antagonist UFP-101 in stressed rats increases the number of immature neurons in the dorsal DG of the hippocampus (Vitale et al., 2017).

To monitor adult neurogenesis in the DG of the hippocampus three research tools were used: Ki-67 as a marker of proliferation in the initial phase of adult neurogenesis (neural stem cells and progenitors) (Scholzen and Gerdes, 2000), BrdU injected 4 weeks before brain extraction to mark 4-week-old neurons, and DCX to label early immature neurons (Rao and Shetty, 2004).

Under the present experimental conditions, stress exposure had no effect on the proliferation of neural stem cells and progenitors, measured 24 h later. This result agrees with several reports showing no effect of acute stress on this cell population (Dagyte et al., 2009; Llorens-Martín and Trejo, 2011; Thomas et al., 2007). We then analyzed the survival and maturation of 4-week-old neurons. The choice to focus on this particular population was based on two reasons. First, this time point corresponds to a critical period of enhanced excitability, synaptic plasticity and sensitivity to environmental changes (Bergami et al., 2015). Second, the maturation of 4-week-old neurons has been shown to be inhibited by acute stress (Llorens-Martín and Trejo, 2011). In the present study, stress exposure did not decrease the number of 4-week-old BrdU-labelled cells and did not increase the percentage of 4-week-old neurons still expressing the immaturity marker DCX. This discrepancy with Llorens-Martin and Trejo data might be due to the different stressful stimulus used (electric footshock vs forced swim) and/or to sex related reasons (male vs female mice). Interestingly, under the present experimental conditions, the acute exposure to an unescapable and unpredictable stress lead to an increase of DCX+ immature neurons. Since the study of 4-week-old neurons did not suggest any slow-down of the maturation process, the higher number of DCX+ cells suggests that LH stress accelerated the differentiation of neuronal progenitors. The density of DCX+ cells positively correlates with the helpless behavior in the vehicle treated group. This correlation was specific for the ventral part of the hippocampus. This is in line with previous studies demonstrating the involvement of new neurons from the ventral DG in stress resilience (Anacker et al., 2018; Levone et al., 2014). To the best of our knowledge, this is the first evidence of a positive correlation between the density of immature neurons and the helpless behavior. Previous LH studies performed in rats

focused on later time points, from one week to 10 days after inescapable stress, and showed an overall reduction in proliferation irrespective of the helpless status (Ho and Wang, 2010; Malberg and Duman, 2003; Vollmayr et al., 2003). In addition, Ho and Wang found a reduction in the survival of cells that had been labeled with BrdU one day before stress, specifically in helpless rats (Ho and Wang, 2010). Altogether, these results suggest that helplessness is associated with a biphasic effect on adult neurogenesis: a transient increase in neuronal differentiation followed by a protracted loss of newborn neurons. Clearly this hypothesis needs to be challenged by further studies that should systematically compare different stressful stimuli and their effects at different time points after stress exposure.

Finally, we observed that BTRX-246040 treatment for 2 consecutive days did not modify the density of immature neurons in naive animals and did not inhibit stress-induced changes in immature neuron density. The mechanism by which NOP antagonists prevent the acquisition of helplessness is therefore independent of hippocampal adult neurogenesis, at least under the present experimental conditions. Of note, these results apparently disagree with the study performed by Vitale and colleagues that reported that chronic administration of the NOP antagonist UFP-101 increases the number of DCX+ cells in the DG of rats hippocampus (Vitale et al., 2017). This discrepancy can be probably explained considering the different model used (rat chronic mild stress) and the chronic administration of the NOP antagonist in the Vitale et al. study.

5. Conclusions

In conclusion, the present study demonstrated that the NOP receptor antagonist BTRX-246040 produces antidepressant-like effect in the FST and in the LH model of depression. Additionally, BTRX-246040 was able to prevent the development of the LH phenotype in mice subjected to the unescapable stress procedures. This effect of the NOP antagonist is not related to adult neurogenesis in the DG of the hippocampus. This research, demonstrating with a clinically viable NOP antagonist that the NOP blockage reduces stress susceptibility, paves the way for clinical proof of concept studies in patients vulnerable to stress triggered diseases.

6. Acknowledgment

We would like to thank BlackThorn Therapeutics for providing BTRX-246040.

Funding

This work was supported by the University of Ferrara (FAR and FIR grant to CR), the CNRS (to LM), the French Ministry of Research (to FD), and the Brazilian National Council Research (CNPq; Grant No. 303072/2019-9, and No. 401837/2016-5 to ECG).

Conflict of interest

The authors declare no conflict of interest

References

Alder, J., Kallman, S., Palmieri, A., Khadim, F., Ayer, J.J., Kumar, S., Tsung, K., Grinberg, I., Thakker-Varia, S., 2013. Neuropeptide orphanin FQ inhibits dendritic morphogenesis through activation of RhoA. Dev. Neurobiol. 73, 769–784. https://doi.org/10.1002/DNEU.22101

Anacker, C., Luna, V.M., Stevens, G.S., Millette, A., Shores, R., Jimenez, J.C., Chen, B., Hen, R., 2018. Hippocampal neurogenesis confers stress resilience by inhibiting the ventral dentate gyrus. Nat. 2018 5597712 559, 98–102. https://doi.org/10.1038/s41586-018-0262-4

Autry, A.E., Adachi, M., Nosyreva, E., Na, E.S., Los, M.F., Cheng, P.F., Kavalali, E.T., Monteggia, L.M., 2011. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. Nat. 2011 4757354 475, 91–95. https://doi.org/10.1038/nature10130

Browne, C.A., Lucki, I., 2019. Targeting opioid dysregulation in depression for the development of novel therapeutics. Pharmacol. Ther. 201, 51. https://doi.org/10.1016/J.PHARMTHERA.2019.04.009

Dawson, G.R., Post, A., Smart, T.S., Browning, M., Harmer, C.J., 2021. Accuracy in recognising happy facial expressions is associated with antidepressant response to a NOP receptor antagonist but not placebo treatment. J. Psychopharmacol. https://doi.org/10.1177/02698811211044684

Der-Avakian, A., D'Souza, M.S., Potter, D.N., Chartoff, E.H., Carlezon, W.A., Pizzagalli, D.A., Markou, A., 2017. Social defeat disrupts reward learning and potentiates striatal nociceptin/orphanin FQ mRNA in rats. Psychopharmacology (Berl). 234, 1603–1614. https://doi.org/10.1007/s00213-017-4584-y

Ferrari, F., Rizzo, S., Ruzza, C., Calo, G., 2020. Detailed in vitro pharmacological characterization of the clinically viable nociceptin/orphanin FQ peptide receptor antagonist BTRX-246040. J. Pharmacol. Exp. Ther. 373, 34–43. https://doi.org/10.1124/jpet.119.262865

Gavioli, E.C., Calo', G., 2013. Nociceptin/orphanin FQ receptor antagonists as innovative antidepressant drugs, Pharmacology and Therapeutics. Elsevier Inc. https://doi.org/10.1016/j.pharmthera.2013.05.008

Gavioli, E.C., Holanda, V.A.D., Calo, G., Ruzza, C., 2021. Nociceptin/orphanin FQ receptor system blockade as an innovative strategy for increasing resilience to stress. Peptides 141, 170548. https://doi.org/10.1016/J.PEPTIDES.2021.170548

Gavioli, E.C., Holanda, V.A.D., Ruzza, C., 2019. NOP ligands for the treatment of anxiety and mood disorders, in: Handbook of Experimental Pharmacology. Springer New York LLC, pp. 233–257. https://doi.org/10.1007/164_2018_188

Gavioli, E.C., Marzola, G., Guerrini, R., Bertorelli, R., Zucchini, S., De Lima, T.C.M., Rae, G.A.,

Salvadori, S., Regoli, D., Calo, G., 2003. Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: pharmacological and genetic evidences from the mouse forced swimming test. Eur. J. Neurosci. 17, 1987–1990. https://doi.org/10.1046/J.1460-9568.2003.02603.X

Goeldner, C., Reiss, D., Kieffer, B.L., Ouagazzal, A.M., 2010. Endogenous nociceptin/orphanin-FQ in the dorsal hippocampus facilitates despair-related behavior. Hippocampus 20, 911–916. https://doi.org/10.1002/HIPO.20760

Ho, Y.C., Wang, S., 2010. Adult neurogenesis is reduced in the dorsal hippocampus of rats displaying learned helplessness behavior. Neuroscience 171, 153–161. https://doi.org/10.1016/J.NEUROSCIENCE.2010.08.062

Holanda, V.A.D., Medeiros, I.U., Asth, L., Guerrini, R., Calo', G., Gavioli, E.C., 2016. Antidepressant activity of nociceptin/orphanin FQ receptor antagonists in the mouse learned helplessness. Psychopharmacology (Berl). 233, 2525–2532. https://doi.org/10.1007/s00213-016-4310-1

Holanda, V.A.D., Oliveira, M.C., Da Silva Junior, E.D., Calo', G., Ruzza, C., Gavioli, E.C., 2020. Blockade of nociceptin/orphanin FQ signaling facilitates an active copying strategy due to acute and repeated stressful stimuli in mice. Neurobiol. Stress 13, 100255. https://doi.org/10.1016/J.YNSTR.2020.100255

Holanda, V.A.D., Pacifico, S., Azevedo Neto, J., Finetti, L., Lobão-Soares, B., Calo, G., Gavioli, E.C., Ruzza, C., 2019a. Modulation of the NOP receptor signaling affects resilience to acute stress. J. Psychopharmacol. https://doi.org/10.1177/0269881119864942

Holanda, V.A.D., Pacifico, S., Azevedo Neto, J., Finetti, L., Lobão-Soares, B., Calo, G., Gavioli, E.C., Ruzza, C., 2019b. Modulation of the NOP receptor signaling affects resilience to acute stress. J. Psychopharmacol. 33, 1540–1549. https://doi.org/10.1177/0269881119864942

