Università degli Studi di Ferrara

DOCTORAL COURSE IN

"Translational Neurosciences and Neurotechnologies"

CYCLE XXXV

COORDINATOR Prof. Luciano Fadiga

Exploring novel therapeutic strategies for the treatment of epilepsy-associated neuroinflammation

Scientific/disciplinary sector (SDS): BIO/14

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Abstract (English)

Mesial temporal lobe epilepsy is one of the most common forms of epilepsy which is often accompained by several comorbidities that can significantly impact the quality of life. This often originates from a precipitating event followed by epileptogenesis, in which multiple pathophysiological phenomena occur, including neuroinflammation. Seizures themselves can induce neuroinflammation and spontaneous recurrent seizures (SRSs) can perpetuate chronic neuroinflammation. Moreover, a pre-existing neuroinflammation enhances the pre-disposition to seizures, linked with alterations in the neuronal excitability as well as increased neuropathology induced by seizures. Both astrocytes and microglial cells exacerbate the inflammatory state and create a very unfavourable environment for neuronal cells, resulting in loss of synapses and neuronal death.

During my PhD I aimed to investigate possible therapeutic strategies for the treatment of epilepsy-associated neuroinflammation:

- Exploring the hypothesis that 7,8 dihydroxyflavone (7,8- DHF), an agonist of the BDNF-trkB receptor that may also exert anti-inflammatory effects, can impact epileptogenesis. To avail this goal, the lithium-pilocarpine rat model was used. The effect of this compound on astrogliosis was evaluated by immunohistochemical analysis of ex-vivo brain samples using the GFAP antibody. We found that low 7,8- DHF doses (5 mg/kg) display a clear (even if barely significant) tendency to reduce the neuroinflammation.

- Testing if a combination of neurotrophic factors (namely FGF-2 and BDNF) can attenuate parameters of neuroinflammation, including microcytosis, astrocytosis, and the expression of IL-1β. We aimed to test if the attenuation of the neuroinflammation obtained in vivo with BDNF and FGF-2 (Bovolenta et al., 2010) was direct or secondary to other effects, for example the reduction in spontaneous recurrent seizures severity and frequency. For this, we have used an in vitro model of neuroinflammation induced by lipopolysaccharide (LPS, 100 ng/ml) in a mouse primary mixed glial culture. The release of cytokines and NO was analysed. Our results suggested that the anti-inflammatory effect of BDNF and FGF-2 in vivo in an epilepsy model was indirect and likely due to a reduction in seizure frequency and severity.

Abstract (Italian)

L'epilessia del lobo temporale mesiale (mesial temporal lobe epilepsy, mTLE) è una delle forme più comuni di epilessia ed è spesso accompagnata da diverse comorbidità che possono avere un impatto significativo sulla qualità della vita. Spesso la mTLE ha origine da un evento traumatico seguito poi da epilettogenesi, processo durante il quale si verificano molteplici fenomeni fisiopatologici, inclusa la neuroinfiammazione. Le stesse crisi epilettiche possono indurre neuroinfiammazione e le crisi ricorrenti spontanee (sponatneous recurrent seizures, SRSs) possono causare una neuroinfiammazione cronica. Una neuroinfiammazione preesistente può inoltre anche aumentare la predisposizione alle crisi, con alterazioni dell'eccitabilità neuronale e peggioramento della neuropatologia. In queste condizioni, sia gli astrociti che le cellule microgliali aumentano lo stato infiammatorio creando un ambiente sfavorevole per le cellule neuronali, con conseguente perdita di contatti sinaptici e morte neuronale.

Durante il mio dottorato di ricerca ho voluto indagare le possibili strategie terapeutiche per il trattamento della neuroinfiammazione associata all'epilessia:

- Esplorare l'ipotesi che il 7,8 diidrossiflavone (7,8-DHF), un agonista del recettore BDNF-trkB con effetti antinfiammatori, possa influenzare l'epilettogenesi. Per raggiungere questo obiettivo, è stato utilizzato il modello della litio-pilocarpina. L'effetto del 7,8-DHF sull'astrogliosi è stato valutato mediante analisi immunoistochimica di campioni di cervello ex vivo utilizzando l'anticorpo GFAP. Abbiamo scoperto che basse dosi di 7,8-DHF (5 mg/kg) mostrano una chiara (anche se poco significativa) tendenza a ridurre la neuroinfiammazione.
- Testare se una combinazione di fattori neurotrofici (FGF-2 e BDNF) può attenuare la neuroinfiammazione, nello specifico la microcitosi, l'astrocitosi e l'espressione di IL-1β. Abbiamo mirato a verificare se l'attenuazione della neuroinfiammazione ottenuta in vivo con BDNF e FGF-2 (Bovolenta et al., 2010) fosse diretta o secondaria ad altri effetti, ad esempio la riduzione della gravità e della frequenza delle crisi ricorrenti spontanee. Per questo obiettivo, abbiamo utilizzato un modello in vitro di neuroinfiammazione indotta da lipopolisaccaride (LPS, 100 ng/ml) in una coltura gliale mista primaria di topo. È stato analizzato il rilascio di citochine e NO. I nostri risultati suggeriscono che l'effetto antinfiammatorio di BDNF e FGF-2 in vivo in un modello di epilessia sia indiretto e probabilmente dovuto a una riduzione della frequenza e della gravità delle crisi.

TABLE OF CONTENT

ABBREVIATIONS

CNS = central nervous system

TBI = traumatic brain injury

MS = multiple sclerosis

ROS = reactive oxygen species

TNF = tumor necrosis factor

NDDs = neurodegenerative diseases

IL-6 = interleukin-6

ERK = extracellular signal-regulated kinase

JNK = c-Jun-N-terminal-kinase

MAPK = p38-mitogen activated-protein kinase

FADD = Fas-associated death-domain

ALS = amyotrophic lateral sclerosis

IFNγ = interferons gamma

mTOR = mammalian target of rapamycin

COX-2 = cyclooxygenase-2

MyD88 = myeloid differentiating factor 88

NTFs = neurotropic factors

BDNF = brain-derived neurotrophic factor

NT-3 = neurotrophins-3

PLCγ = phospholipase C

PKC = protein kinase C

IP3 = inositol 1,4,5-triphosphate

TLE = temporal lobe epilepsy

TgFGF2 = transgenic FGF-2

BBB = blood brain barrier

CVD = cerebrovascular disease

AD = Alzheimer's disease

IL-1β = interleukin-1β

iNOS = inducible nitric oxide synthase

IL-1RA = interleukin-1 receptor antagonist

RNS = reactive nitrogen species

TNFR = TNF receptor

IKK = IκB kinase

GP130 = glycoprotein-130

ALD = X-linked adrenoleukodystrophy

NFκB = nuclear factor kappa B

TLR4 = toll-like receptor 4

mPGES-1 = prostaglandin E synthase-1

LPS = lipopolysaccharide

P2Y = purinergic receptors

HD = Huntington's disease

NGF = nerve growth factor

CPE = carboxy peptidase E

PIP2 = phosphatidylinositol 4,5-bisphosphate

DAG = diacylglycerol

FGF-2 = fibroblast growth factor-2

i.c.v. = intracerebroventricular

CA = cornu ammonis

WT = wild type

HS = hippocampal sclerosis

nTLE = neocortical temporal lobe epilepsy

HIC = high-income countries

VEGF = vascular endothelial growth factor

SRSs = spontaneous recurrent seizures

PRRs = pattern recognition receptors

mtDNA = mutant mitochondrial DNA

BD = PTX BD4-3

STING = stimulator of interferon genes

PAMPs = pathogen-associated molecular patterns

DAMPs = damage-associated molecular patterns

MPTP = mitochondrial permeability transition pore

RLRs = retinoic acid inducible gene-I-like receptors

GABA = gamma amino butyric acid

SE = status epilepticus

LMIC = low/middle-income countries

NMDARs = N-methyl-d-aspartate receptors

TGF-β1 = transforming growth factor- beta 1

IL-1R1 = IL-1 receptor-1

NLRs = NOD-like receptors

cGAS = cyclic GMP-AMP synthase

7,8 DHF = 7,8 Dihydroxyflavone

LTG = Lamotrigine

CHAPTER 1: EPILEPSY

1.1 Definition and epidemiology

Epilepsy is 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. According to International League Against Epilepsy (ILAE), an epileptic seizure is "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Beghi, 2020). Epilepsy can be diagnosed by: (1) at least 2 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 after 2 unprovoked seizures (at least 60%) (Falco-Walter et al., 2018). Epilepsy is considered "resolved" under the following circumstances: (1) in a patient with an age-dependent epilepsy syndrome who is older than the age in which this syndrome is active, or (2) in a patient who has been seizure free for ≥10 years and has been off all anti-seizure medications for ≥5 years (Fisher et al., 2014).

The prevalence of epilepsy differs significantly among countries depending on the local distribution of risk and etiologic factors, the number of seizures at diagnosis, and if considering only active epilepsy (active prevalence) or including also cases in remission (lifetime prevalence). In a systematic review and meta-analysis of incidence studies, the pooled incidence rate of epilepsy was 61.4 per 100,000 person-years (95% CI 50.7–74.4). The incidence was higher in low/middle-income countries (LMIC) than in high-income countries (HIC): 139.0 (95% CI 69.4–278.2) vs. 48.9 (95% CI 39.0–61.1) (Beghi, 2020).

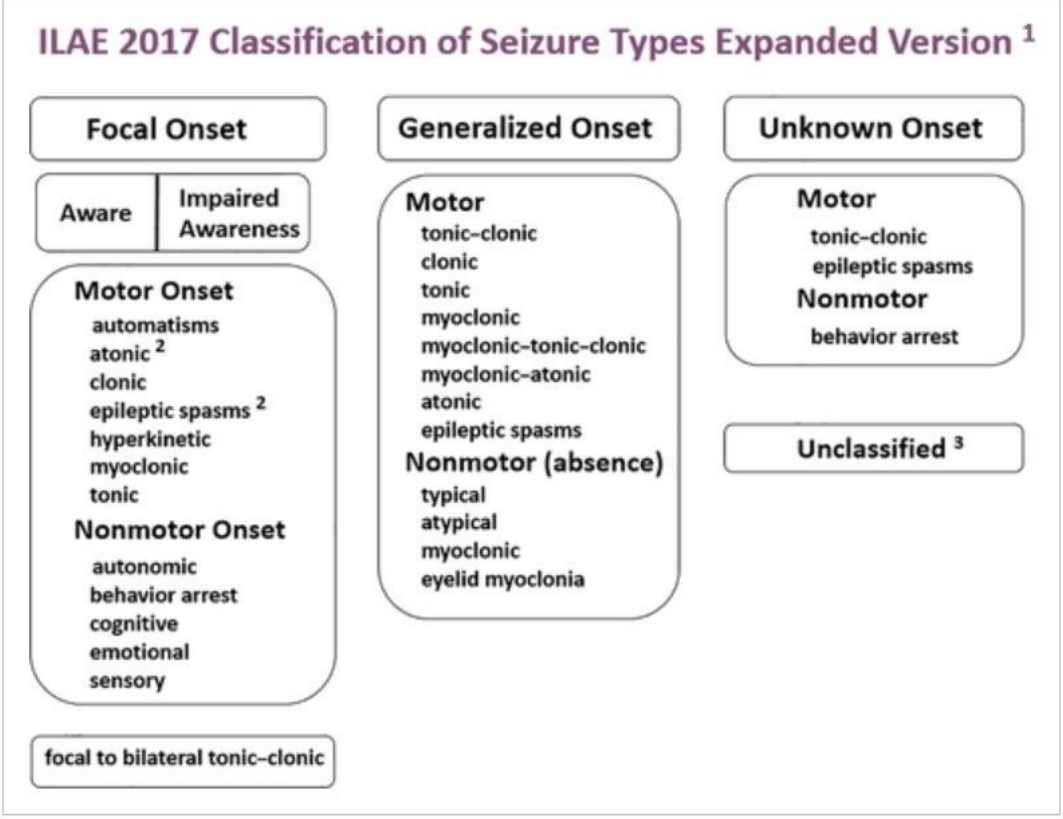
1.2 Seizure classification

Seizures are classified by the onset: Focal onset seizures, Generalized, and unknown onset seizures.

Focal onset seizures are "originating within networks limited to one hemisphere. They may be discretely localized or more widely distributed. Focal seizures may originate in subcortical structures."

Generalized onset seizures are "originating at some point within, and rapidly engaging, bilaterally distributed networks."

A seizure of unknown onset may still evidence certain defining motor (e.g., tonic–clonic) or non-motor (e.g., behavior arrest) characteristics.



ILAE classification of seizures type (Fisher et al., 2017)

1.3 Temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is a chronic condition of the nervous system characterized by recurrent, unprovoked focal seizures originating in the temporal lobe, lasting for about one or two minutes. A focal seizure within the temporal lobe can also spread to the other brain areas when it might become a focal to bilateral seizure (Communications and Public Liaison - NIH, 2015). TLE is the most

common type of focal epilepsies. According to the ILAE classification, it can be divided into two types; neocortical temporal lobe epilepsy (nTLE) and mesial temporal lobe epilepsy. The most common subtype is mTLE, and the seizures are originated from the para-hippocampal gyrus, amygdala, hippocampus, and hippocampus, but the most common pathology is hippocampal sclerosis (HS) (Maillard et al., 2004; Querol Pascual, 2007). ILAE defines HS as a severe segmental loss of pyramidal neurons of the CA1 region and less neuronal loss can be observed in the CA3 and CA4 regions of pyramidal cells. Experimental models depict that activation of N-methyl-d-aspartate receptors (NMDARs) can produce neuronal damage in TLE.

Moreover, electrical stimulation of afferent pathways to the hippocampus of the healthy animal brains replicates cell loss that is like with the TLE, and it has been shown that repetitive seizures lead to persistent recurrent inhibition loss and irreversibly damage the adjacent interneurons (Sloviter, 1987). Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter that inhibits neuronal firing by activating two different classes of receptors, GABAA and GABAB, via calcium influx into the CNS. Consequently, the GABAergic interneuronal damage, in turn, cause continuous dysregulated neuronal firing, leading to seizures. However, a growing shred of evidence shows that TLE can be developed even with minimal neuronal loss (Ong, 2019).

1.4 Experimental models of TLE

To better understand the pathophysiology of TLE and thus to develop new pharmacological treatments, animal models that present features similar to those seen in patients with TLE have been developed during the last four decades. Some of the models are based on the systemic administration of chemoconvulsants to induce SE that is followed by the occurrence of spontaneous recurrent seizures (SRSs) originating from limbic structures. The chemoconvulsant models widely employed in basic epilepsy research are the pilocarpine and the kainic acid (Adachi, 2014). Other models include electrical (kindling model) (Rolston et al., 2011) and genetic models (Serikawa et al., 2015).