Houtani, T., Nishi, M., Takeshima, H., Sato, K., Sakuma, S., Kakimoto, S., Ueyama, T., Noda, T., Sugimoto, T., 2000. Distribution of Nociceptin/Orphanin FQ Precursor Protein and Receptor in Brain and Spinal Cord: A Study Using In Situ Hybridization and X-Gal Histochemistry in Receptor-Deficient Mice THE JOURNAL OF COMPARATIVE NEUROLOGY 424:489-508 (2000). J. Comp. Neurol 424, 489–508. https://doi.org/10.1002/1096-9861

Jacobson, L., Sapolsky, R., 1991. The Role of the Hippocampus in Feedback Regulation of the Hypothalamic-Pituitary-Adrenocortical Axis. Endocr. Rev. 12, 118–134. https://doi.org/10.1210/EDRV-12-2-118

Levone, B.R., Cryan, J.F., O'Leary, O.F., 2014. Role of adult hippocampal neurogenesis in stress resilience. Neurobiol. Stress 1, 147–155. https://doi.org/10.1016/J.YNSTR.2014.11.003

Li, N., Lee, B., Liu, R.J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.Y., Aghajanian, G., Duman, R.S., 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science (80-.). 329, 959–964. https://doi.org/10.1126/SCIENCE.1190287/SUPPL_FILE/LI.SOM.PDF

Llorens-Martín, M., Trejo, J.L., 2011. Mifepristone prevents stress-induced apoptosis in newborn neurons and increases AMPA receptor expression in the dentate gyrus of C57/BL6 mice. PLoS One 6. https://doi.org/10.1371/JOURNAL.PONE.0028376

Ly, C., Greb, A.C., Cameron, L.P., Wong, J.M., Barragan, E. V., Wilson, P.C., Burbach, K.F., Soltanzadeh Zarandi, S., Sood, A., Paddy, M.R., Duim, W.C., Dennis, M.Y., McAllister, A.K., Ori-McKenney, K.M., Gray, J.A., Olson, D.E., 2018. Psychedelics Promote Structural and Functional Neural Plasticity. Cell Rep. 23, 3170–3182. https://doi.org/10.1016/J.CELREP.2018.05.022

Malberg, J., Schechter, L., 2005. Increasing Hippocampal Neurogenesis: A Novel Mechanism for Antidepressant Drugs. Curr. Pharm. Des. 11, 145–155. https://doi.org/10.2174/1381612053382223

Malberg, J.E., Duman, R.S., 2003. Cell Proliferation in Adult Hippocampus is Decreased by Inescapable Stress: Reversal by Fluoxetine Treatment. Neuropsychopharmacol. 2003 289 28, 1562–1571. https://doi.org/10.1038/sj.npp.1300234

Malberg, J.E., Eisch, A.J., Nestler, E.J., Duman, R.S., 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J. Neurosci. 20, 9104–9110. https://doi.org/10.1523/JNEUROSCI.20-24-09104.2000

Malberg, J.E., Hen, R., Madsen, T.M., 2021. Adult Neurogenesis and Antidepressant Treatment: The Surprise Finding by Ron Duman and the Field 20 Years Later. Biol. Psychiatry 90, 96–101. https://doi.org/10.1016/J.BIOPSYCH.2021.01.010

Manabe, T., Noda, Y., Mamiya, T., Katagiri, H., Houtani, T., Nishi, M., Noda, T., Takahashi, T., Sugimoto, T., Nabeshima, T., Takeshima, H., 1998. Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors. Nat. 1998 3946693 394, 577–581. https://doi.org/10.1038/29073

Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentler, M., Costentin, J., 1995. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature. https://doi.org/10.1038/377532a0

Nativio, P., Pascale, E., Maffei, A., Scaccianoce, S., Passarelli, F., 2012. Effect of stress on hippocampal nociceptin expression in the rat. Stress 15, 378–384.

https://doi.org/10.3109/10253890.2011.627071

Neal, C.R., Mansour, A., Reinscheid, R., Nothacker, H.-P., Civelli, O., Watson, S.J., 1999. Localization of Orphanin FQ (Nociceptin) Peptide and Messenger RNA in the Central Nervous System of the Rat. J. Comp. Neurol 406, 503–547. https://doi.org/10.1002/(SICI)1096-9861(19990419)406:4

Ozawa, A., Brunori, G., Mercatelli, D., Wu, J., Cippitelli, A., Zou, B., Xie, X.S., Williams, M., Zaveri, N.T., Low, S., Scherrer, G., Kieffer, B.L., Toll, L., 2015. Knock-In Mice with NOP-eGFP Receptors Identify Receptor Cellular and Regional Localization. J. Neurosci. 35, 11682–11693. https://doi.org/10.1523/JNEUROSCI.5122-14.2015

Post, A., Smart, T.S., Jackson, K., Mann, J., Mohs, R., Rorick-Kehn, L., Statnick, M., Anton, R., O'Malley, S.S., Wong, C.J., 2016a. Proof-of-Concept Study to Assess the Nociceptin Receptor Antagonist LY2940094 as a New Treatment for Alcohol Dependence. Alcohol. Clin. Exp. Res. 40, 1935–1944. https://doi.org/10.1111/acer.13147

Post, A., Smart, T.S., Krikke-Workel, J., Dawson, G.R., Harmer, C.J., Browning, M., Jackson, K., Kakar, R., Mohs, R., Statnick, M., Wafford, K., McCarthy, A., Barth, V., Witkin, J.M., 2016b. A Selective Nociceptin Receptor Antagonist to Treat Depression: Evidence from Preclinical and Clinical Studies. Neuropsychopharmacology 41, 1803–1812. https://doi.org/10.1038/npp.2015.348

Rao, M.S., Shetty, A.K., 2004. Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. Eur. J. Neurosci. 19, 234–246. https://doi.org/10.1111/J.0953-816X.2003.03123.X

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J., Civelli, O., 1995. Orphanin FQ: A neuropeptide that activates an opioidlike G protein-coupled receptor. Science (80-.). 270, 792–794. https://doi.org/10.1126/science.270.5237.792

Rorick-Kehn, L.M., Ciccocioppo, R., Wong, C.J., Witkin, J.M., Martinez-Grau, M.A., Stopponi, S., Adams, B.L., Katner, J.S., Perry, K.W., Toledo, M.A., Diaz, N., Lafuente, C., Jiménez, A., Benito, A., Pedregal, C., Weiss, F., Statnick, M.A., 2016. A Novel, Orally Bioavailable Nociceptin Receptor Antagonist, LY2940094, Reduces Ethanol Self-Administration and Ethanol Seeking in Animal Models. Alcohol. Clin. Exp. Res. 40, 945–954. https://doi.org/10.1111/acer.13052

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., Hen, R., 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301, 805–809.

https://doi.org/10.1126/SCIENCE.1083328

Schoenfeld, T.J., Gould, E., 2012. Stress, Stress Hormones, and Adult Neurogenesis. Exp. Neurol. 233, 12. https://doi.org/10.1016/J.EXPNEUROL.2011.01.008

Scholzen, T., Gerdes, J., 2000. The Ki-67 Protein: From the Known and the Unknown. https://doi.org/10.1002/(SICI)1097-4652(200003)182:3

Snyder, J.S., Soumier, A., Brewer, M., Pickel, J., Cameron, H.A., 2011. Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nat. 2011 4767361 476, 458–461. https://doi.org/10.1038/nature10287

Statnick, M.A., Chen, Y., Ansonoff, M., Witkin, J.M., Rorick-Kehn, L., Suter, T.M., Song, M., Hu, C., Lafuente, C., Jiménez, A., Benito, A., Diaz, N., Martínez-Grau, M.A., Toledo, M.A., Pintar, J.E., 2016. A novel nociceptin receptor antagonist LY2940094 inhibits excessive feeding behavior in rodents: A possible mechanism for the treatment of binge eating disorder. J. Pharmacol. Exp. Ther. 356, 493–502. https://doi.org/10.1124/jpet.115.228221

Surget, A., Tanti, A., Leonardo, E.D., Laugeray, A., Rainer, Q., Touma, C., Palme, R., Griebel, G., Ibarguen-Vargas, Y., Hen, R., Belzung, C., 2011. Antidepressants recruit new neurons to improve stress response regulation. Mol. Psychiatry 2011 1612 16, 1177–1188. https://doi.org/10.1038/mp.2011.48

Toledo, M.A., Pedregal, C., Lafuente, C., Diaz, N., Martinez-Grau, M.A., Jiménez, A., Benito, A., Torrado, A., Mateos, C., Joshi, E.M., Kahl, S.D., Rash, K.S., Mudra, D.R., Barth, V.N., Shaw, D.B., McKinzie, D., Witkin, J.M., Statnick, M.A., 2014. Discovery of a novel series of orally active nociceptin/orphanin FQ (NOP) receptor antagonists based on a dihydrospiro(piperidine-4,7′-thieno[2,3- C]pyran) scaffold. J. Med. Chem. 57, 3418–3429. https://doi.org/10.1021/jm500117r

Ubaldi, M., Cannella, N., Borruto, A.M., Petrella, M., Di Bonaventura, M.V.M., Soverchia, L., Stopponi, S., Weiss, F., Cifani, C., Ciccocioppo, R., 2021. Role of Nociceptin/Orphanin FQ-NOP Receptor System in the Regulation of Stress-Related Disorders. Int. J. Mol. Sci. 22. https://doi.org/10.3390/IJMS222312956

Vitale, G., Filaferro, M., Micioni Di Bonaventura, M.V., Ruggieri, V., Cifani, C., Guerrini, R., Simonato, M., Zucchini, S., 2017. Effects of [Nphe 1, Arg 14, Lys 15] N/OFQ-NH 2 (UFP-101), a potent NOP receptor antagonist, on molecular, cellular and behavioural alterations associated with chronic mild stress. J. Psychopharmacol. 31, 691–703. https://doi.org/10.1177/0269881117691456

Vollmayr, B., Simonis, C., Weber, S., Gass, P., Henn, F., 2003. Reduced cell proliferation in the dentate gyrus is not correlated with the development of learned helplessness. Biol. Psychiatry