Pilocarpine model

The pilocarpine animal model of status epilepticus (SE) is a clinically translatable and well-established model that satisfies all of the criterions essential for a SE animal model: SRS following a latency period, replication of electrographic, behavioral, neuropathological, and metabolic changes, as well as, pharmacoresistant to anti-epileptic drugs quite similar to that observed in human SE (Ahmed Juvale & Che Has, 2020). Pilocarpine is an M1 muscarinic acetylcholine receptors agonist that are mainly found in the striatum, cortex, and hippocampus. It promotes continuous excitatory activity leading to brain tissue damage. Pilocarpine-induced seizures initially begin in the ventral forebrain, and the nucleus accumbens is most likely the primary site of injury because muscarinic receptors are highly dense in this region (Kobayashi et al., 1978; Nonaka & Moroji, 1984). The most significant injuries can be found in the neocortex, characterized by swollen cell bodies and dendrites, as well as axonal pruning (Clifford et al., 1987). Animals often suffer a 10–20 percent loss of body weight after SE, which eventually recover to the baseline values after one week (Turski et al., 1989). The preferred routes of drug administration are intraperitoneal (i.p.) and intrahippocampal injections; both routes elicit identical behavioral and electrographic effects and similar histopathological alterations. Intrahippocampal injections, however, have a higher survival rate (71%) as compared to i.p. injections (Furtado et al., 2002). The termination of chemoconvulsants induced SE is shown to be more difficult than electrically induced models (Bankstahl & Löscher, 2008). Thus, several studies utilize a repetitive dose or a combination of anti-epileptic drugs (AEDs) for the termination of SE and to reduce mortality rates (Lee et al., 2017). As the mortality rate is very high in pilocarpine models, the lithium-pilocarpine model of SE is a new hope to counter this issue. The lithium-pilocarpine model of SE is generally characterized by the administration of lithium chloride, followed by pilocarpine administration 18 h later (Fan et al., 2020). The use of LiCl allows for a substantial reduction in the pilocarpine dose required, along with a reduction in the time to SE onset. Both the LiCl-pilocarpine and high-dose pilocarpine models follow the same pattern in seizure initiation and propagation. Both models also generate similar electrographic, behavioural, neuropathological, and

metabolic symptoms (Clifford et al., 1987; Müller et al., 2009). The LiCl-pilocarpine model has a higher SE induction rate than pilocarpine alone, with some investigators even reporting 100% SE induction compared with pilocarpine, which only results in 60% SE induction (Ahmad, 2013; Goffin et al., 2007), although the numbers may vary among laboratories. Moreover, the LiCl-pilocarpine model has a lower mortality rate and has been shown to generate more consistent and prolonged seizures with reproducible results, thus making it a promising model for studying SE (Ahmed Juvale & Che Has, 2020).

Kainic acid model

Kainate/ kainic acid (KA) is a glutamate analog that is a Digenea simplex-derived potent neurotoxin. Initially, it was used to induce electrophysiological and behavioral seizures to establish a TLE model, where the seizures originated in the CA3 region of the hippocampus. Indeed, this toxin produces the tissue lesions and damage that can be concomitant with those perceived in human individuals with TLE (Victor Nadler, 1981). The KA receptors (KARs) are localized throughout the brain, such as the cerebellum, entorhinal cortex, amygdala, basal ganglia, and most abundantly in the hippocampus. The KARs are the ionotropic-glutamate receptors that can either be pre-synaptic or post-synaptic. The pre-synaptic KARs are the bidirectional receptors, eliciting their action as excitatory via ionotropic activity and inhibition through "non-canonical" signaling, termed as metabotropic signalling (Lerma & Marques, 2013). The excitatory neurotransmission is elicited by post-synaptic receptors. These receptors can also contribute to both the pre and post-synaptic GABAergic neurotransmission. The subunits of KAR are GluK1 (GluR5), GluK2 (GluR6), GluK3 (GluR7), GluK4 (KA1), and GluK5 (KA2). The GluK4 is highly expressed in the CA3 region of the hippocampus, while GluK5 is expressed in both the CA3 and CA1 areas. GluK4 and GluK5 are primarily concentrated in the CA3 field and have a higher affinity for KA, contributing to the excitotoxic damaging pattern in this area (Bloss & Hunter, 2010).

A process of severe neuronal damage reaching the point of death due to over-stimulation using excitatory neurotransmitters, like glutamate, is called excitotoxicity. A cascade of molecular

interactions leads to excessive depolarization and osmotic imbalance, resulting in the post-synaptic membrane rupture. Among several ongoing mechanisms, intracellular calcium accumulation plays a central role, followed by over-activation of glutamate receptors. The calcium rise can strongly impact the endoplasmic reticulum and mitochondria. The ionic imbalance (Na+,Cl-, K+) is also involved in excitotoxicity induced by KA (Friedman, 2006; Ha et al., 2002). The standard routes of administration of KA are intracerebral (supra-hippocampal, intra-hippocampal, intra-amygdala), intranasal, and systemic. The basic parameters that should be considered to select the route of injection are labor-intensity, mortality rate, lesion control, gender, strain, and age of the animal (Rusina et al., 2021). Compared with pilocarpine, it is more challenging to induce SE in mice with systemic KA administration (McKhann et al., 2003). Studies in mouse models have shown that the C57BL/6, C57BL/10, and F1 C57BL/6∗CBA/J strains are resistant to systemic KA administration, while the FVB/N, ICR, and DBA/2 J strains are vulnerable (McLin & Steward, 2006). KA models have also proven impractical for pharmacoresistant studies because of differences in AED reactions. Another limitation is the excitotoxic effect of KA, which makes separating direct neuronal damage from seizure-induced neuronal damage difficult (Reddy & Kuruba, 2013).

Electrical model

Electrical-kindling models in rodents are well-established and moderate throughput models of chronic SE that are frequently used for the drug discovery purposes. Electrical kindling is particularly a valuable technique used to study the chronic stage of epilepsy because the stimulation site is known. In addition, there are no other disturbances, in contrast to the SE models that are linked with widespread uni or bilateral limbic damages caused by the cell death (Batten et al., 2016). Importantly, these electrical stimulation models often exhibit many features linked with clinical epilepsy: neurological damage, cognitive deficits, and chronic seizure activity (Pitkänen et al., 2017). The amygdala-kindling model in rodents is one of the most extensively used models of TLE, and the only chronic epilepsy model that has been clinically validated. In most studies on kindling in rats, outbred strains, such as Wistar have been used (Löscher et al., 2017). The lamotrigine (LTG) resistant

amygdala-kindled rat model is useful for not only identifying the compounds effective against secondarily generalized partial seizures, but also allows for the differentiation of compounds that may be effective in the therapy-resistant patient population (Pitkänen et al., 2017).

These models of TLE are induced by repeated (most often daily) stimulation pulses in the limbic structures, most commonly in the amygdala, which results in the gradual induction of limbic system seizures, allowing the evaluation of network mechanisms. The network structures that are recruited in these forms of limbic seizures in animals include perirhinal, piriform, and entorhinal areas (Faingold & Blumenfeld, 2014).

Genetic model

The genetic models involve manipulation (knockin/ knockout) of the animal's genetic material to determine the involvement of a particular gene in epilepsy.

CHAPTER 2: NEUROINFLAMMATION

2.1 Introduction

Neuroinflammation is a well-orchestrated and complex process involving glial cells, particularly astrocytes and microglia. It is a defensive mechanism that initially aids in inhibiting or removing diverse pathogens. This neuroinflammatory response elicits beneficial effects via the removal of cellular debris, thus promoting neural tissue repair. However, sustained and persistent inflammation is detrimental and inhibits the tissue regeneration (Yang & Zhou, 2019). Neuroinflammation can be initiated by various endogenous and exogenous factors, including infection, toxic metabolites, injury, aging, autoimmune diseases, passive smoke, and air pollution. These factors stimulate the pro-inflammatory chemokines and cytokines that activate microglial cells eliciting primary immune responses in CNS. Sustained microglial activation allows the recruitment of B or T lymphocytes and macrophages that can cross the compromised BBB, amplifying the chronic inflammation and, ultimately neurodegeneration (Pracucci et al., 2021). Excessive and prolonged neuroinflammation acts as a pivotal driver of various neurological disorders, including traumatic brain injury, cerebrovascular disease (CVD), multiple sclerosis (MS), Alzheimer's disease (AD), psychological disorders, epilepsy, and chronic pain. It is a common mechanism, connecting the ischemic, traumatic, epileptic, psychiatric, demyelinating, and degenerative pathologies (Gorji, 2022).

2.2 Positive and negative aspects of neuroinflammation

There are multiple immune, biochemical, psychological, and physiological consequences of the neuroinflammatory responses prompted by the production of chemokines, cytokines, secondary messengers, and reactive oxygen species (ROS) from glial cells (microglia and astrocytes). The degree of neuroinflammation depends upon the duration, context, and primary insult or stimulus course. Brief and controlled neuroinflammatory responses are functional to brain development and

physiology. Several lines of evidence suggested the involvement of cytokines production and activated microglia in the early development of the brain. Activated microglia provide synaptic pruning and support the immunological activities in the CNS. Moreover, the increased neuroinflammatory signals b/w CNS resident cells and T-cells are involved in normal learning and memory. The IL-1 signaling is significant in re-populating the depleted microglia from the local progenitor sources (DiSabato et al., 2016). Conversely, highly pathological or destructive neuroinflammation is linked with the glia activation with significant chemokines and cytokines production, edema, enhanced permeability of blood-brain-barrier (BBB) & breakdown. High-degree neuroinflammation leads to both primary as well as secondary damage that can elicit chronic neuroinflammatory responses that might never resolve. This degree is linked with the autoimmune disease-induced immune responses, which are characterized by the glia over-activation and production of chemokines and cytokines (Linker et al., 2002) (Figure 1).

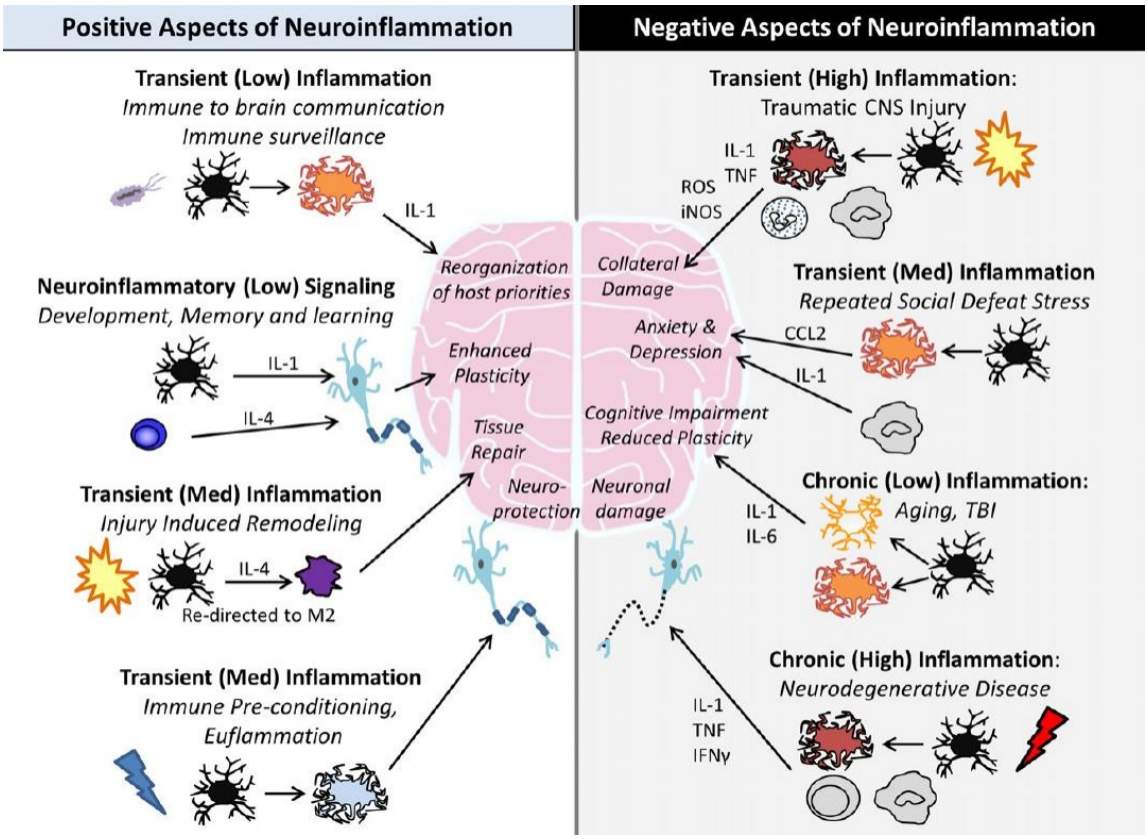


Figure 1. Positive & negative impacts of neuroinflammation (DiSabato et al., 2016)

The duration as well as intensity of the neuroinflammation, in particular, accounts for the destructive or supportive immune signals to CNS. ***Positive aspects****: After infection, immune to the brain signals results in subsequent re-organization of the host priorities & and the stimulation of sickness behaviours. IL-1 & and IL-4 plays a significant role in the maintenance of memory and learning. IL-4 derives the macrophages (M2) re-polarization that effectively promotes the recovery as well as axonal re-growth after traumatic injury to CNS. Euflammation or immune pre-conditioning serves as training method for innate immune system to more neuro-protective phenotypes.* ***Negative aspects****: Uncontrolled chronic neuroinflammation leads to the increased cytokines production such as IL-1 and TNF), ROS, and iNOS). The transient neuroinflammation also allows the macrophage and monocytes recruitment which results in depression and anxiety. In addition, IL-1 and IL-6 driven low and chronic neuroinflammatory reactions are the leading cause of cognitive impairments and decreased neuronal plasticity. Chronic neuroinflammation at higher degree greatly damages the nervous system which is the characteristic of NDDs.* ***Abbreviations****: Interleukin (IL), tumor necrosis factor (TNF), reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), neurodegenerative diseases (NDDs).*

2.3 Factors involved in the neuroinflammatory process

Pro-inflammatory cytokines

The cytokines are loose and broad category of peptides with low molecular weight (~5–25 kDa) that are important in cell signaling. They are produced by the activated/reactive immune cells exerting biological activities. The cytokines are secreted by a various cellular types, particularly leukocytes and glial cells in the brain, which regulate the physiological and pathological processes, including acquired immunity, innate immunity, and inflammation. The most abundant pro-inflammatory cytokines are IL-1β, TNF-α, and IL-6.

- *Interleukin-1*

IL-1 belongs to the cytokines family, including IL-1β, IL-1α, and its receptors antagonist (IL-1RA). They are present as both the soluble forms and membrane-bounded that are involved in autocrine and paracrine signaling. Both the IL-1β and IL-1α coordinate host defence and inflammatory responses within the body. IL-1 contributes to the activation of other inflammation concomitant molecules, chemokines, and pro-inflammatory cytokines. IL-1β and IL-1α exert their effects by binding to two specific receptors, IL-1RI and IL-1RII. The ligands and the receptor components of IL-1 family, IL-1β, IL-1α, IL-1RI, IL-1RII, and IL-1RA are expressed at a low level in the brains of healthy

individuals. The initial IL-1 primary source is in the microglia in response to the injury or infections. The caspase-1 is expressed by the microglial cells. The role of active caspase-1 is to cleave pro-IL-1β to its activated form IL-1β. IL-1 elicits both the maladaptive and adaptive responses from microglia cells that possibility prompt neuroinflammation, injury, and infection by the release of reactive nitrogen species (RNS), reactive oxygen species (ROS), chemokines, cytokines, prostaglandins, and proteases. Excessive and prolonged inflammation switch microglia from M2 (anti-inflammatory) to M1 (pro-inflammatory) state, resulting in neuronal damage and, ultimately neurodegeneration (Kaur et al., 2020). Studies have provided the evidence that neuroinflammation and the subsequent neurodegeneration can be ameliorated by blocking the IL-1 signaling, particularly IL-1β. Moreover, IL-1β exhibits a prominent role in the development and aggravation of chronic neurodegeneration concomitant with acute neuroinflammation reactions, including ischemia, brain injury, and stroke (Basu et al., 2005; Hara et al., 1997).

- *Tumor necrosis factor-α*

TNF-α is a neuroinflammation mediating cytokine that was discovered in 1975 by Carswell. It regulates the synaptic plasticity, water intake, sleep, food, and memory and learning under a normal healthy state. While under pathological conditions, glial and neurons release excessive TNF-α, resulting in neuroinflammatory responses. It induces multiple signals activation making its prominent role in neuroinflammation. These neuroinflammatory signals include extracellular signal-regulated kinase (ERK), NFκB activation, c-Jun-N-terminal-kinase (JNK), and p38-mitogen activated-protein kinase (MAPK) (Muhammad, 2020). Neurons, microglia, and astrocytes play a significant role in the production and release of TNF-α both in free and membrane-bounded forms. TNF-α signaling is transmitted via two receptors TNF receptor 1 (TNFR1) that are expressed by several cell, and TNF receptor 2 (TNFR2) that is found in glial cells and some specific neurons subtypes (Wertz & Dixit, 2008). One of the most significant early neuroinflammation initiators, TNF-α, interacts with TNFR1 to mediate the extrinsic apoptotic-death signals through Fas-associated death-domain (FADD) while

it interacts with TNFR2 to mediate neuroinflammation via activation of NFκB (Sedger & McDermott, 2014).

NFκB, during dormant state, resides in cytosol bounded with NFκB inhibitory proteins (IκBα). Inflammatory stimulus results from various pathological processes, leading to the degradation of IκBα upon phosphorylation by a kinase known as IκB kinase (IKK). This allows the migration of NFκB from cytosol to the nucleus, where it activates the transcription of several pro-inflammatory genes. Hence, TNF-α either activated by NFκB or it activates it, creating a positive feedback loop which intensify and perpetuates the inflammation by releasing other cytokines like IL-1β and IL-6 (Sedger & McDermott, 2014). Another signaling pathway by which TNF-α persuades inflammatory cytokines is the JAK-STAT signaling pathway. TNF-α signaling, in particular, initiates this pathway when it binds to its receptors. The activated STAT translocates into the nucleus, allowing the synthesis of several cytokines (Tanabe et al., 2010).

- *Interleukin-6*

Like other cytokines, IL-6 is also upregulated in response to neuroinflammation. In the CNS, IL-6 may be released by neurons, astrocytes, microglia, and endothelial cells. Stimulators for the production of IL-6 are, for instance, IL-1β, TNFα, and transmitters such as norepinephrine, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP38) and bradykinin. Membrane depolarization is also one of the primary mechanisms for neuronal production of IL-6 (Erta et al., 2012). The cellular effects of IL-6 are exerted by the activation of IL-6-receptor (IL-6R). This can be a membrane-bound receptor, expressed only in few cells, or a soluble receptor. To produce a signal, the IL-6R needs to interact with glycoprotein-130 (gp130), that is a membrane protein ubiquitously expressed. The pro-inflammatory pathway mediating neurodegeneration is known as trans-signaling, which relies on IL-6R soluble form (Rothaug et al., 2016).

Nitric oxide (NO)

A bioactive free radical, NO, plays a crucial role as a neuromodulator and neurotransmitter in the CNS. It executes both the physiological as well as pathological processes. Its physiological functions depend upon its local concentration, availability, and the downstream target compound's nature. Particularly, it is implicated in physiological functions of vascular and neuronal cells, while higher concentrations are involved in pathogenesis of multiple neurological disease such as neuroinflammatory diseases, stroke, demyelination, and neurodegenerative diseases. This Janus-faced pleiotropic molecule is enzymatically synthesized by the activity of nitric oxide synthase (NOS). The sustained inflammatory signals evoke the excessive production of NO, leading to the reactive nitrogen species (RNS) formation and neuronal death (Ghasemi & Fatemi, 2014). The inducible NOS (iNOS) is expressed by microglia and astrocytes under pathological conditions, including inflammatory and neurotoxic damage, trauma, and ischemia. Shreds of evidence have suggested that glial NO is also involved in the pathology of multiple sclerosis, X-linked adrenoleukodystrophy (ALD), experimental allergic encephalopathy, trauma, Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and many other neurological diseases (Nakamura et al., 2012). Briefly, the production of NO is correlated with the microglial activation and the neuroinflammation within the brain. The activated glial cell prompt iNOS and then produce NO, triggering the mobilization of calcium from the endoplasmic reticulum. This, in turn, activates the vesicular glutamate release from the astrocytes leading to neuronal death and contribute to neurodegenerative diseases (Yuste et al., 2015). The involvement of RNS and NO in neurotoxicity is shown in figure 2.

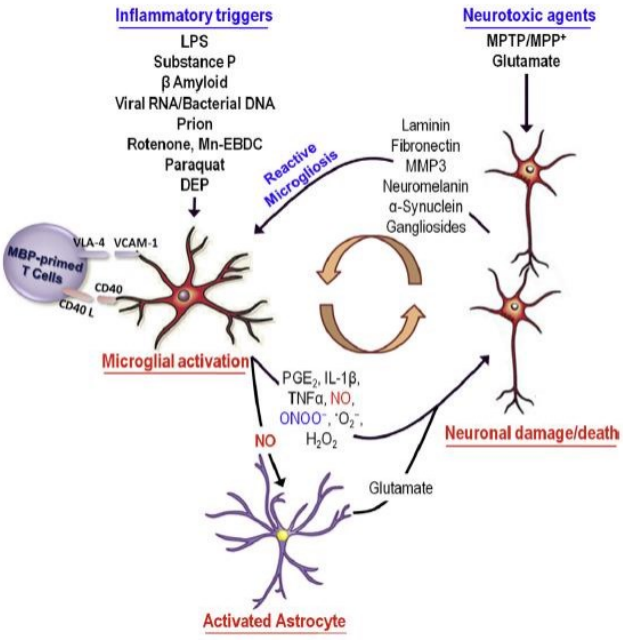


Figure 2. Involvement of RNS and NO in progressive brain toxicity (Ghasemi & Fatemi, 2014)

Over activated microglia induce neurotoxicity via two mechanisms. ***(1)*** *Activated microglia initiate neuronal damage through pro-inflammatory stimuli, including LPS, bacterial DNA, viral RNA, β-amyloid, Mn-EBDC, rotenone, prion, DEP, paraquat as well as cellular contact with the MBP-primed T-cell, resulting in production of pro-inflammatory factors (neurotoxins), like $ONOO^-$, NO, H_2O_2, PGE_2, and various cytokines.* ***(2)*** *Over-activation of microglia as a result of neuronal damage through a number of factors being release by dead or damaged neurons (reactive microgliosis), which is toxic for other neighbouring neurons, leading to perpetuating cycle of the neuronal loss/death. NO derived from iNOS allows the intracellular stores Ca^{2+} release which causes the release of glutamate from astrocytes, thus stimulating the exocytosis of the vesicular glutamate. The crosstalk b/w neuronal and glial cells in pathological condition forms a feedback loop and NO plays a significant role contributing to the aggravation of these events. NO plays both the efferent and afferent inflammatory stimulator in neuronal damage/injury.* ***Abbreviations****: lipopolysaccharide (LPS), manganese ethylene bisdithiocarbamate (Mn-EBDC), diesel exhaust particles (DEP), myelin basic protein (MBP), prostaglandin E_2 (PGE_2), matrix metalloproteinase3 (MMP3), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Interlukin-1β (IL-1β), Tumor necrosis factor-α (TNF-α)*

Oxidative stress

The oxygen-derived reactive molecules (ROS) are critical to trigger the NDDs. They are naturally produced in the biological systems and have important functions in the cellular homeostasis, inflammation, response to stressors, and cell survival. In chronic oxidative stress, these species could be harmful as they oxidize lipids and proteins and damage mitochondrial DNA (mtDNA).

Simultaneously, ROS higher concentrations can activate pro-inflammatory signaling pathways and create a feedback cycle aiding in sustenance of high concentration of chemokines and cytokines. Pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, and IFNs stimulate ROS generation and, in turn, ROS could also activate the NFκB promoting more production of pro-inflammatory cytokines (Monkkonen & Debnath, 2018). Several lines of evidence suggest that various factors like gene mutation, iron accumulation, and mitochondrial dysfunction can generate ROS and, thus neuroinflammation. Once the tissue is severely inflamed, it causes the accumulation of ROS, leading to cell death (Weng et al., 2018). In chronic loss of redox balance, signaling pathways that modulate the immune system are altered with immune response dysregulation and pre-dominance of the pro-inflammatory responses.

In chronic states, ROS can be harmful as they oxidize proteins and lipids and mediate signaling that leads to the astrocytes-microglia activation. Simultaneously, the presence of high ROS concentrations can activate pro-inflammatory signaling pathways and create the vicious cycles to maintain increased secretions of chemokines and pro-inflammatory cytokines. Pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and interferons (IFNs) induce ROS, in turn, ROS can also activate the NFκB and allow the production of more pro-inflammatory cytokines (Monkkonen & Debnath, 2018). Thus, oxidative stress and inflammation are closely interrelated and often coexist.

2.4 Neuroinflammatory signalling pathways

PI3K/AKT/mTOR Pathway

The (phosphatidylinositide 3-kinase) PI3K pathway is activated by diverse stimuli that include also inflammatory stimuli, such as lipopolysaccharide (LPS), a bacterial toxin that activates toll-like receptor-4 (TLR4)/CD14 receptor in microglia. Phosphatidylinositol 4,5-bisphosphate is phosphorylated by the PI3K after the activation to generate phosphatidylinositol-3,4,5-trisphosphate. Phosphatidylinositol-3,4,5-trisphosphate then binds with pleckstrin homology domain of one of the Akt (also known as protein kinase B) isoforms and facilitates phosphorylation of Akt1, Akt2, or Akt3

at, by the phosphatidylinositol dependent kinases 1 and 2, respectively, at Thr308/309/305 and Ser273/474/472 (Laine et al., 2002). The phosphorylation on respective residues of the Akt results in the further catalytic activity changes of the downstream targets, including mammalian target of rapamycin (mTOR) and glycogen synthasekinase-3 (GSK-3) (Cantley, 2002; Fukao & Koyasu, 2003). PI3K plays a vital role in the inflammation and activation of microglia. In particular, the PI3K inhibitor LY294002 produces down-regulation of prostaglandin E synthase-1 (mPGES-1) and upregulation of cyclooxygenase-2 (COX-2) by (de Oliveira et al., 2008).

Mitogen activated protein kinase (MAPK) Pathway

The MAPKs belong to the class of serine/threonine kinases that are activated as a result of mitogens, growth factors, and stress and play a significant role in the growth, differentiation, and survival of the mammalian cells. The MAPKs group includes JNK, ERKs, and p38 MAPK. The p38 MAPK pathways activation has a crucial role in the development of neuroinflammation. Its signaling cascade regulates the chemokines, adhesion molecules, and cytokines expression that control the inflammatory responses. IL-1, TNF-α, IL-8, IL-6, and CCL-2 depend on the p38 signaling (Brook et al., 2000). Cytokine-mediated and glutamate-induced apoptosis is regulated by the p38 MAPK within the brain. The p38 MAPK signaling and expression of pro-inflammatory cytokines have been reported in the glutamate-regulated neuronal loss of neonatal rats. Hence, its inhibition promoted the neuroprotection (Torres et al., 2006). Inconsistencies in the p38 MAPK signaling pathway in neuronal cells coupled with the neuroinflammatory processes and chronic activation of microglia pose an over-all damaging effect (Streit et al., 2004). The sustained glial activation leads to the increased pro-inflammatory cytokines release which results in the amplified inflammatory responses causing neuroinflammation (Chao et al., 1995; Skaper, 2007).

Nuclear factor kappa B (NFκB)

The TLRs play an essential role as a signal transduction membrane protein for generating of inflammatory responses. They recognize the specific ligands for initiating the inflammatory process, thereby resulting in activation of NFκB to promote the cytokines release, microglial phagocytosis,

and the regulation of the other molecules needed for the adaptive immune responses. The myeloid differentiating factor 88 (MyD88) activates the TLR4 which leads to the activation of astrocytes in response to infection, thereby leading to neuroinflammation (Shastri et al., 2013; Su et al., 2016). Activation of TLR4 promotes the translocation of NFκB from cytoplasm into the nucleus, which then stimulates the release of pro-inflammatory cytokines, such as IL-1β.

2.5 NEUROGLIA

Neuroglial cells are the non-neuronal cells in the nervous system. Glial cells are oligodendrocytes, microglia, and astrocytes. Oligodendrocytes are specialized in myelin production, microglia are macrophages resident in the CNS, and astrocytes are traditionally viewed as cells involved in supporting neurons. Anyway, as stated below, it is now clear that astrocytes perform a broad array of functions. Neuroglia is involved in several CNS functions, among these, microglia and astrocytes represent the brain defence system (Verkhratsky et al., 2014). Neuroglia is vital both in physiological (preservation of brain homeostasis) as well as in pathological context (repair and restoration of brain homeostasis) (Verkhratsky & Butt, 2013; Verkhratsky & Nedergaard, 2016). The homeostatic support appears at all the levels of nervous system organization: molecular level (maintenance of homeostasis of neurotransmitters, ions, protons, metabolites, ROS, etc.), at network level (regulation of synaptogenesis, maturation, and regulation by microglia and astrocytes), at cellular level (involvement of astrocytes in neurogenesis), at organ level (maintenance of BBB and glymphatic flow and regulation of functional hyperaemia by astrocytes), at systemic level (ionic homeostasis, energy metabolism, and central chemoceptors), and at connectome level (maintenance of myelin sheath by Schwann cells and oligodendrocytes) (Verkhratsky et al., 2019). Failure to do so results in the development and progression of neurological and neurodegenerative diseases, resulting in nervous tissue damage.