54, 1035–1040. https://doi.org/10.1016/S0006-3223(03)00527-4

Witkin, J.M., Rorick-Kehn, L.M., Benvenga, M.J., Adams, B.L., Gleason, S.D., Knitowski, K.M., Li, X., Chaney, S., Falcone, J.F., Smith, J.W., Foss, J., Lloyd, K., Catlow, J.T., McKinzie, D.L., Svensson, K.A., Barth, V.N., Toledo, M.A., Diaz, N., Lafuente, C., Jiménez, A., Benito, A., Pedregal, C., Martínez-Grau, M.A., Post, A., Ansonoff, M.A., Pintar, J.E., Statnick, M.A., 2016. Preclinical findings predicting efficacy and side-effect profile of LY2940094, an antagonist of nociceptin receptors. Pharmacol. Res. Perspect. 4. https://doi.org/10.1002/prp2.275

Witkin, J.M., Statnick, M.A., Rorick-Kehn, L.M., Pintar, J.E., Ansonoff, M., Chen, Y., Tucker, R.C., Ciccocioppo, R., 2014. The biology of Nociceptin/Orphanin FQ (N/OFQ) related to obesity, stress, anxiety, mood, and drug dependence. Pharmacol. Ther. 141, 283–299. https://doi.org/10.1016/J.PHARMTHERA.2013.10.011

Witkin, J.M., Wallace, T.L., Martin, W.J., 2019. Therapeutic Approaches for NOP Receptor Antagonists in Neurobehavioral Disorders: Clinical Studies in Major Depressive Disorder and Alcohol Use Disorder with BTRX-246040 (LY2940094). Handb. Exp. Pharmacol. 254, 399–415. https://doi.org/10.1007/164_2018_186

Yamada, J., Jinno, S., 2019. Potential link between antidepressant-like effects of ketamine and promotion of adult neurogenesis in the ventral hippocampus of mice. Neuropharmacology 158, 107710. https://doi.org/10.1016/J.NEUROPHARM.2019.107710

Figure legends

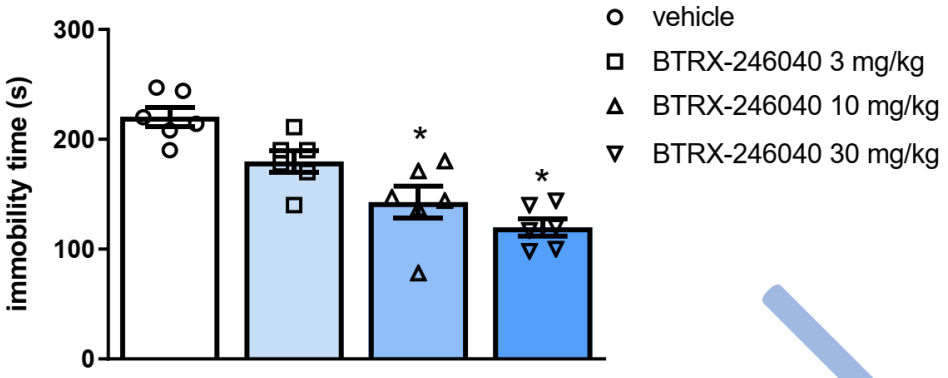


Figure 1. Forced swimming test. Dose-response curve to BTRX-246040 (i.p., 60 min pretreatment). Data are the mean ± sem of 6 mice/group. * $p < 0.05$ according to one-way followed by Bonferroni's post hoc test.

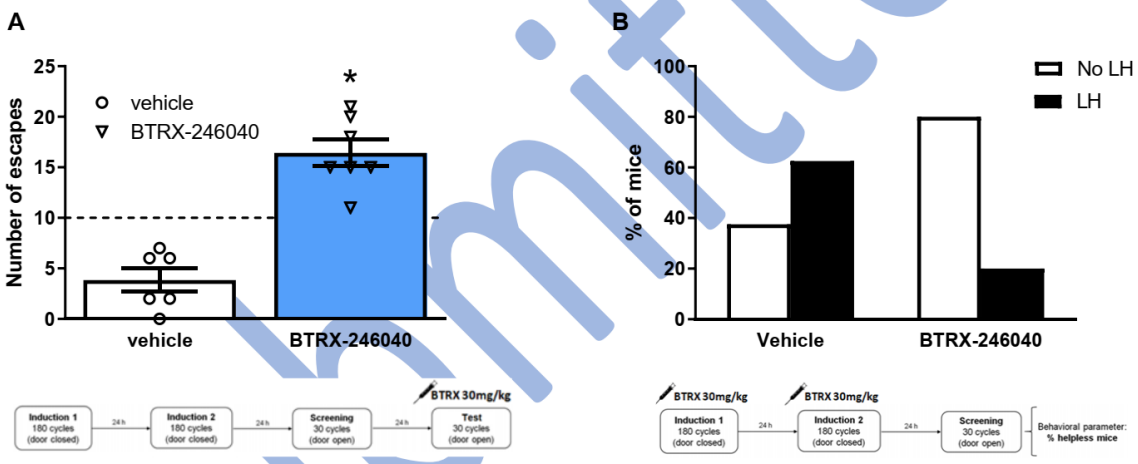


Figure 2. Learned helplessness test. Panel A: effect of BTRX-246040 30 mg/kg given i.p. 60 min before the test session on the number of escapes. Data are the mean ± sem of 6 (vehicle) or 7 (BTRX-246040) mice/group. Panel B: effect of BTRX-246040 30 mg/kg given i.p. 60 min before the induction sessions on the % of mice developing helpless behavior. 16 mice were treated with vehicle and 15 mice were treated with BTRX-246040. * $p < 0.05$ Student's t test (panel A) or chi-square test (panel B).

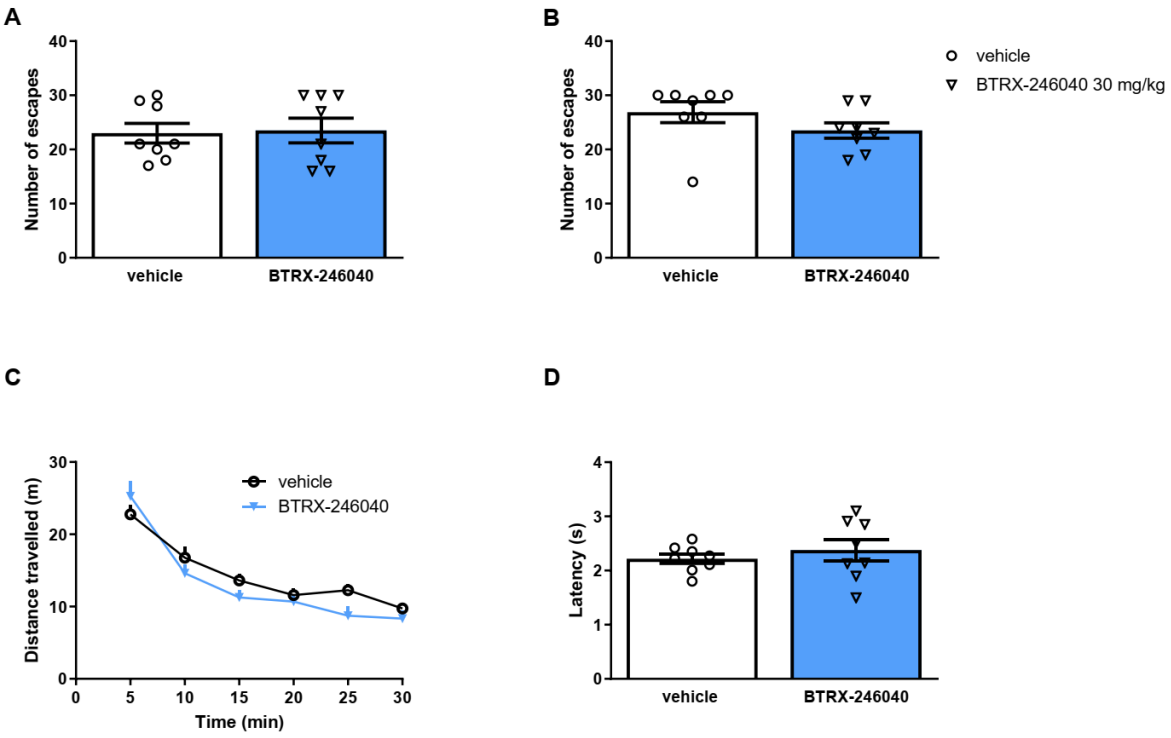


Figure 3. Escapable electric footshock task, locomotor activity and tail withdrawal assay. Panel A: effects of BTRX-246040, 30 mg/kg i.p. given 60 min before the test session (protocol 1) in the escapable electric footshock task. Data are the mean ± sem of 8 mice/group. Panel B: effects of BTRX-246040, 30 mg/kg i.p. given 60 min before the two induction sessions (protocol 2) in the escapable electric footshock task. Data are the mean ± sem of 8 mice/group. Panel C: effects of BTRX-246040, 30 mg/kg i.p. given 60 min before the test in the locomotor activity assay. Data are the mean ± sem of 8 mice/group. Panel D: effects of BTRX-246040, 30 mg/kg i.p. given 60 min before the test in the tail withdrawal assay. Data are the mean ± sem of 8 mice/group.