Astrocytes

The astrocytes are considerably heterogeneous in appearance and functions, displaying a significant adaptive plasticity that allows the maintenance of CNS functions in development and aging. They are highly integrated into the neural networks and control the CNS homeostasis at all the levels of organization. They maintain the CNS molecular homeostasis by transporting of major protons and ions, catabolizing and removing neurotransmitters, and releasing precursors of neurotransmitters and scavengers of ROS. Neurotransmission is sustained by astrocytes by supplying neurons with the precursors of neurotransmitters and control homeostasis of cell via embryonic and adult neurogenesis (Verkhratsky & Nedergaard, 2018). Astrocytes also transport growth factors to the neurons, support the formation of synapses, and help to regulate the extracellular balance of neurotransmitters and ions, making these essential for the homeostasis of the brain. Following disease and CNS injury, these glial cells undergo a process known as astrogliosis, resulting in secretion of either pro-inflammatory or anti-inflammatory factors. Functionally, the astrogliosis results in molecules expression that provides support to the injured neurons and isolate CNS inflammation and damaged area from the healthy CNS tissues, re-build and maintain compromised BBB, and contribute to circuitry re-modelling around lesioned regions. These functional reactions are, therefore, beneficiary to the CNS. However, prolonged neuroinflammation and increased injury severity results in the augmentation of a pro-inflammatory state by astrocytes (Soung & S. Klein, 2020).

The reactive astrocytes release several molecular signals contributing to the inflammatory state of CNS post-disease or injury via direct activation of immune defence along-with the release of chemokines, cytokines, and other growth factors. Studies have revealed that injury or infection significantly contribute to the production of neurotoxic and pro-inflammatory molecular profile by astrocytes. Activation of STAT3 and NFκB functions as a transcriptional regulator of the pro-inflammatory astrocytes during autoimmune inflammation. Pieces of evidence have also suggested that astrocytes contribute to triggering the inflammatory responses as a result of increased neuronal activity in stress, neuropathic pain, and epilepsy (Colombo & Farina, 2016; Soung & S. Klein, 2020).

Microglia

Several lines of evidence have elicited a strong association b/w neurodegeneration and microglial activity. Microglial cells are resident macrophages within the brain and are the primary immune cells of CNS that play a significant role in neuroinflammatory mechanisms (Zhao et al., 2019). Microglia display a unique molecular-homeostatic 'signature', comprised of a specific surface protein expression pattern and a transcriptional profile that differs from macrophages of peripheral tissue (Butovsky & Weiner, 2018). In adults, they are an independent self-renewing population that is renewed multiple times throughout life. Typically, they support the synapse elimination and synaptic homeostasis. Data shows that microglia are essential to support the brain development, efficiently pruning synapses throughout life during learning, altering CNS about threats, acting as the antigen-presenting cells, and surveying tissue for immunogens (S. W. Lee et al., 2019; Yong et al., 2019). Acute neurodegenerative diseases, including hypoxia, trauma, and stroke, compromise the neuronal survival, indirectly triggering neuroinflammation. The microglial activation is termed as M1 (pro-inflammatory, potentiate neuronal injury) and M2 (anti-inflammatory, phagocytic, and pro-regenerative). Signaling cascade initiates along-with the activation of microglia that induces the pro-inflammatory mediators release and increase the permeability of BBB, facilitates the infiltration through leukocytes, including macrophages and T-cells, that have several similar functional characteristics of microglia, including TLRs (Chen et al., 2016; Ostanin et al., 2009). Although microglial activation is critical and essential for the host defence, prolonged and excessive activation of microglia results in increased production and release of pro-inflammatory cytokines leading to neuronal death (Zhao et al., 2019). Hence, acute neuroinflammation has protective effects, while chronic neuroinflammation is damaging and detrimental. So, the duration of inflammatory reactions and the type of activation of microglia is the leading cause of either beneficial or harmful outcomes to the brain.

2.6 Crosstalk between astrocytes and microglia in neuroinflammation

Microglia-astrocytes along-with the glutamatergic neurons compose a “quad-partite” synapse. It is mandatory for the circuit operations based on the neuro-immune communications (Macht, 2016). The astrocytes are abundantly distributed in a multifaceted network, which is connected through the gap junctions and is present in almost all the operational regions of the spinal cord and the brain, thereby supporting and influencing the indirectly connected neural circuits. Conversely, microglia are the restless cells that constantly move their processes throughout the brain (Davalos et al., 2005; Nimmerjahn et al., 2005). The brain-gut axis is another route for microglia-astrocytes crosstalk by which the dietary tryptophan metabolites directly act on microglia (resident in CNS), which then regulate the pathogenic activity of astrocytes during neurodegeneration and neuroinflammation (Rothhammer et al., 2018). The purinergic receptors (P2Y) are expressed in microglia and astrocytes, which might play a crucial role in the microglia-astrocytes communication during a neuroinflammatory response. For instance, the astrocyte derivative ATP binds to the P2Y6 and P2Y12 receptors that are expressed by microglia, allowing the microglial processes extension and phagocytosis. The binding of ATP with microglia and astrocytes may also evoke the Ca^{2+} currents and promote the inflammatory cytokines production and release by microglia (Koizumi et al., 2007; P. W. Liu et al., 2017).

As neuroinflammation plays a significant role in multiple neurodegenerative disorders, it is pretty evident that astrocytes and microglial cells are engaged constantly in a fine crosstalk. After the injury, the microglial cells are the first to respond dynamically to the pro-inflammatory stimuli and derive the astrocytes to secrete an anti-inflammatory cytokine, interleukin 10 (IL-10). If the inflammation is not resolved, then microglial cells proliferate and swap to their reactive phenotype M1, termed microgliosis. The reactive state of microglial cells then releases cytokines, prostaglandins, IL-1β, and TNF-α in the brain. The astrocytes sense these molecules as potent neuroinflammation signals, switch to astrogliosis (a reactive astrocyte state A1), and ultimately secrete more pro-inflammatory stimuli. Hence, a reactive loop is initiated (K. Li et al., 2019).

Moreover, the crosstalk b/w microglia and astrocytes is also maintained through the secreted mediators, including neurotransmitters, glio-transmitters, chmokines, cytokines, ATP, NO, ROS, RNS, mitogenic factors, metabolic factors, and growth factors that could mediate the changes in tissue and cell metabolism (Drago et al., 2017). The production of NO by M1 leads to an elevation of the glycolytic enzymes, resulting in production of ROS, NO, and the release of hypoxia-inducible factor-1 alpha by astrocytes. As a result of NO production by astrocytes, IL-1β production is also enhanced by microglia, which is perceived as pro-inflammatory cytokine by the astrocytes, which also enhances the NO production and so on. As a consequence of these interactive responses, both the astrocytes and microglial cells acquire the perilous neuroinflammatory state, which is characterized by pro-inflammatory molecules upregulation, including TNF-α, IL-6, IL-1β, ROS, and NO. It creates a very unfavorable environment for neuronal cells, resulting in loss of synapse and neuronal death (Matejuk & Ransohoff, 2020).

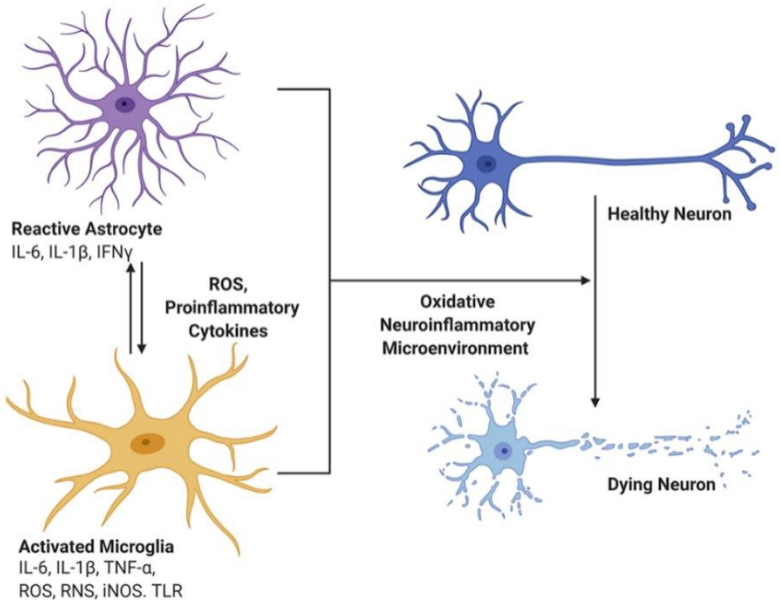


Figure 3. Crosstalk between astrocytes and microglia (Matejuk & Ransohoff, 2020)

The production of pro-inflammatory mediators including chemokines and cytokines plays significant role in the development of inflammatory microenvironment in demyelinating diseases and NDDs. This microenvironment leads to pathological condition within CNS allowing the microglial activation. Abbreviations: neurodegenerative diseases (NDDs), central nervous system (CNS), interleukin (IL), tumor necrosis factor-α (TNF-α), reactive oxygen species (ROS), reactive nitrogen species (RNS), inducible nitric oxide synthase (iNOS), toll like receptor (TLR), interferon gamma (IFNγ)

2.7 Neuroinflammation and epilepsy

Several experimental piece of evidence support the association of neuroinflammation with neurological diseases, particularly epilepsy. At the CNS level, neuroinflammatory processes could participate in common pathological mechanisms critical for the onset of seizures and epilepsy. Clinical studies have made it more evident, as shown by the anticonvulsant activities of anti-inflammatory treatments in some drug-resistant epilepsies (French et al., 2017). Experimental data have demonstrated that the seizures themselves can induce neuroinflammation and that the SRSs can perpetuate chronic neuroinflammation. In addition, the neuronal loss associated with the seizures can cause neuroinflammation, but it's not a pre-requisite for the occurrence of neuroinflammation. Though the presence of pre-existing neuroinflammation can enhance seizure susceptibility, linked with alterations in the neuronal excitability as well as increased seizure-induced neuropathology (Vezzani et al., 2011). Both experimental and clinical studies have shown that the level of pro-inflammatory cytokines, including IL-1β, TNFα, and IL-6 increase after the seizure insult. In addition, mRNA expression of pro-inflammatory cytokines is upregulated in the hippocampus along with increased vascular endothelial growth factor (VEGF) and transforming growth factor- beta 1 (TGF-β1) following seizures (Rana & Musto, 2018). After an epileptogenic insult or during the SRSs, excessive amounts of the extracellular High mobility group box-1 protein (HMGB1) are released due to cell death or via active secretion activating TLR4.

It thus seems that mechanistically, the IL-1 receptor-1 (IL-1R1) – toll like receptor4 (TLR-4) signaling pathways initiates the cascade of neuroinflammation in epilepsy. In addition, increased expression of TLR receptors and/or their endogenous agonists, such as HMGB1, which promote activation of the inflammatory process, has been observed in animal models of epileptogenesis (Vezzani et al. 2011). Moreover, the increased release of the endogenous agonists during or post seizures leads to the activation of inflammasomes (NLRP1 and NLRP3) that mediate the IL-1β production in models of temporal lobe epilepsy. Indeed, the release of HMGB1 and production of

pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, by glia and neurons is supported by the activation of NLRPs and, in turn, results in neuronal hyperexcitability (Vezzani et al. 2013). Furthermore clinical data in human epilepsy confirm that IL1-R1 expression is prompted in microglia cells(Vezzani et al., 2019).

2.8 Lipopolysaccharide (LPS) induced neuroinflammation *in vitro* model

Lipopolysaccharide (LPS) is commonly known as endotoxin and is a major component of outer membrane of the Gram-negative bacteria, which induces pronounced inflammatory responses. It is a heat-stable glycolipid that significantly reinforces the outer membrane's asymmetric phospholipid bilayer, providing structural integrity as well as permeability barrier to a bacterial cell (Bertani & Ruiz, 2018). To identify and respond to toxic insults, immune cells have pattern recognition receptors (PRRs) that comprise TLRs, NOD-like receptors (NLRs), and retinoic acid inducible gene-I-like receptors (RLRs). PRRs become activated upon recognition of pathogen-associated molecular patterns (PAMPs), which include intact pathogens or their components, such as LPS, and damage-associated molecular patterns (DAMPs) containing host cells molecules related to cellular damage or death. Binding a ligand to PRR initiates a downstream signaling pathway that triggers an inflammatory cascade. The LPS molecule is a potent PAMP, able to induce a pronounced inflammatory response; it binds to TLR4 activating a cascade of downstream signaling pathways, resulting in the production of cytokines and chemokines, including IL-1β, IL-6, TNF-α, ROS, and NO (Skrzypczak-Wiercioch & Sałat, 2022).

2.9 Mitochondrial dysfunction and neuroinflammation

Mitochondria are intensly involved in regulating cell death, a critical event in the pathogenesis of epilepsy (Folbergrová & Kunz, 2012). . Indeed, as mitochondria undergo oxidative stress to induce damage, their dysfunction is the leading cause of neuronal death affecting excitability, synaptic

transmission, and apoptosis. Increased ROS production within a cell can alter mitochondrial parameters, including increased ATP concentration, mitochondrial permeability transition pore (MPTP), membrane potentials, and Ca^{2+} adsorption, leading to the irreparable cellular injury (de Araújo Boleti et al., 2020). The mutant mtDNA accumulation induces alterations in the mitochondrial dynamics, increases the ROS production, and loss of mitochondrial membrane potential leading to exacerbation of the mitochondrial dysfunction. The defective mitochondria and the mtDNA are degraded by specific catabolic process as mitophagy, but after the extensive mitochondrial damage, mtDNA can escape this pathway. Furthermore, the damaged mtDNA acts as a DAMP for regulating the inflammatory response by triggering the interferon (IFN) genes, TLR9, and the NLRP3 inflammasome (X. Zhang et al., 2019). The mitochondrial stress and injury allow the mtDNA release into the cytoplasm, activation of the DNA-induced cyclic GMP-AMP synthase (cGAS)-cyclic GMPAMP (cGAMP)-stimulator of interferon genes (STING) pathway and promotion of inflammatory responses (Bai et al., 2017). The mutant mtDNA also triggers the activation of NLRP3 inflammasome, which stimulates the production of pro-inflammatory cytokines (Zhong et al., 2019). Overall, mitochondrial dysfunction contributes to several neurologic disorders, including acquired epilepsies such as temporal lobe epilepsy (TLE) (Waldbaum & Patel, 2010).

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

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Review

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

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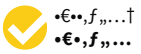

Abstract: Innate immune response is one of our primary defenses against pathogens infection, although, if dysregulated, it represents the leading cause of chronic tissue inflammation. This dualism is even more present in the central nervous system, where neuroinflammation is both important for the activation 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 (TNF-), 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 dierent 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 ecient 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 dierent 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.

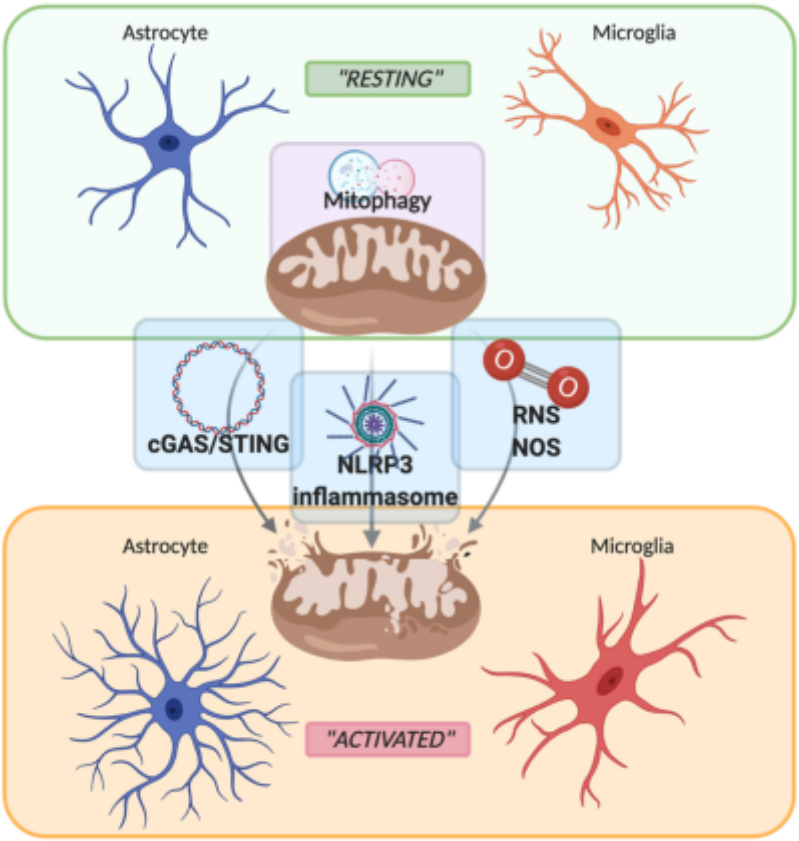

Figure 1. Schematic representation of the switch between "resting" and "activated" state of astrocyte and microglia mediated by mitochondria. Mitochondria maintain their healthy and physiological state by mitophagy. Upon stressful condition, such as inflammatory stimuli, mitochondria are disrupted with 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 dierent 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 suer 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 2^0,3^0-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-1 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 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, dierent 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 -amyloid protein (A) and -synuclein (-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 eects observed in dierent 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), hydroxyl (OH), peroxyl (RO_2), 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 dierent 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 formation is increased by the loss of electrons, leading to the formation of H_2O_2. O_2 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 eciency 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 (Ym), 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 Ym, 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 dierent 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 eectively 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 sucient 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.

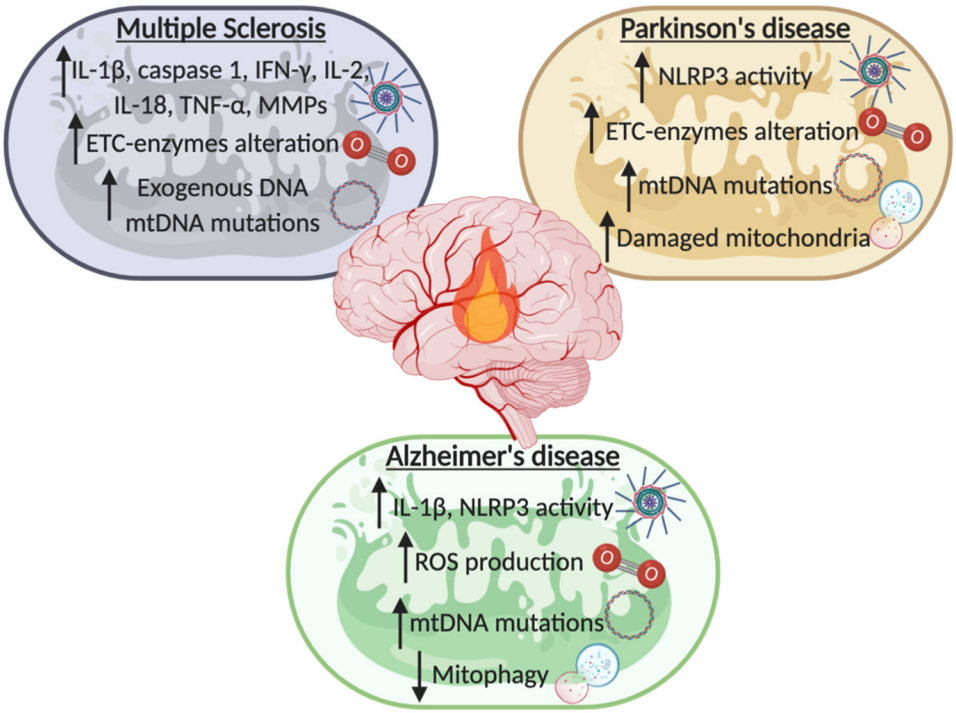

Figure 2. Schematic list of mitochondrial contribution to the recurrence of neuroinflammation during the development of neurodegenerative diseases (NDDs). In multiple sclerosis, Parkinson's disease, and Alzheimer's disease mitochondria have a fundamental role in the induction of a neuroinflammatory state 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 aected 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-, IL-2, IL-18, and TNF-, have been found in human samples

obtained from MS patients [95,96]. To become biologically active TNF- has to be cleaved by a disintegrin and metalloproteinase (ADAM-17) called TNF--converting enzyme (TACE). Interestingly, elevated levels of TNF- 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- processing [98]. Consistent with this, the most frequently used treatment for MS, namely IFN-, 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 dierent 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 aect 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-1 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- 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 aected 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 -Syn forming the Lewy bodies. Accordingly, autosomal dominant mutation of the gene encoding the -Syn protein (SNCA) determines familial PD [120]. Interestingly, -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 aects mitochondrial behaviors: to date dierent 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 Ym, 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 -Syn aggregates [134]. Lastly, also carbenoxolone, a heat shock protein inducer, was found to exert beneficial eects 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 aected 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 A. As described for the other NDDs, mitochondria play a key role also in the pathophysiology of AD. Interestingly, A 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 A interacts with dierent 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 A 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 aected 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, A was found sucient to activate NLRP3 inflammasome. Accordingly, NLRP3 knockout ameliorated A-related pathology and the development of cognitive decline [153]. Interestingly, mice expressing human tau mutations as well as patients aected 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 eects 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 eector molecule IL-1 were found in serum, CSF, and brain of patients with AD as well as other types of dementia [155]. Once secreted, IL-1 enhances the production of A by neurons and induces the phosphorylation of the tau protein [156,157]. Accordingly, IL-1 brain injection upregulates amyloid deposits levels and provokes amyloidogenesis, while IL-1 blockade reduces neuroinflammation by decreasing fibrillar A 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 3 and the cyclin dependent kinase 5, which are the mediators of the hyperphosphorylation of tau protein [163].

Proinflammatory cytokines production, including IL-1, 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 A 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-aected 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 aected 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 dierent outcomes [176–178]. The failure of blood supply dierentially aects 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 eects of reperfusion, identified NLRP3 mediated neuroinflammation as an eligible target [185–188]. Although the expression of dierent inflammasome components including NLRP1, NLRP3, NLRC4, ASC, caspase-1, and the proinflammatory cytokines IL-1 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 aects 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-1 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^+ eux, 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.

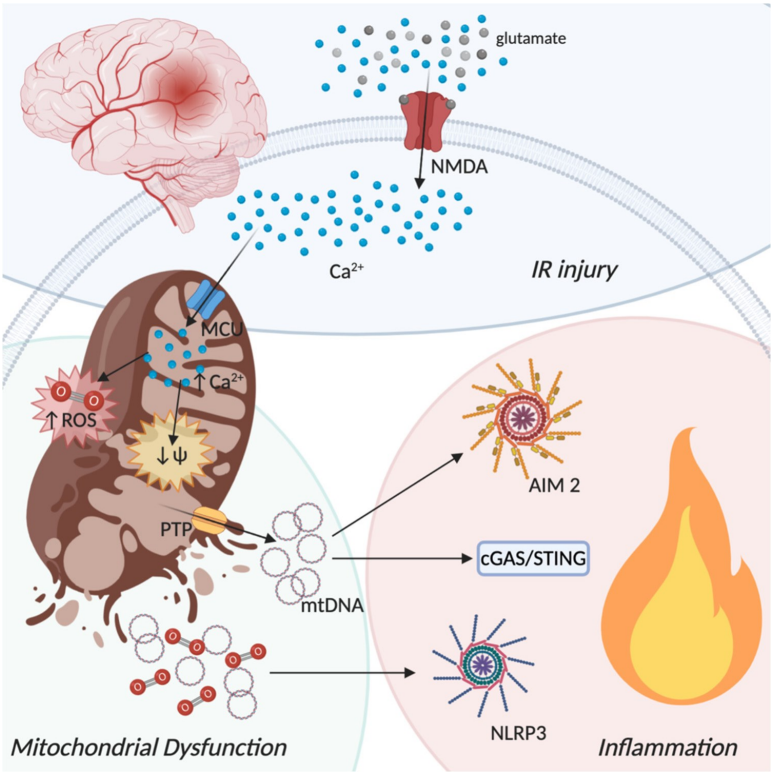

Figure 3. Ischemic-reperfusion injury from a mitochondrial perspective. Oxygen depletion occurring during an ischemic event lead to neuronal membrane depolarization with glutamate release in the extracellular space. Glutamate binds to N-methyl-D-aspartate (NMDA) receptors promoting a strong influx of 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 dierent 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 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 that leads to IR injury [216]. In agreement, the treatment with rotenone, a mitochondrial complex I inhibitor, causes the loss of Ym and thus increases ROS production, enhancing NLRP3-dependent IL-1 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 aecting 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 aected 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-1 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 aected 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].

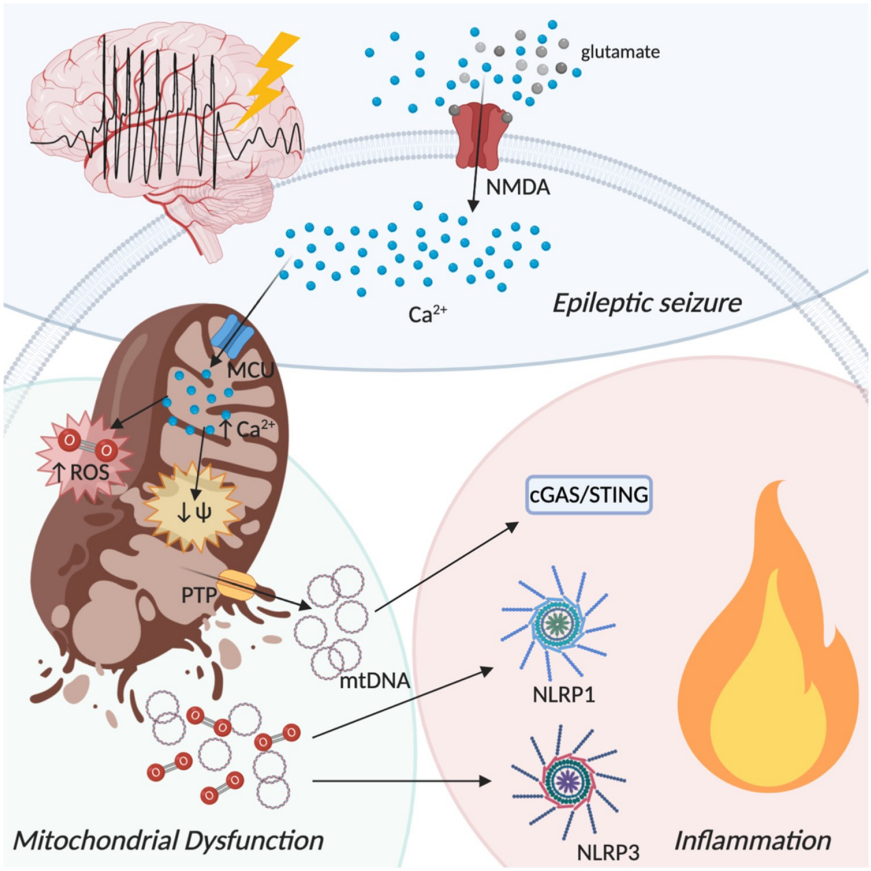

Figure 4. Epileptic seizure from a mitochondrial perspective. Repeated epileptic seizures activate NMDA receptors with the consequent influx of Ca^{2+} into the cytoplasm. Additionally, in this scenario, increased cytoplasmic Ca^{2+} concentration activates MCU. Augmented mitochondrial Ca^{2+} level triggers the activation of dierent 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-1 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-1, 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 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 , 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 eect 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 eect 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 dierent 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-1, 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-1 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- 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-, 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 (TGF-) [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-1 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 eective 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 eect 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 eect on dopaminergic neurons by downregulating microglial activation, thus decreasing TNF- and IL-1 production [284]. Another compound with promising eect 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 eects 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 eect in Parkinsonian patients (NCT04232969). As previously described, oxidative stress has a crucial role in triggering the degeneration of dopaminergic neurons. Accordingly, studies on dierent 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 eect 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 A-dependent injury, and its administration both in in vitro and in vivo models resulted in a strong inhibition of IL-1 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 eects in a mouse model of AD [289], while a pioglitazone phase III clinical trial was terminated because of lack of ecacy [290]. Chronic treatment with AF710B (ANAVEX 3-71), a selective allosteric M1 muscarinic and sigma-1 receptor agonist, showed anti-amyloid and anti-neuroinflammatory eects 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 eects 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 ecacious [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 ecacy in preventing brain tissue damage (NCT03320018). Lastly, the ecacy 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 eects in vitro [302]. Cannabidiol has also been investigated for both its anti-inflammatory and antioxidant properties [303]. Even though dierent phase III clinical trials are currently assessing the ecacy 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 eects.

Therapy	Disease	Eects	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 dierent 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 ecacy. 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 ecacy 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.