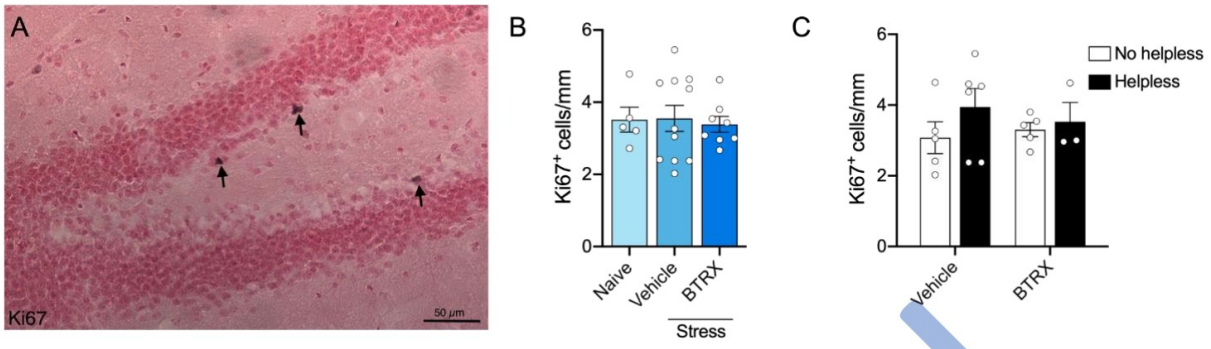


Figure 4. Effect of stress exposure and BTRX-246040 on stem cell proliferation. Panel A: representative image of Ki67+ cells (indicated by white arrows) in the dentate gyrus after immunolabelling and Fast Red counterstaining. GCL, granular cell layer. Panel B: effect of stress exposure and BTRX on Ki67+ cell density. Panel C: Ki67+ cell density in LH *vs* no LH animals. All results are presented as mean ± sem of 5-11 mice/group.

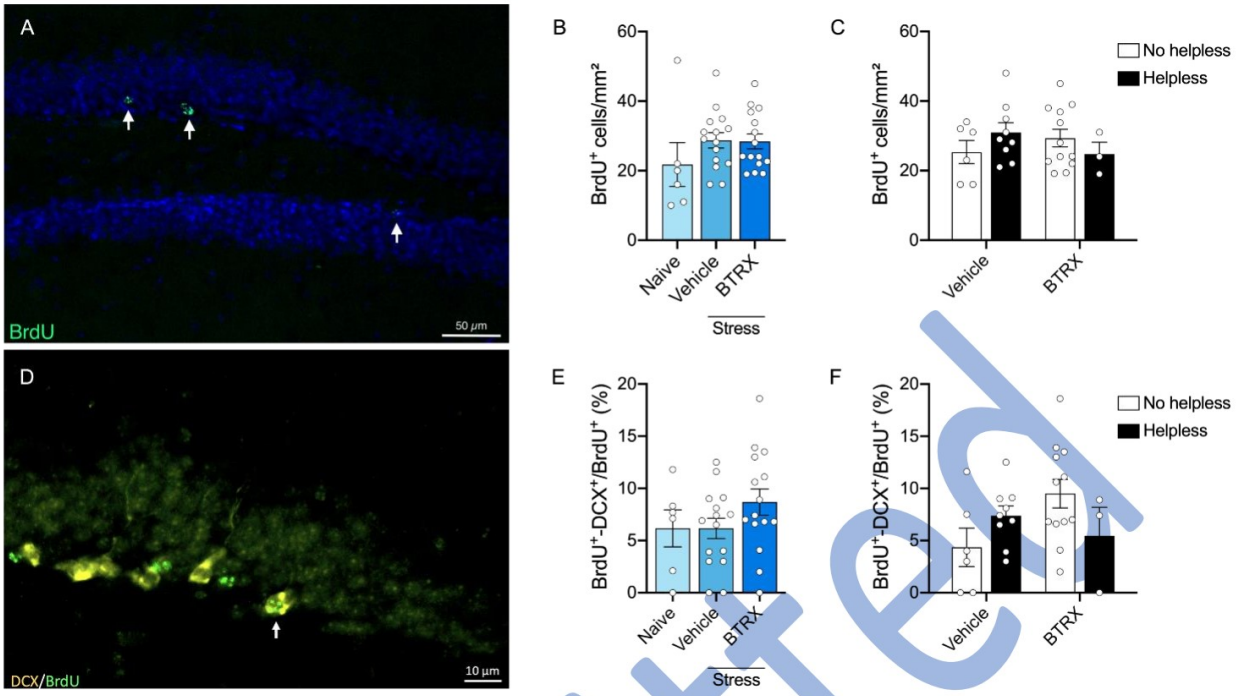


Figure 5. Effect of stress exposure and BTRX-246040 on the survival and maturation of 4-week-old neurons. Panel A: representative image of BrdU+ cells (in green, indicated by white arrows) in the dentate gyrus after immunolabelling and Hoechst staining (in blue). Panel B: effect of stress exposure and BTRX-246040 on BrdU+ cell density. Panel C: BrdU+ cell density in LH *vs* no LH animals. Panel D: representative image of BrdU+ cells (in green) and DCX+ cells (in yellow) in the dentate gyrus. The white arrow indicates a cell co-expressing the two markers. Panel E: effect of stress exposure and BTRX-246040 on the percentage of BrdU+ cells that express DCX. Panel F: Percentage of BrdU+ cells that express DCX in LH *vs* no LH animals. All results are presented as mean ± sem of 5-15 mice/group.

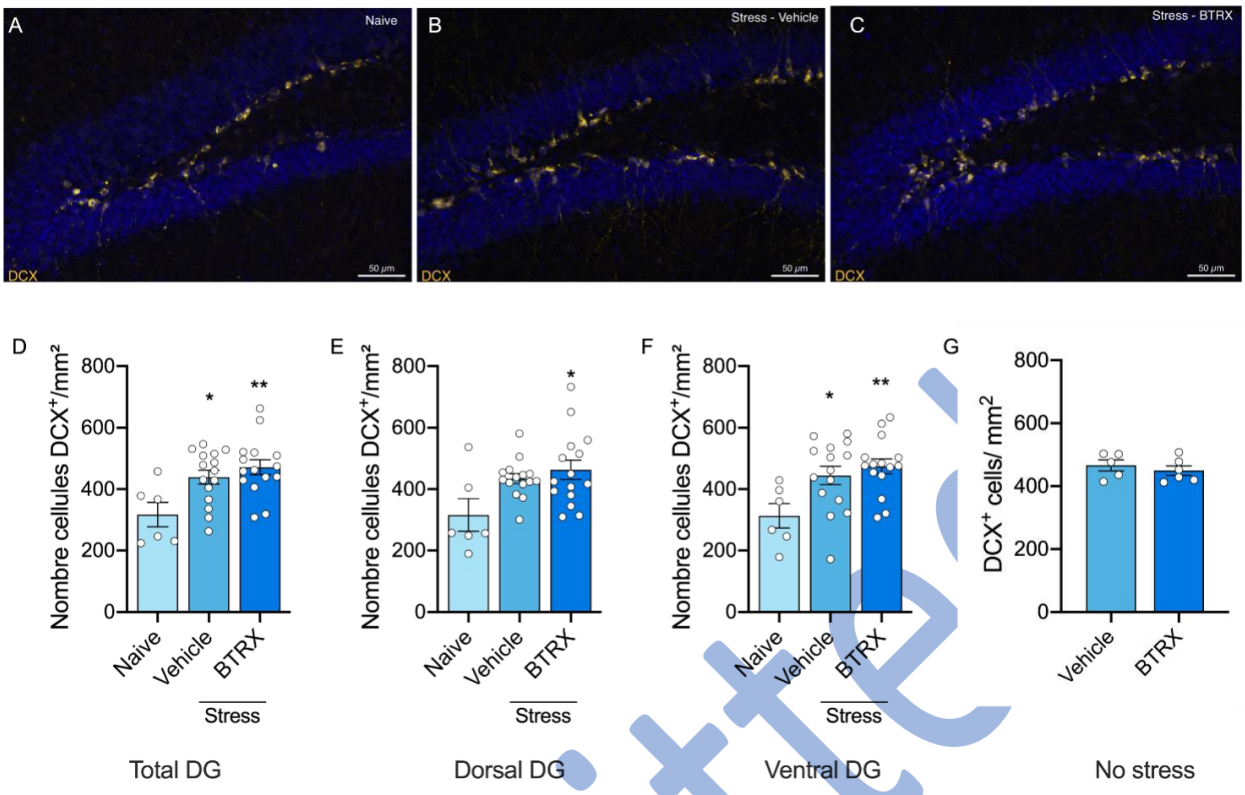


Figure 6. Effect of stress exposure and BTRX-246040 on the density of immature neurons. Panel A: representative image of DCX+ cells (in yellow) in the dentate gyrus after immunolabelling and Hoechst staining (in blue) in naive mice. Panel B: representative image of DCX+ cells in vehicle mice exposed to stress procedure. Panel C: representative image of DCX+ cells in BTRX-246040 treated mice exposed to stress procedure. Panel D: effect of stress exposure and BTRX-246040 on DCX+ cell density in the whole dentate gyrus. Panel E: effect of stress exposure and BTRX-246040 on DCX+ cell density in the dorsal dentate gyrus. Panel F: effect of stress exposure and BTRX-246040 on DCX+ cell density in the ventral dentate gyrus. Panel G: effect of BTRX-246040 in naive mice on DCX+ cell density in the whole dentate gyrus. All results are presented as mean ± sem of 5-15 mice/group. *, $p<0.05$, **, $p<0.01$ *vs* naïve, according to Tukey's post hoc test.

REFERENCES

Abraham, J., P. D. Fox, C. Condello, A. Bartolini, and S. Koh. 2012. 'Minocycline attenuates microglia activation and blocks the long-term epileptogenic effects of early-life seizures', *Neurobiol Dis*, 46: 425-30.

Agrawal, R., E. Noble, E. Tyagi, Y. Zhuang, Z. Ying, and F. Gomez-Pinilla. 2015. 'Flavonoid derivative 7,8- DHF attenuates TBI pathology via TrkB activation', *Biochim Biophys Acta*, 1852: 862-72.

Allone, C., V. Lo Buono, F. Corallo, L. R. Pisani, P. Pollicino, P. Bramanti, and S. Marino. 2017. 'Neuroimaging and cognitive functions in temporal lobe epilepsy: A review of the literature', *J Neurol Sci*, 381: 7-15.

Arellano, J. I., A. Munoz, I. Ballesteros-Yanez, R. G. Sola, and J. DeFelipe. 2004. 'Histopathology and reorganization of chandelier cells in the human epileptic sclerotic hippocampus', *Brain*, 127: 45-64.

Barde, Y. A., D. Edgar, and H. Thoenen. 1982. 'Purification of a new neurotrophic factor from mammalian brain', *EMBO J*, 1: 549-53.

Barker-Haliski, M., and H. S. White. 2015. 'Glutamatergic Mechanisms Associated with Seizures and Epilepsy', *Cold Spring Harb Perspect Med*, 5: a022863.

Bazil, C. W. 2004. 'Comprehensive care of the epilepsy patient--control, comorbidity, and cost', *Epilepsia*, 45 Suppl 6: 3-12.

Bialer, M., and H. S. White. 2010. 'Key factors in the discovery and development of new antiepileptic drugs', *Nat Rev Drug Discov*, 9: 68-82.

Binder, D. K., M. J. Routbort, and J. O. McNamara. 1999. 'Immunohistochemical evidence of seizure-induced activation of trk receptors in the mossy fiber pathway of adult rat hippocampus', *J Neurosci*, 19: 4616-26.

Blair, R. D. 2012. 'Temporal lobe epilepsy semiology', *Epilepsy Res Treat*, 2012: 751510.

Blumcke, I., M. Thom, E. Aronica, D. D. Armstrong, F. Bartolomei, A. Bernasconi, N. Bernasconi, C. G. Bien, F. Cendes, R. Coras, J. H. Cross, T. S. Jacques, P. Kahane, G. W. Mathern, H. Miyata, S. L. Moshe, B. Oz, C. Ozkara, E. Perucca, S. Sisodiya, S. Wiebe, and R. Spreafico. 2013. 'International consensus classification of hippocampal sclerosis in temporal lobe epilepsy: a Task Force report from the ILAE Commission on Diagnostic Methods', *Epilepsia*, 54: 1315-29.

Blumenfeld, H. 2014. 'What is a seizure network? Long-range network consequences of focal seizures', *Adv Exp Med Biol*, 813: 63-70.

Bollen, E., T. Vanmierlo, S. Akkerman, C. Wouters, H. M. Steinbusch, and J. Prickaerts. 2013. '7,8-Dihydroxyflavone improves memory consolidation processes in rats and mice', *Behav Brain Res*, 257: 8-12.

Bothwell, M. 2014. 'NGF, BDNF, NT3, and NT4', *Handb Exp Pharmacol*, 220: 3-15.

Brandt, C., K. Tollner, R. Klee, S. Broer, and W. Loscher. 2015. 'Effective termination of status epilepticus by rational polypharmacy in the lithium-pilocarpine model in rats: Window of opportunity to prevent epilepsy and prediction of epilepsy by biomarkers', *Neurobiol Dis*, 75: 78-90.

Bucci, C., P. Alifano, and L. Cogli. 2014. 'The role of rab proteins in neuronal cells and in the trafficking of neurotrophin receptors', *Membranes (Basel)*, 4: 642-77.

Castello, N. A., M. H. Nguyen, J. D. Tran, D. Cheng, K. N. Green, and F. M. LaFerla. 2014. '7,8-Dihydroxyflavone, a small molecule TrkB agonist, improves spatial memory and increases thin spine density in a mouse model of Alzheimer disease-like neuronal loss', *PLoS One*, 9: e91453.

Cattaneo, A., N. Cattane, V. Begni, C. M. Pariante, and M. A. Riva. 2016. 'The human BDNF gene: peripheral gene expression and protein levels as biomarkers for psychiatric disorders', *Transl Psychiatry*, 6: e958.

Cavalheiro, E. A. 1995. 'The pilocarpine model of epilepsy', *Ital J Neurol Sci*, 16: 33-7.
Cavazos, J. E., G. Golarai, and T. P. Sutula. 1991. 'Mossy fiber synaptic reorganization induced by kindling: time course of development, progression, and permanence', *J Neurosci*, 11: 2795-803.
Cavus, I., J. W. Pan, H. P. Hetherington, W. Abi-Saab, H. P. Zaveri, K. P. Vives, J. H. Krystal, S. S. Spencer, and D. D. Spencer. 2008. 'Decreased hippocampal volume on MRI is associated with increased extracellular glutamate in epilepsy patients', *Epilepsia*, 49: 1358-66.
Chao, M. V. 2003. 'Neurotrophins and their receptors: a convergence point for many signalling pathways', *Nat Rev Neurosci*, 4: 299-309.
Chen, J., K. W. Chua, C. C. Chua, H. Yu, A. Pei, B. H. Chua, R. C. Hamdy, X. Xu, and C. F. Liu. 2011. 'Antioxidant activity of 7,8-dihydroxyflavone provides neuroprotection against glutamate-induced toxicity', *Neurosci Lett*, 499: 181-5.
Chen, L., X. Gao, S. Zhao, W. Hu, and J. Chen. 2015. 'The Small-Molecule TrkB Agonist 7, 8-Dihydroxyflavone Decreases Hippocampal Newborn Neuron Death After Traumatic Brain Injury', *J Neuropathol Exp Neurol*, 74: 557-67.
Chen, L., Y. Wang, and Z. Chen. 2020. 'Adult Neurogenesis in Epileptogenesis: An Update for Preclinical Finding and Potential Clinical Translation', *Curr Neuropharmacol*, 18: 464-84.
Chen, X., H. Ye, R. Kuruvilla, N. Ramanan, K. W. Scangos, C. Zhang, N. M. Johnson, P. M. England, K. M. Shokat, and D. D. Ginty. 2005. 'A chemical-genetic approach to studying neurotrophin signaling', *Neuron*, 46: 13-21.
Cisternas, F. A., J. B. Vincent, S. W. Scherer, and P. N. Ray. 2003. 'Cloning and characterization of human CADPS and CADPS2, new members of the Ca2+-dependent activator for secretion protein family', *Genomics*, 81: 279-91.
Clarkson, C., R. M. Smeal, M. G. Hasenoehrl, J. A. White, M. E. Rubio, and K. S. Wilcox. 2020. 'Ultrastructural and functional changes at the tripartite synapse during epileptogenesis in a model of temporal lobe epilepsy', *Exp Neurol*, 326: 113196.
Clifford, D. B., J. W. Olney, A. Maniotis, R. C. Collins, and C. F. Zorumski. 1987. 'The functional anatomy and pathology of lithium-pilocarpine and high-dose pilocarpine seizures', *Neuroscience*, 23: 953-68.
Clossen, B. L., and D. S. Reddy. 2017. 'Novel therapeutic approaches for disease-modification of epileptogenesis for curing epilepsy', *Biochim Biophys Acta Mol Basis Dis*, 1863: 1519-38.
Cohen, S., R. Levi-Montalcini, and V. Hamburger. 1954. 'A Nerve Growth-Stimulating Factor Isolated from Sarcom as 37 and 180', *Proc Natl Acad Sci U S A*, 40: 1014-8.
Conner, J. M., J. C. Lauterborn, Q. Yan, C. M. Gall, and S. Varon. 1997. 'Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport', *J Neurosci*, 17: 2295-313.
Curia, G., D. Longo, G. Biagini, R. S. Jones, and M. Avoli. 2008. 'The pilocarpine model of temporal lobe epilepsy', *J Neurosci Methods*, 172: 143-57.
Curia, G., C. Lucchi, J. Vinet, F. Gualtieri, C. Marinelli, A. Torsello, L. Costantino, and G. Biagini. 2014. 'Pathophysiogenesis of mesial temporal lobe epilepsy: is prevention of damage antiepileptogenic?', *Curr Med Chem*, 21: 663-88.
De Deyn, P. P., R. D'Hooge, B. Marescau, and Y. Q. Pei. 1992. 'Chemical models of epilepsy with some reference to their applicability in the development of anticonvulsants', *Epilepsy Res*, 12: 87-110.
de Tisi, J., G. S. Bell, J. L. Peacock, A. W. McEvoy, W. F. Harkness, J. W. Sander, and J. S. Duncan. 2011. 'The long-term outcome of adult epilepsy surgery, patterns of seizure remission, and relapse: a cohort study', *Lancet*, 378: 1388-95.
Dechant, G., and H. Neumann. 2002. 'Neurotrophins', *Adv Exp Med Biol*, 513: 303-34.

Dezsi, G., F. Sieg, M. Thomas, T. J. O'Brien, M. van der Hart, and N. C. Jones. 2017. 'Disease-Modifying Effects of Neural Regeneration Peptide 2945 in the GAERS Model of Absence Epilepsy', *Neurochem Res*, 42: 2055-64.

Dossi, E., F. Vasile, and N. Rouach. 2018. 'Human astrocytes in the diseased brain', *Brain Res Bull*, 136: 139-56.

Dudek, F. E., and K. J. Staley. 2012. 'The Time Course and Circuit Mechanisms of Acquired Epileptogenesis.' in th, J. L. Noebels, M. Avoli, M. A. Rogawski, R. W. Olsen and A. V. Delgado-Escueta (eds.), *Jasper's Basic Mechanisms of the Epilepsies* (Bethesda (MD)).

Ehrlich, T., A. Reyes, B. M. Paul, V. Uttarwar, S. Hartman, K. Mathur, Y. A. Chang, M. Hegde, J. J. Shih, and C. R. McDonald. 2019. 'Beyond depression: The impact of executive functioning on quality of life in patients with temporal lobe epilepsy', *Epilepsy Res*, 149: 30-36.

Emili, M., S. Guidi, B. Uguagliati, A. Giacomini, R. Bartesaghi, and F. Stagni. 2022. 'Treatment with the flavonoid 7,8-Dihydroxyflavone: a promising strategy for a constellation of body and brain disorders', *Crit Rev Food Sci Nutr*, 62: 13-50.

Falcicchia, C., G. Paolone, D. F. Emerich, F. Lovisari, W. J. Bell, T. Fradet, L. U. Wahlberg, and M. Simonato. 2018. 'Seizure-Suppressant and Neuroprotective Effects of Encapsulated BDNF-Producing Cells in a Rat Model of Temporal Lobe Epilepsy', *Mol Ther Methods Clin Dev*, 9: 211-24.