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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 A	adenosine triphosphate
BBB	-amyloid protein
BNIP3	blood–brain barrier
BNIP3L	B-cell lymphoma 2 nineteen kilodalton interacting protein 3
cGAMP	BNIP3-like
cGAS	$2^0,3^0$-cyclic GMP-AMP
CNS	cyclic GMP-AMP synthetase
CSF	central nervous system
CXC3CR1	cerebrospinal fluid
DAMPs	CX3C chemokine receptor 1
	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-1	interleukin-1
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- TLE	transforming growth factor
TLR	temporal lobe epilepsy
TNF-	Toll-like receptor
TREM2	tumor necrosis factor
-Syn	triggering receptor expressed on myeloid cells-2
Ym	-synuclein
	mitochondrial membrane potential

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CHAPTER 3: RESEARCH WORK

A crucial medical need in epilepsy is thoroughly investigating the mechanisms underlying the epileptogenesis process. Focusing on the neurobiological processes that can be induced by modulating the expression of specific genes, such as neurotrophic factors, cellular damage, and neuroinflammation may prompt the identification of effective targets. Addressing the need to discover effective anti-epileptogenic therapies to reduce all these hallmarks of the disease progression is a direct implication of this research focus.

3.1 Neurotropic factors

The neurotropic factors (NTFs) are involved in the growth and enhance neuronal survival. They play a significant role in both the development and maturation of nervous system, where they promote synaptic plasticity, neuronal survival, and the long-lasting memory formation. Released NTFs bind to their specific receptors, which are then internalized and transported by the retrograde axonal transportation to the soma of cell, where multiple survival-promoting effects are initiated (Machaliński et al., 2012). The expression of NTFs remains higher in the regions with ongoing plasticity, like the hippocampus but declines with age; indeed, NTFs can increase the axonal regeneration and the neuronal survival. They also promote the protective effects against the oxidative stress through a mitigating effect on ROS or interventions in the signal cascade induced by the ROS formation (Gardiner et al., 2009). Moreover, a reduction in NTFs expression can lead to an increased ROS production and cellular death via apoptotic pathways. They can also reduce the intracellular Ca^{2+} level derived by oxidative stress by induced antioxidant enzymes or calcium binding proteins. NTFs have been shown to improve neural regeneration in models of Alzheimer's disease (AD), Parkinson’s disease (PD), and Huntington’s disease (HD) (Xiao & Le, 2016).

3.2 Brain derived neurotropic factor (BDNF)

The Brain-derived neurotrophic factor (BDNF) promotes the differentiation, maturation, and neuronal survival in the CNS, exerting neuroprotective effects under pathological conditions like glutamatergic stimulation, hypoglycaemia, neurotoxicity, and cerebral ischemia. Indeed, it has been observed that BDNF expression levels drop in several NDDs, including Parkinson's disease (PD), multiple sclerosis (MS), and Huntington's disease (Mattson et al., 2004; Murer et al., 2001; Zuccato et al., 2010; Zuccato & Cattaneo, 2009). Besides the neuroprotective effects, it also plays a significant role in the energy homeostasis. Moreover, BDNF stimulates and controls neurogenesis from the neural stem cells, and the protein as well as the mRNA are widely expressed in the brain, in particular in the cortex, olfactory bulb, basal forebrain, hippocampus, mesencephalon, brainstem, hypothalamus, and the spinal cord. (Bathina & Das, 2015).

BDNF is one of the members of the neurotrophin family of the growth factors along-with the nerve growth factor (NGF), including neurotrophins-3 (NT-3), NT4/5, and NT-6. All the neurotropins bind with the high-affinity receptor tyrosine kinases, such as trkA, trkB, and trkC, and low-affinity receptors, such as p75NTR. NGF preferentially binds trkA, BDNF and NT-4/5 with trkB, and NT-3 with trkC. The interaction between receptors and their respective ligands can activate various signaling pathways, including MAPK-ERK, STAT, and PI3K-AKT. The human BDNF gene is localized on chromosome 11, region p13–14, that spans ∼70 kb. The gene structure is comprised of 11 exons (I–IX, plus Vh and VIIIh) in 5′ end and has 9 functional promoters which are used in the tissue and the brain regions, named exon I, II (with the transcripts IIa, IIb, and IIc), III, IV, V (with the transcripts Va, Vb, Vc, and V–VIII–VIIIh), VI (with the transcripts VIb, VIb–IXabd, and VIb–IXbd), VII (with transcripts VIIa and VIIb) and IX (with transcripts IXabd and IXabcd). Although the BDNF gene comprises nine exons, the coding sequence resides in exon 9, with eight upstream exons that encode promoters regulating spatial and cell-type-specific expression (Adachi, 2014). Each of the 5′ exons can generate a specific transcript or isoform through alternative splicing, which is all characterized by the presence of a common coding region at 3′ end. This 3′ coding area, located at exon IX, contains the common sequence which encodes for the pro-BDNF protein.

Accordingly, by alternative promoters and the splicing mechanisms, different transcripts of BDNF can be generated and all transcripts can encode for the same pro-BDNF protein product (Cattaneo et al., 2016).

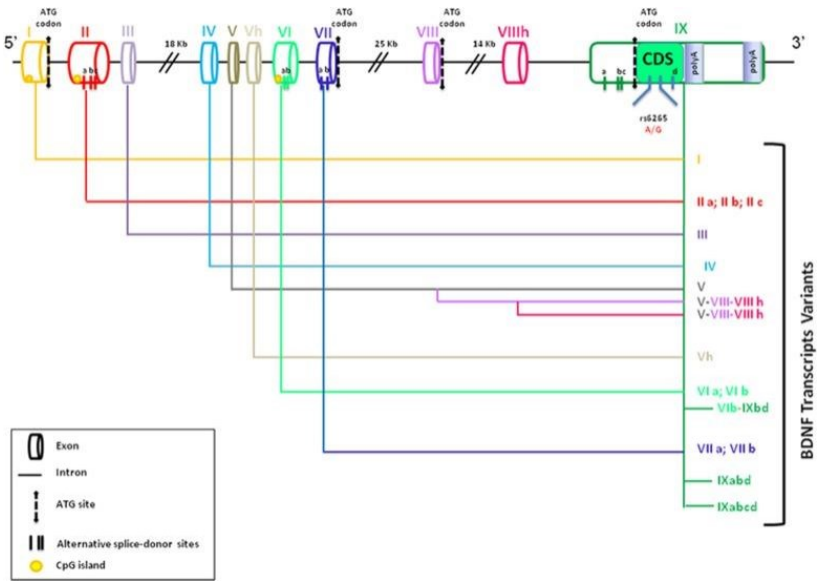

Figure 4: Structure of human BDNF gene (Cattaneo et al., 2016)

The gene contains 11 exons (I–IX, plus Vh and VIIIh), that combine with various different transcripts.

Coding DNA sequence (CDS) is within exon IX.

Abbreviations: Brain-derived neurotrophic factor (BDNF); Coding DNA sequence (CDS)

Indeed, BDNF is initially synthesized in the endoplasmic reticulum as a precursor protein (32–35 kDa); pre-pro-BDNF, and the signal peptide is subsequently then cleaved to produce a pro-BDNF, consisting of a pro-domain of 129 amino acids and a mature domain of 118 amino acids, which then moves through the Golgi apparatus and sorted into either regulated or the constitutive regulatory pathway. The pro-BDNF is sorted by vesicles in the presence of lipid raft-associated sorting receptor carboxypeptidase E (CPE). The vesicular proBDNF is either secreted as pro-BDNF and cleaved at the extracellular level for generating mature (mBDNF) or proteolytically cleaved intracellularly for generating the mBDNF. Its terminal domain is cleaved by a distinct protein convertase enzyme to form mature BDNF (mBDNF; 13 kDa), which is biologically active. The vesicular secretions can have both the pro-BDNF and mBDNF, with the specific amount of secreted mBDNF solely

depending upon the type and the activity of convertases enzymes (Bathina & Das, 2015; Cattaneo et al., 2016).

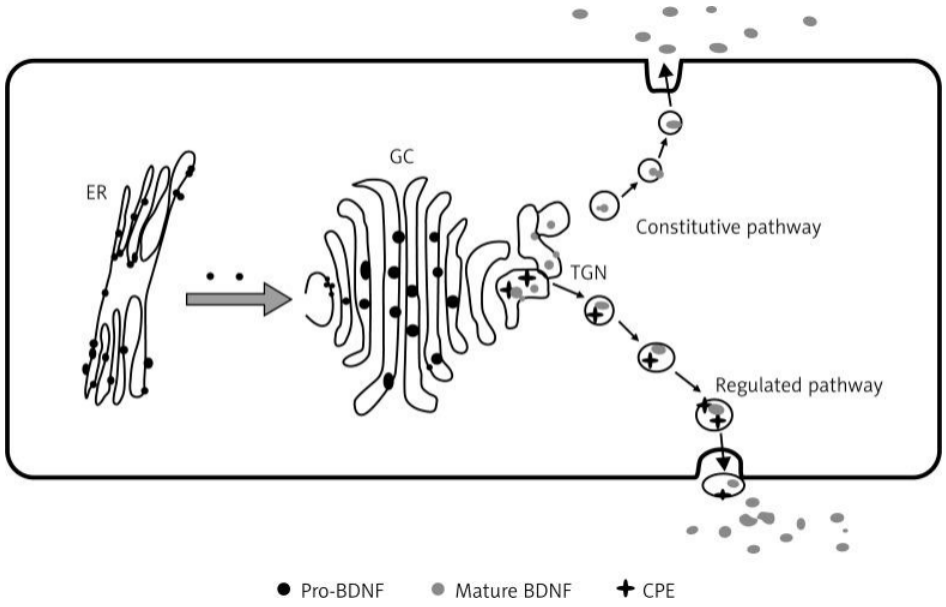

Figure 5: Synthesis, transport and release of mature BDNF (Bathina & Das, 2015)

BDNF executes its function by binding with 2 types of receptors: the TrkB and $p75^{NTR}$. Once activated, the receptor undergoes auto-phosphorylation of the intracellular domain in correspondence with tyrosine residues. The phosphorylated form recruits adapter proteins (Shc), and subsequently, the other proteins are necessary to activate their respective signaling pathways:

- *Mitogen-activated protein kinase (MAP) pathway*:

  The adapter protein initially recruits Grb2 and allows its binding to Sos, that lead to the activation of monomeric G protein Ras. Then, it activates Ser/Thr kinase Raf, which phosphorylates MEK kinase (MAP/ERK kinase), activating it. MAP kinase ERK (extracellular regulated kinase) is the target of MEK, which once in the nucleus, regulates gene expression by activating some transcription factors.

- *Phosphatidyl-inositol-3-kinase (PI3K) pathway*:

  The adapter protein recruits Grb2 and binds it to GAB1 and Sos by activating the PI3K kinase. Then, phosphorylates PDK1, which activates Akt (also called PKB), that once in the nucleus, regulates gene expression by activating some transcription factors.

- *PLCγ (phospholipase C) pathway:*

  Unlike other pathways, the intervention of adapter proteins is not required by PLCγ. Membrane phospholipid PIP2 (phosphatidylinositol 4,5-bisphosphate) is the substrate of this phospholipase, which is hydrolyzed to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Later, once it reaches to the endoplasmic reticulum level, it favors the entry of Ca^{2+} into the cytoplasm. In the presence of Ca^{2+}, DAG activates protein kinase C (PKC).

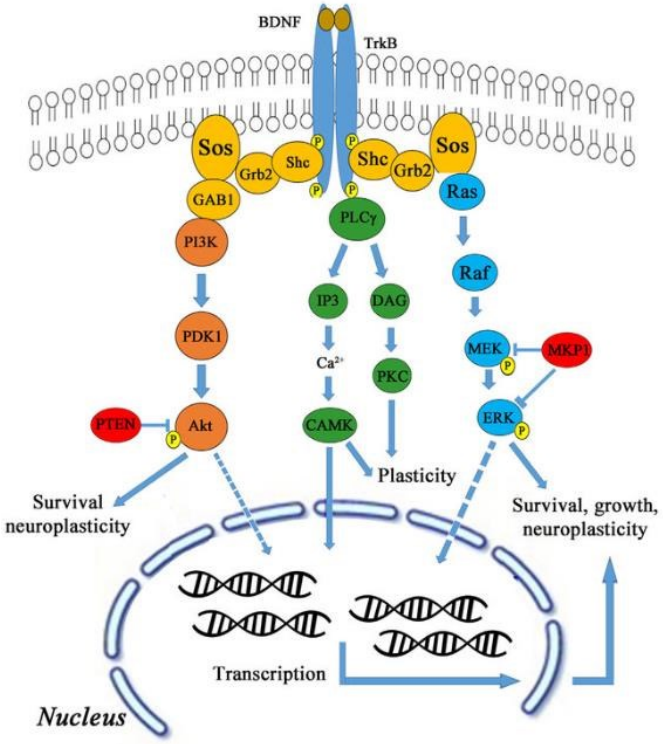

Figure 6**. **Activation of Signalling pathway by the binding of BDNF to TrkB receptor (Alam et al., 2016)

3.3 TrkB receptor and neuroinflammation

Some evidence in different neurological disease model has suggested that the activation of the TrkB receptor can attenuate neuroinflammation. A small partial agonist of the BDNF-TrkB receptor, named PTX BD4-3 (BD), has significantly reduced the astrogliosis and microgliosis in an animal model of focal cortical photothrombotic stroke (Gu et al., 2021). Luo et al., 2019 have shown that the upregulation of the BDNF/TrkB pathway can attenuate neuroinflammation and thus improve the

cognitive deficits in a streptozotocin-Induced AD rat model. Another study demonstrated that the activation of the BDNF/TrkB/ERK/CREB signaling pathway attenuated oxidative stress and neuroinflammation (B. Zhang et al., 2020). In addition, upregulation of BDNF/TrkB signaling can inhibit microglial activation, thereby reversing the aging-associated microglial activation by reducing the release of pro-inflammatory cytokines (Wu et al., 2020).

All these promising data led us to test the hypothesis that a TrkB agonist 7,8 dihydroxyflavone (7,8-DHF) could exert an anti-inflammatory effect in an in vivo model of temporal lobe epilepsy. The anti-inflammatory efficacy of 7,8-DHF was evaluated by analysing the level of astrocytosis using immunofluorescence in ex-vivo samples. Moreover, the effect on seizure severity and frequency and the associated co-morbidities were also evaluated. In our study, the administration of low- dose of 7,8-DHF (5 mg/kg) displayed a tendency to reduce markers of neuroinflammation, but these results were not significant.

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

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ORIGINAL ARTICLE

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

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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). The mechanism of these different dose-related effects remains to be elucidated. Nonetheless, considering its excellent safety profile and antioxidant properties, as well as its putative effects on TrkB receptors, 7,8-DHF represents an interesting template for the development of effective and well-tolerated anti-epileptogenic drugs.

Keywords Brain-derived neurotrophic factor · TrkB receptor · Epileptogenesis · Neuronal death

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 [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 dificult, 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 [1]. This implies the practical impossibility of clinically testing potential preventive therapies [2, 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 [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 [5–7] and causes enhanced activation of the BDNF high-afinity receptor, TrkB [8, 9]. Less clear is whether TrkB hyper-activation plays a pro- or anti-epileptogenic role. A local supplementation of

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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 [10]. 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 [11].

However, other lines of evidence suggest that BDNF is instead pro-epileptogenic: transgenic overexpression of BDNF is suficient to cause mTLE in adult mice [12, 13], and BDNF heterozygotes exhibit impairments in kindling epileptogenesis [14], an effect observed also after intraventricular administration of proteins that selectively scavenge BDNF [15]. 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 2 weeks after intra-amygdala kainate (KA)-induced SE prevents development of mTLE and comorbid anxiety-like behavior in $TrkB^{F616A}$ transgenic animals [16]. However, this also exacerbates SE-induced neuronal degeneration [17]. 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 [18]. In addition to its effect on TrkB, this compound is known to exert antioxidant effects [19]. Because reactive oxygen species are rapidly induced in the brain after epileptogenic insults, and antioxidant drugs have been reported to exert anti-epileptogenic effects [20], 7,8-DHF is expected to exert favorable effects. However, its actions on BDNF may represent a double-edged sword.

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 [21, 22].

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 [23]: 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 [24]. 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 99 rats that underwent this procedure, 15 (i.e., 15%) did not enter SE and 11 (11%) died during SE or within 24 h. The remaining 73 rats were assigned to the 3 experimental groups: vehicle, 7,8-DHF 5 mg/kg, and 7,8-DHF 10 mg/kg. Fifty have been employed for the video-EEG, behavioral and histological experiments, and the other 23 for Western blot. 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, except those used for Western blot that were killed 3, 7, or 21 days after SE. For the immunofluorescence and the Western blot studies, we also employed a group of 24 naïve animals.

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 [25, 26].

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 [23], 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 [27]. 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). Seizures were detected by visual inspection of the EEG by investigators that were blind of the group to which animals belonged. 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, and (iii) more than 5 spikes/s [28]. Video-EEG monitoring was performed 24 h/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 as follows: 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 [29]. 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 snifing or observing the object at less than 2-cm distance [30]. 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 [27], and mounted onto polarized slides (Superfrost slides, Diapath Martinengo, Bergamo, Italy). Sections were dewaxed and rehydrated as previously described [10]: 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 [27]. 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 [31], and an algorithm tailored to measure percent of suprathreshold pixels according to the IsoData method [32]. 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 [28]), 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.

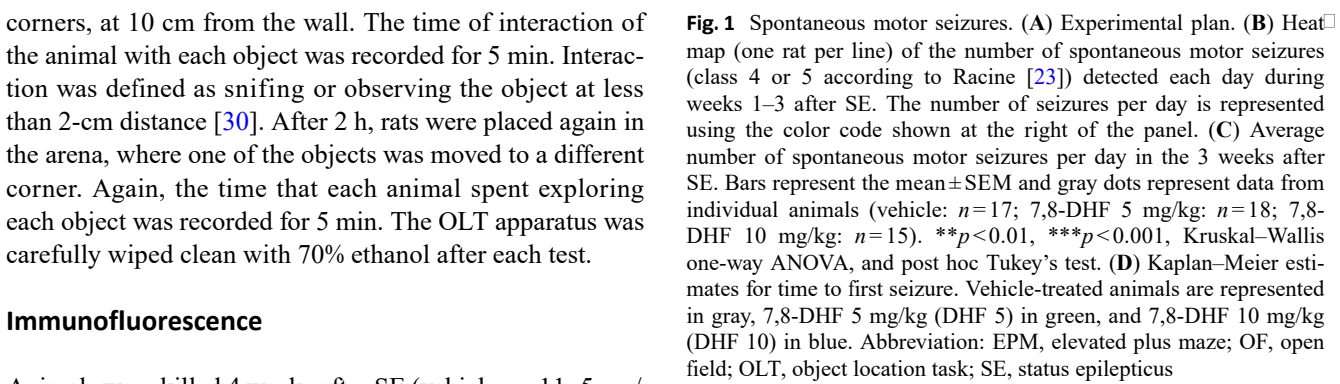
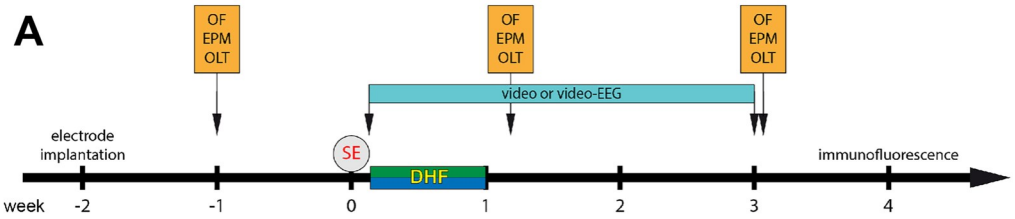


Fig. 1 Spontaneous motor seizures. (**A**) Experimental plan. (**B**) Heat map (one rat per line) of the number of spontaneous motor seizures (class 4 or 5 according to Racine [23]) detected each day during weeks 1–3 after SE. The number of seizures per day is represented using the color code shown at 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 (vehicle: $n=17$; 7,8-DHF 5 mg/kg: $n=18$; 7,8-DHF 10 mg/kg: $n=15$). $^{**}p<0.01$, $^{***}p<0.001$, 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, and 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

Western Blot Analysis

Tissue homogenization was performed as previously described [33]. 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).



B

rat # \ dpSE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0	0	0	0	0	1	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
7	0	0	0	0	0	0	0	1	0	0	2	1	0	1	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	1	2	0	0
10	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1
12	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
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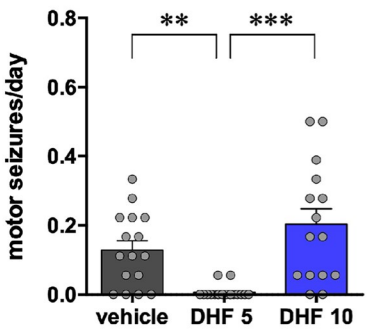
C



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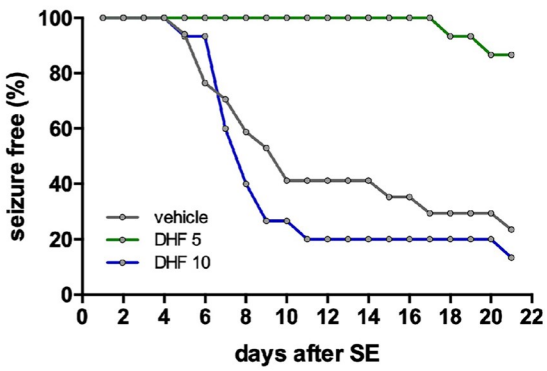


For loading controls, membranes were stripped in acidic buffer (0.2 M glycine, 0.1% SDS, 1% Tween-20, pH 2.2) and re-probed with the appropriate antibody. In the cases where stripping was not possible, the same lysates were run simultaneously on duplicate gels, and probed with phospho- and total antibodies. 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 Supplementary Table S1.

Statistical Analysis and Data Availability

Statistical analyses performed in this study are reported in Supplementary Table S2. 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 1 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

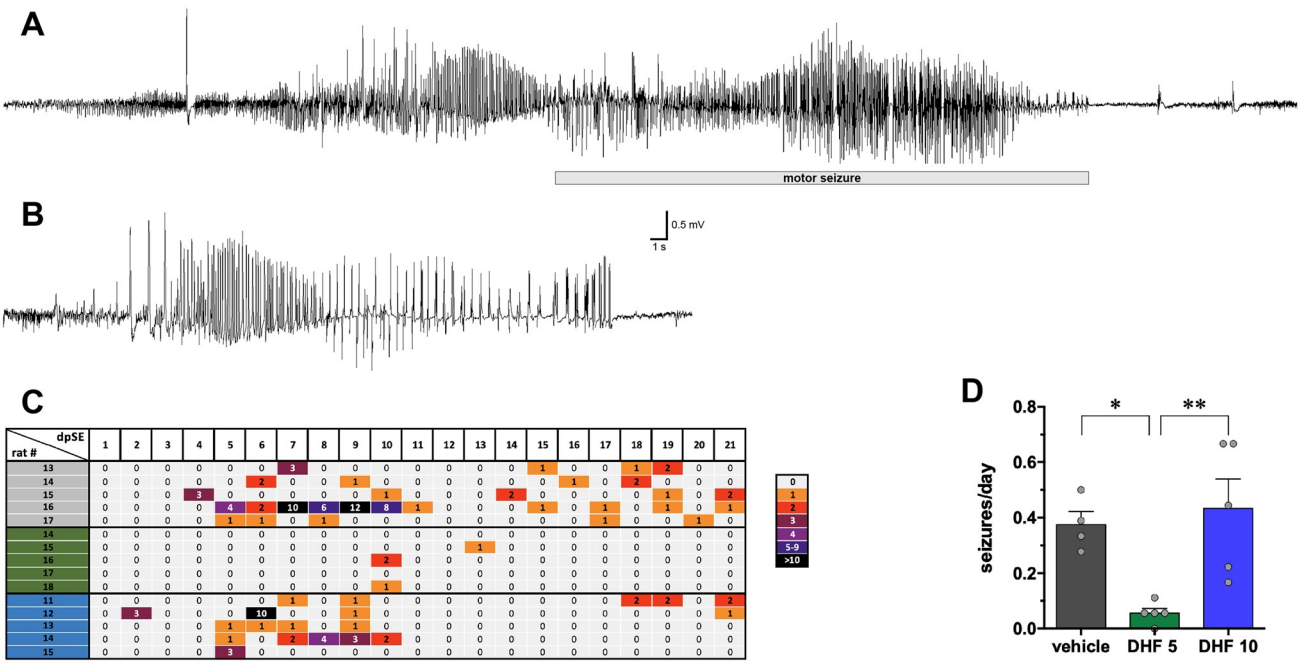


Fig. 2 EEG. Representative EEG patterns in the hippocampus during motor (**A**) and nonmotor (**B**) seizures in vehicle-treated animals. Identical patterns were observed in animals treated with 7,8-DHF 5 or 10 mg/kg. The horizontal bar in (**A**) indicates the motor part of the seizure. (**C**) Heat map (one rat per line) of the number of spontaneous EEG seizures (motor and nonmotor) 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. (**D**) 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 (vehicle: $n = 5$; 7,8-DHF 5 mg/kg: $n = 5$; 7,8-DHF 10 mg/kg: $n = 5$). *$p < 0.05$, **$p < 0.01$, 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, and 7,8-DHF 10 mg/kg (DHF 10) in blue

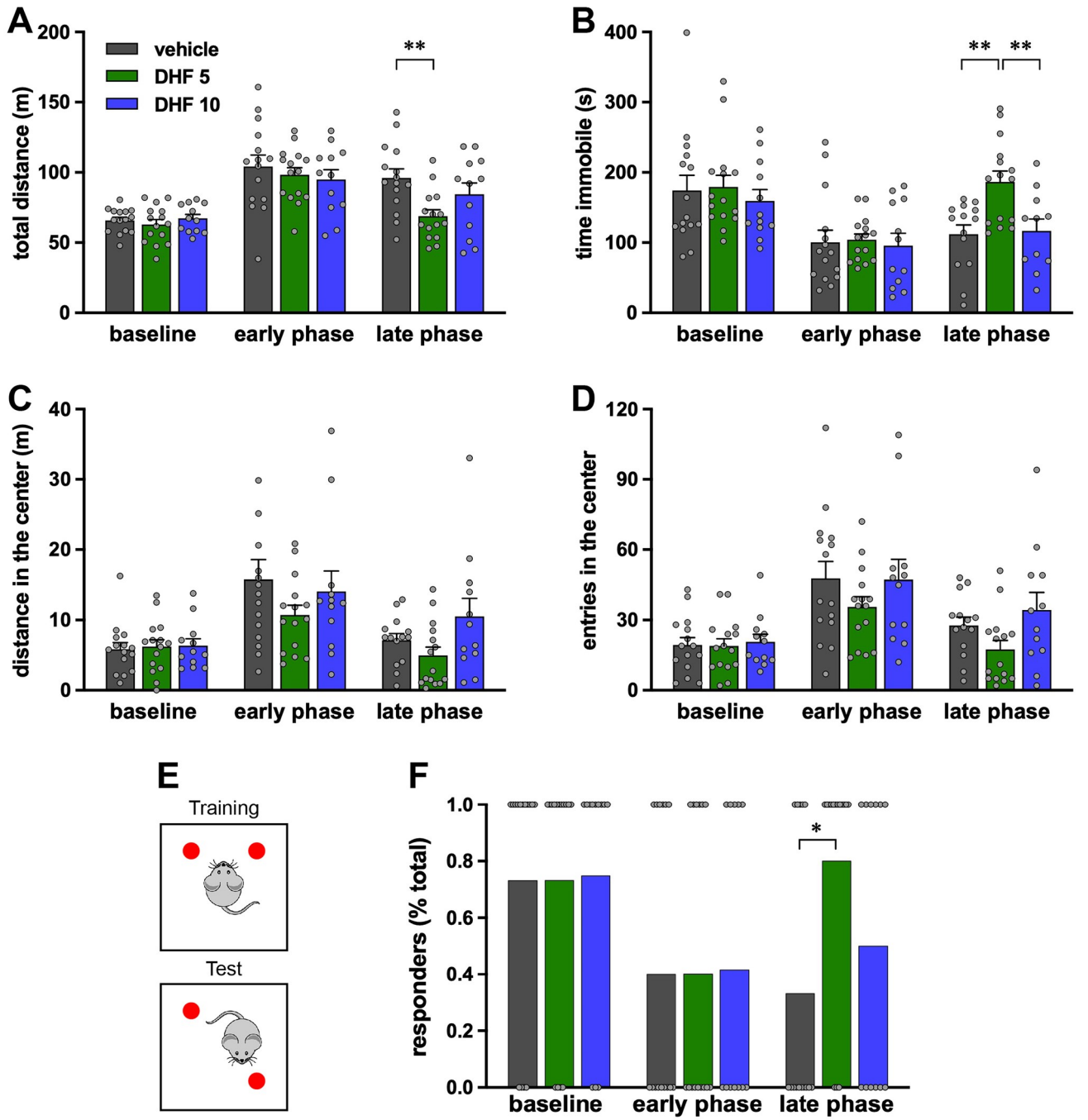


Fig. 3 Open field (OF) test and object location task (OLT). OF was performed before SE (baseline), 8 days (early phase), or 21 days (late phase) after SE. (**A**) Total distance run by each rat. (**B**) Time spent immobile. (**C**) Distance run in center quadrants. (**D**) Number of entries in the center quadrants of the arena. Bars represent the mean ± SEM and gray dots represent data from individual animals (vehicle: $n = 15$; 7,8-DHF 5 mg/kg: $n = 15$; 7,8-DHF 10 mg/kg: $n = 12$). $^{**}p < 0.01$, 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, and 7,8-DHF 10 mg/kg (DHF 10) in blue. (**E**) Schematic representation of the OLT test (see "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 2-h 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). (**F**) 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, and 7,8-DHF 10 mg/kg (DHF 10) in blue. $^{*}p < 0.01$, Fisher's exact test

(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 C), 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 comorbidities 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 D). 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 B), but was not affected by 10 mg/kg DHF (Fig. 3A–D).