Fiest, K. M., J. Dykeman, S. B. Patten, S. Wiebe, G. G. Kaplan, C. J. Maxwell, A. G. Bulloch, and N. Jette. 2013. 'Depression in epilepsy: a systematic review and meta-analysis', *Neurology*, 80: 590-9.

Fiest, K. M., K. M. Sauro, S. Wiebe, S. B. Patten, C. S. Kwon, J. Dykeman, T. Pringsheim, D. L. Lorenzetti, and N. Jette. 2017. 'Prevalence and incidence of epilepsy: A systematic review and meta-analysis of international studies', *Neurology*, 88: 296-303.

Fisher, R. S. 2017. 'An overview of the 2017 ILAE operational classification of seizure types', *Epilepsy Behav*, 70: 271-73.

Fisher, R. S., C. Acevedo, A. Arzimanoglou, A. Bogacz, J. H. Cross, C. E. Elger, J. Engel, Jr., L. Forsgren, J. A. French, M. Glynn, D. C. Hesdorffer, B. I. Lee, G. W. Mathern, S. L. Moshe, E. Perucca, I. E. Scheffer, T. Tomson, M. Watanabe, and S. Wiebe. 2014. 'ILAE official report: a practical clinical definition of epilepsy', *Epilepsia*, 55: 475-82.

Fisher, R. S., J. H. Cross, J. A. French, N. Higurashi, E. Hirsch, F. E. Jansen, L. Lagae, S. L. Moshe, J. Peltola, E. Roulet Perez, I. E. Scheffer, and S. M. Zuberi. 2017. 'Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology', *Epilepsia*, 58: 522-30.

Fisher, R. S., W. van Emde Boas, W. Blume, C. Elger, P. Genton, P. Lee, and J. Engel, Jr. 2005. 'Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE)', *Epilepsia*, 46: 470-2.

French, J. A. 2007. 'Refractory epilepsy: clinical overview', *Epilepsia*, 48 Suppl 1: 3-7.

Garcia-Diaz Barriga, G., A. Giralt, M. Anglada-Huguet, N. Gaja-Capdevila, J. G. Orlandi, J. Soriano, J. M. Canals, and J. Alberch. 2017. '7,8-dihydroxyflavone ameliorates cognitive and motor deficits in a Huntington's disease mouse model through specific activation of the PLCgamma1 pathway', *Hum Mol Genet*, 26: 3144-60.

Gastaut, H. 1969. 'Classification of the epilepsies. Proposal for an international classification', *Epilepsia*, 10: Suppl:14-21.

Gibbons, A. S., W. J. Jeon, E. Scarr, and B. Dean. 2016. 'Changes in Muscarinic M2 Receptor Levels in the Cortex of Subjects with Bipolar Disorder and Major

Depressive Disorder and in Rats after Treatment with Mood Stabilisers and Antidepressants', *Int J Neuropsychopharmacol*, 19.
Gill, S. J., S. Lukmanji, K. M. Fiest, S. B. Patten, S. Wiebe, and N. Jette. 2017. 'Depression screening tools in persons with epilepsy: A systematic review of validated tools', *Epilepsia*, 58: 695-705.
Goddard, G. V. 1967. 'Development of epileptic seizures through brain stimulation at low intensity', *Nature*, 214: 1020-1.
Goodman, L. J., J. Valverde, F. Lim, M. D. Geschwind, H. J. Federoff, A. I. Geller, and F. Hefti. 1996. 'Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons', *Mol Cell Neurosci*, 7: 222-38.
Gu, B., Y. Z. Huang, X. P. He, R. B. Joshi, W. Jang, and J. O. McNamara. 2015. 'A Peptide Uncoupling BDNF Receptor TrkB from Phospholipase Cgamma1 Prevents Epilepsy Induced by Status Epilepticus', *Neuron*, 88: 484-91.
Gupta, A. K., P. M. Jeavons, R. C. Hughes, and A. Covanis. 1983. 'Aura in temporal lobe epilepsy: clinical and electroencephalographic correlation', *J Neurol Neurosurg Psychiatry*, 46: 1079-83.
Hallbook, F., C. F. Ibanez, and H. Persson. 1991. 'Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary', *Neuron*, 6: 845-58.
Han, X., S. Zhu, B. Wang, L. Chen, R. Li, W. Yao, and Z. Qu. 2014. 'Antioxidant action of 7,8-dihydroxyflavone protects PC12 cells against 6-hydroxydopamine-induced cytotoxicity', *Neurochem Int*, 64: 18-23.
Harborne, J. B., and C. A. Williams. 2000. 'Advances in flavonoid research since 1992', *Phytochemistry*, 55: 481-504.
Hester, M. S., and S. C. Danzer. 2013. 'Accumulation of abnormal adult-generated hippocampal granule cells predicts seizure frequency and severity', *J Neurosci*, 33: 8926-36.
Hillert, M. H., I. Imran, M. Zimmermann, H. Lau, S. Weinfurter, and J. Klein. 2014. 'Dynamics of hippocampal acetylcholine release during lithium-pilocarpine-induced status epilepticus in rats', *J Neurochem*, 131: 42-52.
Hingray, C., A. McGonigal, I. Kotwas, and J. A. Micoulaud-Franchi. 2019. 'The Relationship Between Epilepsy and Anxiety Disorders', *Curr Psychiatry Rep*, 21: 40.
Honchar, M. P., J. W. Olney, and W. R. Sherman. 1983. 'Systemic cholinergic agents induce seizures and brain damage in lithium-treated rats', *Science*, 220: 323-5.
Huang, Y. Z., X. P. He, K. Krishnamurthy, and J. O. McNamara. 2019. 'TrkB-Shc Signaling Protects against Hippocampal Injury Following Status Epilepticus', *J Neurosci*, 39: 4624-30.
Isgor, C., C. Pare, B. McDole, P. Coombs, and K. Guthrie. 2015. 'Expansion of the dentate mossy fiber-CA3 projection in the brain-derived neurotrophic factor-enriched mouse hippocampus', *Neuroscience*, 288: 10-23.
Jang, S. W., X. Liu, M. Yepes, K. R. Shepherd, G. W. Miller, Y. Liu, W. D. Wilson, G. Xiao, B. Blanchi, Y. E. Sun, and K. Ye. 2010. 'A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone', *Proc Natl Acad Sci U S A*, 107: 2687-92.
Ji, Y., Y. Lu, F. Yang, W. Shen, T. T. Tang, L. Feng, S. Duan, and B. Lu. 2010. 'Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons', *Nat Neurosci*, 13: 302-9.
Jiang, M., Q. Peng, X. Liu, J. Jin, Z. Hou, J. Zhang, S. Mori, C. A. Ross, K. Ye, and W. Duan. 2013. 'Small-molecule TrkB receptor agonists improve motor function and extend survival in a mouse model of Huntington's disease', *Hum Mol Genet*, 22: 2462-70.

Jockusch, W. J., D. Speidel, A. Sigler, J. B. Sorensen, F. Varoqueaux, J. S. Rhee, and N. Brose. 2007. 'CAPS-1 and CAPS-2 are essential synaptic vesicle priming proteins', *Cell*, 131: 796-808.

Johnson, E. L. 2019. 'Seizures and Epilepsy', *Med Clin North Am*, 103: 309-24.

Jung, K. H., K. Chu, M. Kim, S. W. Jeong, Y. M. Song, S. T. Lee, J. Y. Kim, S. K. Lee, and J. K. Roh. 2004. 'Continuous cytosine-b-D-arabinofuranoside infusion reduces ectopic granule cells in adult rat hippocampus with attenuation of spontaneous recurrent seizures following pilocarpine-induced status epilepticus', *Eur J Neurosci*, 19: 3219-26.

Kaculini, C. M., A. J. Tate-Looney, and A. Seifi. 2021. 'The History of Epilepsy: From Ancient Mystery to Modern Misconception', *Cureus*, 13: e13953.

Kang, J. S., I. W. Choi, M. H. Han, G. Y. Kim, S. H. Hong, C. Park, H. J. Hwang, C. M. Kim, B. W. Kim, and Y. H. Choi. 2015. 'The cytoprotective effects of 7,8-dihydroxyflavone against oxidative stress are mediated by the upregulation of Nrf2-dependent HO-1 expression through the activation of the PI3K/Akt and ERK pathways in C2C12 myoblasts', *Int J Mol Med*, 36: 501-10.

Kanner, A. M. 2011. 'Anxiety disorders in epilepsy: the forgotten psychiatric comorbidity', *Epilepsy Curr*, 11: 90-1.

Keezer, M. R., S. M. Sisodiya, and J. W. Sander. 2016. 'Comorbidities of epilepsy: current concepts and future perspectives', *Lancet Neurol*, 15: 106-15.

Klein, S., J. P. Bankstahl, W. Loscher, and M. Bankstahl. 2015. 'Sucrose consumption test reveals pharmacoresistant depression-associated behavior in two mouse models of temporal lobe epilepsy', *Exp Neurol*, 263: 263-71.

Kuzmanovski, I., E. Cvetkovska, M. Babunovska, G. Kiteva Trencevska, B. Kuzmanovska, B. Boshkovski, and R. Isjanovska. 2016. 'Seizure outcome following medical treatment of mesial temporal lobe epilepsy: Clinical phenotypes and prognostic factors', *Clin Neurol Neurosurg*, 144: 91-5.

Kwan, P., A. Arzimanoglou, A. T. Berg, M. J. Brodie, W. Allen Hauser, G. Mathern, S. L. Moshe, E. Perucca, S. Wiebe, and J. French. 2010. 'Definition of drug resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies', *Epilepsia*, 51: 1069-77.

Kwan, P., and M. J. Brodie. 2000. 'Early identification of refractory epilepsy', *N Engl J Med*, 342: 314-9.

Lanteaume, L., S. Khalfa, J. Regis, P. Marquis, P. Chauvel, and F. Bartolomei. 2007. 'Emotion induction after direct intracerebral stimulations of human amygdala', *Cereb Cortex*, 17: 1307-13.

Lee, R., P. Kermani, K. K. Teng, and B. L. Hempstead. 2001. 'Regulation of cell survival by secreted proneurotrophins', *Science*, 294: 1945-8.

Liu, C., C. B. Chan, and K. Ye. 2016. '7,8-dihydroxyflavone, a small molecular TrkB agonist, is useful for treating various BDNF-implicated human disorders', *Transl Neurodegener*, 5: 2.

Liu, G., B. Gu, X. P. He, R. B. Joshi, H. D. Wackerle, R. M. Rodriguiz, W. C. Wetsel, and J. O. McNamara. 2013. 'Transient inhibition of TrkB kinase after status epilepticus prevents development of temporal lobe epilepsy', *Neuron*, 79: 31-8.

Liu, Q. R., L. Lu, X. G. Zhu, J. P. Gong, Y. Shaham, and G. R. Uhl. 2006. 'Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine', *Brain Res*, 1067: 1-12.

Liu, X., C. B. Chan, S. W. Jang, S. Pradoldej, J. Huang, K. He, L. H. Phun, S. France, G. Xiao, Y. Jia, H. R. Luo, and K. Ye. 2010. 'A synthetic 7,8-dihydroxyflavone derivative promotes neurogenesis and exhibits potent antidepressant effect', *J Med Chem*, 53: 8274-86.

Liu, X., O. Obianyo, C. B. Chan, J. Huang, S. Xue, J. J. Yang, F. Zeng, M. Goodman, and K. Ye. 2014. 'Biochemical and biophysical investigation of the brain-derived neurotrophic factor mimetic 7,8-dihydroxyflavone in the binding and activation of the TrkB receptor', *J Biol Chem*, 289: 27571-84.

Loscher, W. 2011. 'Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs', *Seizure*, 20: 359-68.

Loscher, W., H. Potschka, S. M. Sisodiya, and A. Vezzani. 2020. 'Drug Resistance in Epilepsy: Clinical Impact, Potential Mechanisms, and New Innovative Treatment Options', *Pharmacol Rev*, 72: 606-38.

Lum, G. R., C. A. Olson, and E. Y. Hsiao. 2020. 'Emerging roles for the intestinal microbiome in epilepsy', *Neurobiol Dis*, 135: 104576.

Magiorkinis, E., K. Sidiropoulou, and A. Diamantis. 2010. 'Hallmarks in the history of epilepsy: epilepsy in antiquity', *Epilepsy Behav*, 17: 103-8.

Marongiu, D., B. Imbrosci, and T. Mittmann. 2013. 'Modulatory effects of the novel TrkB receptor agonist 7,8-dihydroxyflavone on synaptic transmission and intrinsic neuronal excitability in mouse visual cortex in vitro', *Eur J Pharmacol*, 709: 64-71.

Marosi, K., and M. P. Mattson. 2014. 'BDNF mediates adaptive brain and body responses to energetic challenges', *Trends Endocrinol Metab*, 25: 89-98.

Mazarati, A. M., E. Pineda, D. Shin, D. Tio, A. N. Taylor, and R. Sankar. 2010. 'Comorbidity between epilepsy and depression: role of hippocampal interleukin-1beta', *Neurobiol Dis*, 37: 461-7.

Mazarati, A., P. Siddarth, R. A. Baldwin, D. Shin, R. Caplan, and R. Sankar. 2008. 'Depression after status epilepticus: behavioural and biochemical deficits and effects of fluoxetine', *Brain*, 131: 2071-83.

McRae, P. A., and B. E. Porter. 2012. 'The perineuronal net component of the extracellular matrix in plasticity and epilepsy', *Neurochem Int*, 61: 963-72.

Meeker, R. B., and K. S. Williams. 2015. 'The p75 neurotrophin receptor: at the crossroad of neural repair and death', *Neural Regen Res*, 10: 721-5.

Mesraoua, B., D. Deleu, A. H. Hassan, M. Gayane, A. Lubna, M. A. Ali, T. Tomson, B. A. Khalil, J. H. Cross, and A. A. Asadi-Pooya. 2020. 'Dramatic outcomes in epilepsy: depression, suicide, injuries, and mortality', *Curr Med Res Opin*, 36: 1473-80.

Messenheimer, J. A., E. W. Harris, and O. Steward. 1979. 'Sprouting fibers gain access to circuitry transsynaptically altered by kindling', *Exp Neurol*, 64: 469-81.

Naegele, J. R. 2007. 'Neuroprotective strategies to avert seizure-induced neurodegeneration in epilepsy', *Epilepsia*, 48 Suppl 2: 107-17.

Nawa, H., J. Carnahan, and C. Gall. 1995. 'BDNF protein measured by a novel enzyme immunoassay in normal brain and after seizure: partial disagreement with mRNA levels', *Eur J Neurosci*, 7: 1527-35.

Nelson, M. T., S. M. Todorovic, and E. Perez-Reyes. 2006. 'The role of T-type calcium channels in epilepsy and pain', *Curr Pharm Des*, 12: 2189-97.

Nirwan, N., P. Vyas, and D. Vohora. 2018. 'Animal models of status epilepticus and temporal lobe epilepsy: a narrative review', *Rev Neurosci*, 29: 757-70.

Paradiso, B., P. Marconi, S. Zucchini, E. Berto, A. Binaschi, A. Bozac, A. Buzzi, M. Mazzuferi, E. Magri, G. Navarro Mora, D. Rodi, T. Su, I. Volpi, L. Zanetti, A. Marzola, R. Manservigi, P. F. Fabene, and M. Simonato. 2009. 'Localized delivery of fibroblast growth factor-2 and brain-derived neurotrophic factor reduces spontaneous seizures in an epilepsy model', *Proc Natl Acad Sci U S A*, 106: 7191-6.

Parent, J. M., T. W. Yu, R. T. Leibowitz, D. H. Geschwind, R. S. Sloviter, and D. H. Lowenstein. 1997. 'Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus', *J Neurosci*, 17: 3727-38.

Park, K. M., K. J. Shin, S. Y. Ha, J. Park, S. E. Kim, and S. E. Kim. 2014. 'Response to antiepileptic drugs in partial epilepsy with structural lesions on MRI', *Clin Neurol Neurosurg*, 123: 64-8.
Pietta, P. G. 2000. 'Flavonoids as antioxidants', *J Nat Prod*, 63: 1035-42.
Pitkanen, A. 2010. 'Therapeutic approaches to epileptogenesis--hope on the horizon', *Epilepsia*, 51 Suppl 3: 2-17.
Pitkanen, A., and K. Lukasiuk. 2011. 'Mechanisms of epileptogenesis and potential treatment targets', *Lancet Neurol*, 10: 173-86.
Pitkanen, A., K. Lukasiuk, F. E. Dudek, and K. J. Staley. 2015. 'Epileptogenesis', *Cold Spring Harb Perspect Med*, 5.
Pitkanen, A., and T. P. Sutula. 2002. 'Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy', *Lancet Neurol*, 1: 173-81.
Pressler, R. M., M. R. Cilio, E. M. Mizrahi, S. L. Moshe, M. L. Nunes, P. Plouin, S. Vanhatalo, E. Yozawitz, L. S. de Vries, K. Puthenveettil Vinayan, C. C. Triki, J. M. Wilmshurst, H. Yamamoto, and S. M. Zuberi. 2021. 'The ILAE classification of seizures and the epilepsies: Modification for seizures in the neonate. Position paper by the ILAE Task Force on Neonatal Seizures', *Epilepsia*, 62: 615-28.
Pruunsild, P., A. Kazantseva, T. Aid, K. Palm, and T. Timmusk. 2007. 'Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters', *Genomics*, 90: 397-406.
Pucilowski, O., D. H. Overstreet, A. H. Rezvani, and D. S. Janowsky. 1993. 'Chronic mild stress-induced anhedonia: greater effect in a genetic rat model of depression', *Physiol Behav*, 54: 1215-20.
Reibel, S., Y. Larmet, B. T. Le, J. Carnahan, C. Marescaux, and A. Depaulis. 2000. 'Brain-derived neurotrophic factor delays hippocampal kindling in the rat', *Neuroscience*, 100: 777-88.
Reichardt, L. F. 2006. 'Neurotrophin-regulated signalling pathways', *Philos Trans R Soc Lond B Biol Sci*, 361: 1545-64.
Represa, A., G. Le Gall La Salle, and Y. Ben-Ari. 1989. 'Hippocampal plasticity in the kindling model of epilepsy in rats', *Neurosci Lett*, 99: 345-50.
Rosenow, F., K. M. Klein, and H. M. Hamer. 2015. 'Non-invasive EEG evaluation in epilepsy diagnosis', *Expert Rev Neurother*, 15: 425-44.
Rosenthal, A., D. V. Goeddel, T. Nguyen, M. Lewis, A. Shih, G. R. Laramee, K. Nikolics, and J. W. Winslow. 1990. 'Primary structure and biological activity of a novel human neurotrophic factor', *Neuron*, 4: 767-73.
Rubin, L. L., and J. M. Staddon. 1999. 'The cell biology of the blood-brain barrier', *Annu Rev Neurosci*, 22: 11-28.
Rudge, J. S., P. E. Mather, E. M. Pasnikowski, N. Cai, T. Corcoran, A. Acheson, K. Anderson, R. M. Lindsay, and S. J. Wiegand. 1998. 'Endogenous BDNF protein is increased in adult rat hippocampus after a kainic acid induced excitotoxic insult but exogenous BDNF is not neuroprotective', *Exp Neurol*, 149: 398-410.
Sadakata, T., A. Mizoguchi, Y. Sato, R. Katoh-Semba, M. Fukuda, K. Mikoshiba, and T. Furuichi. 2004. 'The secretory granule-associated protein CAPS2 regulates neurotrophin release and cell survival', *J Neurosci*, 24: 43-52.
Sadakata, T., Y. Shinoda, M. Oka, Y. Sekine, Y. Sato, C. Saruta, H. Miwa, M. Tanaka, S. Itohara, and T. Furuichi. 2012. 'Reduced axonal localization of a Caps2 splice variant impairs axonal release of BDNF and causes autistic-like behavior in mice', *Proc Natl Acad Sci U S A*, 109: 21104-9.
Sajadian, A., S. Esteghamat, F. Karimzadeh, A. Eshaghabadi, F. Sieg, E. J. Speckmann, S. Meuth, T. Seidenbecher, T. Budde, and A. Gorji. 2015. 'Anticonvulsant effect of neural regeneration peptide 2945 on pentylenetetrazol-induced seizures in rats', *Neuropeptides*, 49: 15-23.

Sakas, D. E., S. Korfias, C. L. Nicholson, I. G. Panourias, N. Georgakoulias, S. Gatzonis, and A. Jenkins. 2007. 'Vagus nerve stimulation for intractable epilepsy: outcome in two series combining 90 patients', *Acta Neurochir Suppl*, 97: 287-91.

Savic, I., P. Lindstrom, B. Gulyas, C. Halldin, B. Andree, and L. Farde. 2004. 'Limbic reductions of 5-HT1A receptor binding in human temporal lobe epilepsy', *Neurology*, 62: 1343-51.

Scharfman, H. E. 1997. 'Hyperexcitability in combined entorhinal/hippocampal slices of adult rat after exposure to brain-derived neurotrophic factor', *J Neurophysiol*, 78: 1082-95.

Scharfman, H. E., J. H. Goodman, and A. L. Sollas. 1999. 'Actions of brain-derived neurotrophic factor in slices from rats with spontaneous seizures and mossy fiber sprouting in the dentate gyrus', *J Neurosci*, 19: 5619-31.

Schipper, S., M. W. Aalbers, K. Rijkers, A. Swijsen, J. M. Rigo, G. Hoogland, and J. S. Vles. 2016. 'Tonic GABAA Receptors as Potential Target for the Treatment of Temporal Lobe Epilepsy', *Mol Neurobiol*, 53: 5252-65.

Schultz, C., and M. Engelhardt. 2014. 'Anatomy of the hippocampal formation', *Front Neurol Neurosci*, 34: 6-17.

Segal, M. 1988. 'Synaptic activation of a cholinergic receptor in rat hippocampus', *Brain Res*, 452: 79-86.

Segal, R. A. 2003. 'Selectivity in neurotrophin signaling: theme and variations', *Annu Rev Neurosci*, 26: 299-330.

Sharma, A. K., R. Y. Reams, W. H. Jordan, M. A. Miller, H. L. Thacker, and P. W. Snyder. 2007. 'Mesial temporal lobe epilepsy: pathogenesis, induced rodent models and lesions', *Toxicol Pathol*, 35: 984-99.

Shinoda, Y., T. Sadakata, K. Nakao, R. Katoh-Semba, E. Kinameri, A. Furuya, Y. Yanagawa, H. Hirase, and T. Furuichi. 2011. 'Calcium-dependent activator protein for secretion 2 (CAPS2) promotes BDNF secretion and is critical for the development of GABAergic interneuron network', *Proc Natl Acad Sci U S A*, 108: 373-8.

Simonato, M. 2018. 'Neurotrophic factors and status epilepticus', *Epilepsia*, 59 Suppl 2: 87-91.

Simonato, M., R. Molteni, G. Bregola, A. Muzzolini, M. Piffanelli, L. Beani, G. Racagni, and M. Riva. 1998. 'Different patterns of induction of FGF-2, FGF-1 and BDNF mRNAs during kindling epileptogenesis in the rat', *Eur J Neurosci*, 10: 955-63.

Simonato, M., E. Tongiorgi, and M. Kokaia. 2006. 'Angels and demons: neurotrophic factors and epilepsy', *Trends Pharmacol Sci*, 27: 631-8.

Stafstrom, C. E., and L. Carmant. 2015. 'Seizures and epilepsy: an overview for neuroscientists', *Cold Spring Harb Perspect Med*, 5.

Stafstrom, C. E., and T. P. Sutula. 2005. 'Models of epilepsy in the developing and adult brain: implications for neuroprotection', *Epilepsy Behav*, 7 Suppl 3: S18-24.

Stahl, S. M. 2003. 'Brainstorms: symptoms and circuits, part 2: anxiety disorders', *J Clin Psychiatry*, 64: 1408-9.

Sutula, T. 2002. 'Seizure-Induced Axonal Sprouting: Assessing Connections Between Injury, Local Circuits, and Epileptogenesis', *Epilepsy Curr*, 2: 86-91.

Tanaka, T., H. Saito, and N. Matsuki. 1997. 'Inhibition of GABAA synaptic responses by brain-derived neurotrophic factor (BDNF) in rat hippocampus', *J Neurosci*, 17: 2959-66.

Thoenen, H., and M. Sendtner. 2002. 'Neurotrophins: from enthusiastic expectations through sobering experiences to rational therapeutic approaches', *Nat Neurosci*, 5 Suppl: 1046-50.

Thom, M. 2014. 'Review: Hippocampal sclerosis in epilepsy: a neuropathology review', *Neuropathol Appl Neurobiol*, 40: 520-43.

Todman, D. 2008. 'Epilepsy in the Graeco-Roman world: Hippocratic medicine and Asklepian temple medicine compared', *J Hist Neurosci*, 17: 435-41.
Turski, W. A., E. A. Cavalheiro, M. Schwarz, S. J. Czuczwar, Z. Kleinrok, and L. Turski. 1983. 'Limbic seizures produced by pilocarpine in rats: behavioural, electroencephalographic and neuropathological study', *Behav Brain Res*, 9: 315-35.
Uemori, T., K. Toda, and T. Seki. 2017. 'Seizure severity-dependent selective vulnerability of the granule cell layer and aberrant neurogenesis in the rat hippocampus', *Hippocampus*, 27: 1054-68.
Ulamek-Koziol, M., S. J. Czuczwar, S. Januszewski, and R. Pluta. 2019. 'Ketogenic Diet and Epilepsy', *Nutrients*, 11.
Valente, K. D., and G. Busatto Filho. 2013. 'Depression and temporal lobe epilepsy represent an epiphenomenon sharing similar neural networks: clinical and brain structural evidences', *Arq Neuropsiquiatr*, 71: 183-90.
Vezzani, A., E. Aronica, A. Mazarati, and Q. J. Pittman. 2013. 'Epilepsy and brain inflammation', *Exp Neurol*, 244: 11-21.
Vezzani, A., M. Maroso, S. Balosso, M. A. Sanchez, and T. Bartfai. 2011. 'IL-1 receptor/Toll-like receptor signaling in infection, inflammation, stress and neurodegeneration couples hyperexcitability and seizures', *Brain Behav Immun*, 25: 1281-9.
Vieira, E. L., G. N. de Oliveira, J. M. Lessa, A. P. Goncalves, J. W. Sander, F. Cendes, and A. L. Teixeira. 2015. 'Interleukin-1beta plasma levels are associated with depression in temporal lobe epilepsy', *Epilepsy Behav*, 53: 131-4.
Wada, A., H. Yokoo, T. Yanagita, and H. Kobayashi. 2005. 'Lithium: potential therapeutics against acute brain injuries and chronic neurodegenerative diseases', *J Pharmacol Sci*, 99: 307-21.
Wang, N., X. Mi, B. Gao, J. Gu, W. Wang, Y. Zhang, and X. Wang. 2015. 'Minocycline inhibits brain inflammation and attenuates spontaneous recurrent seizures following pilocarpine-induced status epilepticus', *Neuroscience*, 287: 144-56.
Wheless, J. W., A. J. Gienapp, and P. Ryvlin. 2018. 'Vagus nerve stimulation (VNS) therapy update', *Epilepsy Behav*, 88S: 2-10.
Wiera, G., and J. W. Mozrzymas. 2015. 'Extracellular proteolysis in structural and functional plasticity of mossy fiber synapses in hippocampus', *Front Cell Neurosci*, 9: 427.
Wilcox, K. S., J. M. Gee, M. B. Gibbons, P. Tvrdik, and J. A. White. 2015. 'Altered structure and function of astrocytes following status epilepticus', *Epilepsy Behav*, 49: 17-9.
Wu, C. H., T. H. Hung, C. C. Chen, C. H. Ke, C. Y. Lee, P. Y. Wang, and S. F. Chen. 2014. 'Post-injury treatment with 7,8-dihydroxyflavone, a TrkB receptor agonist, protects against experimental traumatic brain injury via PI3K/Akt signaling', *PLoS One*, 9: e113397.
Wurzelmann, M., J. Romeika, and D. Sun. 2017. 'Therapeutic potential of brain-derived neurotrophic factor (BDNF) and a small molecular mimics of BDNF for traumatic brain injury', *Neural Regen Res*, 12: 7-12.
Yamada, M. K., K. Nakanishi, S. Ohba, T. Nakamura, Y. Ikegaya, N. Nishiyama, and N. Matsuki. 2002. 'Brain-derived neurotrophic factor promotes the maturation of GABAergic mechanisms in cultured hippocampal neurons', *J Neurosci*, 22: 7580-5.
Yoshii, A., and M. Constantine-Paton. 2010. 'Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease', *Dev Neurobiol*, 70: 304-22.
Zeng, Y., F. Lv, L. Li, H. Yu, M. Dong, and Q. Fu. 2012. '7,8-dihydroxyflavone rescues spatial memory and synaptic plasticity in cognitively impaired aged rats', *J Neurochem*, 122: 800-11.
Zhang, Z., X. Liu, J. P. Schroeder, C. B. Chan, M. Song, S. P. Yu, D. Weinshenker, and K. Ye. 2014. '7,8-dihydroxyflavone prevents synaptic loss and memory deficits in a mouse model of Alzheimer's disease', *Neuropsychopharmacology*, 39: 638-50.

Zhao, F., H. Kang, L. You, P. Rastogi, D. Venkatesh, and M. Chandra. 2014. 'Neuropsychological deficits in temporal lobe epilepsy: A comprehensive review', *Ann Indian Acad Neurol*, 17: 374-82.
Zuliani, V., M. Fantini, and M. Rivara. 2012. 'Sodium channel blockers as therapeutic target for treating epilepsy: recent updates', *Curr Top Med Chem*, 12: 962-70.