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 Supplementary Fig. S1). 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 (Supplementary Fig. S1). 7,8-DHF did not modify this phenotype, neither at the early or at the late phase, at 5 or at 10 mg/kg (Supplementary Fig. S1).

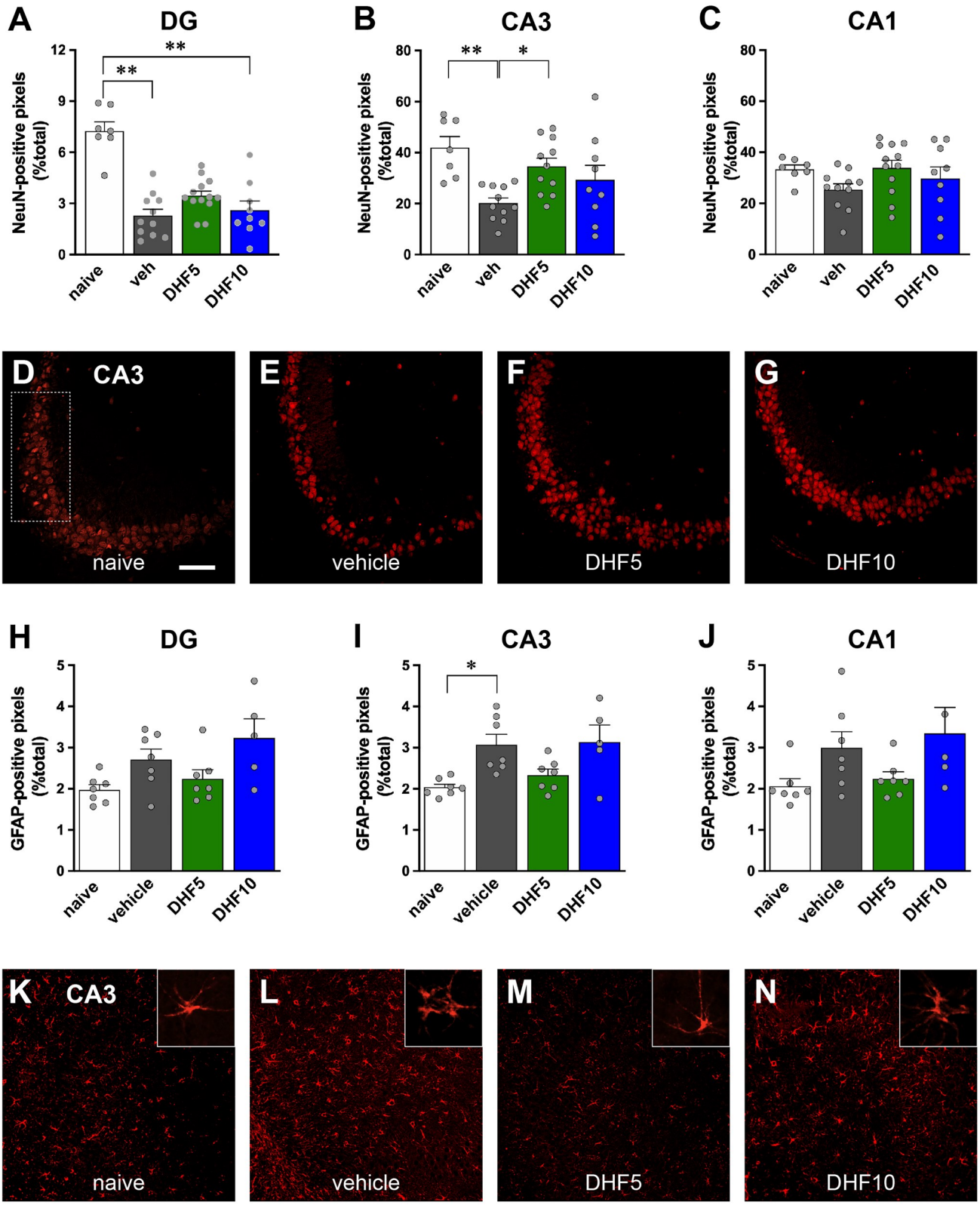
Finally, we explored cognitive abilities using the OLT that evaluates short-term, hippocampal-dependent spatial memory [34]. At baseline, all rats displayed a clear exploratory preference for the re-located novel object, but this preference disappeared with the development of spontaneous seizures (Fig. 3E–F), 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. 3F). 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.

Fig. 4 Immunohistochemical analysis. Quantification of NeuN-positive pixels in the 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 "Methods" for details. Bars represent the mean ± SEM and gray dots represent data from individual animals (naïve: $n=7$; vehicle: $n=11$; 7,8-DHF 5 mg/kg: $n=13$; 7,8-DHF 10 mg/kg: $n=9$). Naïve animals are in white, vehicle-treated animals in gray, 7,8-DHF 5 mg/kg (DHF 5) in green, and 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. Higher-magnification inserts illustrate the changes in the morphology of GFAP-positive cells

Immunofluorescence

To evaluate effects on epilepsy-associated neuronal death, we performed NeuN immunofluorescence [10] in a representative subgroup of epileptic animals in comparison with naïve, non-epileptic controls. A loss in NeuN-positive cells was observed in the hilus of the dentate gyrus (DG) and in the CA3 pyramidal layer of vehicle-treated animals 1 month after pilocarpine-induced SE (Fig. 4A–E). Five mg/kg 7,8-DHF was found to partially protect from damage (Fig. 4A–G). In fact, (i) the loss of NeuN signal in the hilus of the DG was highly significant in vehicle but not in 5 mg/kg 7,8-DHF-treated pilocarpine animals (Fig. 4A); (ii) a nearly complete protection from CA3 neuronal loss was observed in animals treated with 5 mg/kg 7,8-DHF (Fig. 4B and D–G). The higher dose of 7,8-DHF did not provide a significant neuroprotection, but only a tendency to improvement in CA3 and CA1. However, no significant difference was observed between 5 and 10 mg/kg 7,8-DHF in any hippocampal subarea (Fig. 4A–G).

Epilepsy-associated astrocytosis was evaluated using GFAP immunofluorescence in sections adjected to those employed for NeuN. Consistent with previous findings [35], 1 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 DG and in CA1 (Fig. 4H–L). In addition, many of the GFAP-positive cells in epileptic controls displayed short, thick processes, an indication of activated astrocytes (Fig. 4L insert). Once again, 5, but not 10 mg/kg 7,8-DHF prevented all these effects (Fig. 4H–N).


A
DG
NeuN-positive pixels (%total)
naive
veh
DHF5
DHF10
B
CA3
C
CA1
D
CA3
naive
E
vehicle
F
DHF5
G
DHF10
H
DG
GFAP-positive pixels (%total)
I
CA3
J
CA1
K
CA3
naive
L
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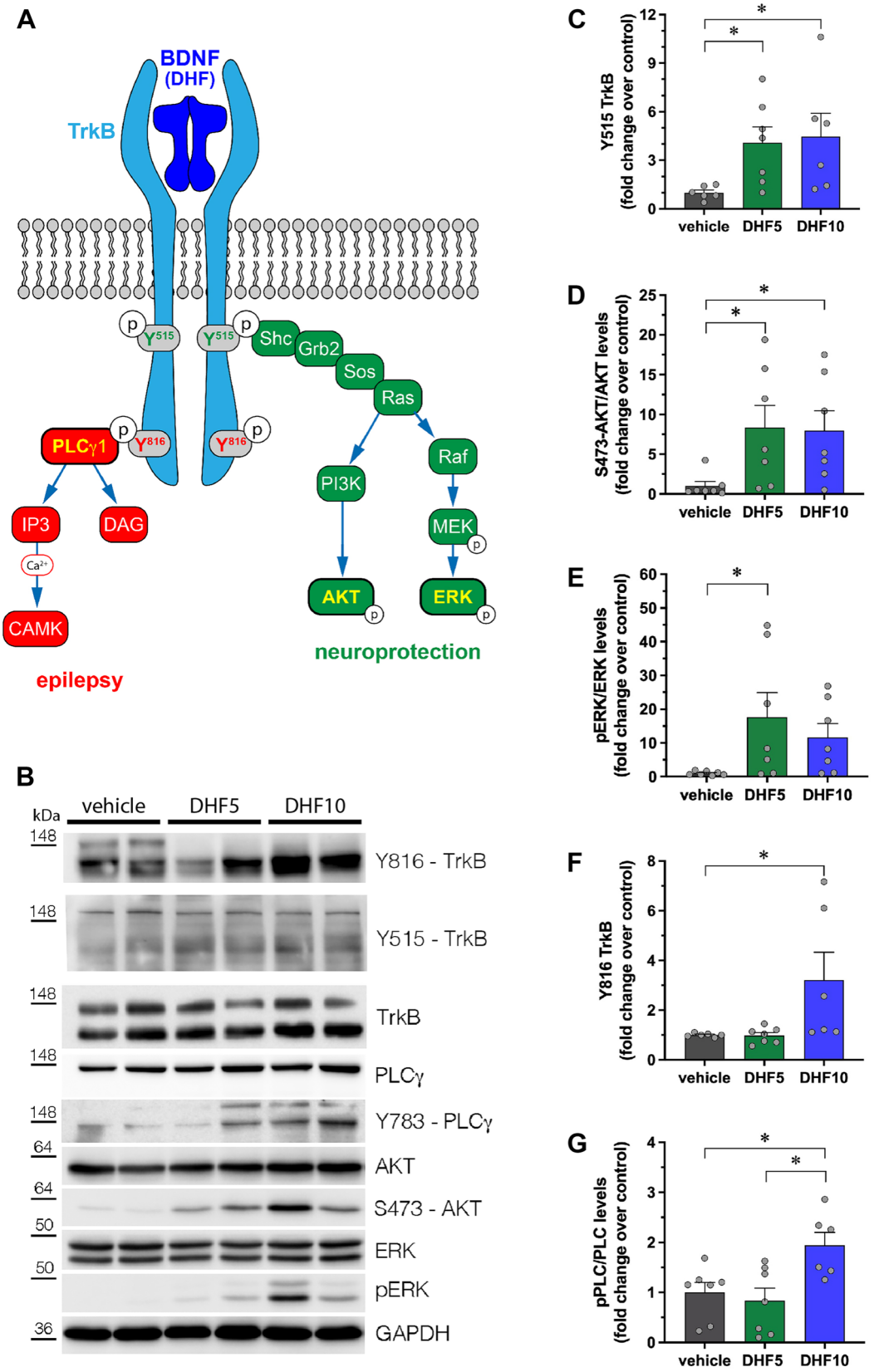
A
BDNF
(DHF)
TrkB
p
Y515
Y515
Shc
Grb2
Sos
Ras
PLCγ1
Y816
Y816
PI3K
Raf
IP3
DAG
MEK
Ca2+
AKT
ERK
CAMK
epilepsy
neuroprotection
B
kDa
vehicle
DHF5
DHF10
148
Y816 - TrkB
148
Y515 - TrkB
148
TrkB
148
PLCγ
148
Y783 - PLCγ
64
AKT
64
S473 - AKT
50
ERK
50
pERK
36
GAPDH
C
Y515 TrkB
(fold change over control)
vehicle
DHF5
DHF10
D
S473-AKT/AKT levels
(fold change over control)
E
pERK/ERK levels
(fold change over control)
F
Y816 TrkB
(fold change over control)
G
pPLC/PLC levels
(fold change over control)

Fig. 5 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). Vehicle: $n=7$; 7,8-DHF 5 mg/kg: $n=7$; 7,8-DHF 10 mg/kg: $n=7$. *$p<0.05$, Kruskal–Wallis one-way ANOVA, and post hoc Tukey's test

Taken together, these data suggest that low 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. To the best of our knowledge, antioxidants have not been reported to lose their antiepileptogenic effect with an increase in dose. We therefore decided to start exploring the alternative hypothesis that may explain this observation is that these effects 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) [36]. Whereas the former pathway has been reported to exert neuroprotective effects, the latter has been suggested to produce pro-epileptogenic effects (Fig. 5A) [37].

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. First, hippocampal homogenates isolated from vehicle or 7,8-DHF-treated-naïve rats were analyzed by western blot, as shown in Fig. 5B. After 7 days of treatment, we found that, whereas 7,8-DHF increased TrkB Y515, AKT, and ERK phosphorylation to similar levels at both doses (Fig. 5C–E), only the dose of 10 mg/kg increased (by ~ two-fold) the levels of phosphorylated TrkB Y816 and of PLCγ1 (Fig. 5F and G). Level of all phosphorylations was not significantly different from control values after 3 days of 7,8-DHF treatment or 14 days after its discontinuation (data not shown).

We then attempted to extend these observations to epileptic rats. Seven days after SE, all phosphorylations were dramatically increased, and returned to near baseline levels after 21 days (Supplementary Fig. S2). We could not detect any significant further increase with 7,8-DHF treatment, except for a tendency to increased levels of phospho-AKT in both low and high dose-treated animals, and a tendency to increased levels of phosphor-PLCγ only in those treated with high-dose 7,8-DHF (Fig. 6). These changes, however, were also non-significant.

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 [18], however, we found highly significant dose-dependent differences in many respects (in particular SRSs and cell death).

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 [38]. In fact, 7,8-DHF is a flavonoid originally known for its antioxidant properties [19], and more recently recognized as a high-afinity 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) [18]. 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 [18]. However, no study was performed thus far in epilepsy models except one [39] in which 5 mg/kg 7,8-DHF was administered for three times only (one every other day), the last time 21 days *before* kainate-induced SE. In other words, this is the first study in which 7,8-DHF was administered under clinically-relevant conditions, i.e. *after* SE and during epileptogenesis.

The puzzling observation that 7,8-DHF exerts highly favorable effects at a low, but not at a higher dose, is difficult to interpret. Antioxidant drugs are well known to exert anti-epileptogenic effects and, even if dose–response studies are not yet available, no evidence thus far supports the possibility that they may lose effect with an increase in dose [20]. However, this event cannot be excluded, because several anti-oxidant dietary phytochemicals have been reported to exert pro-oxidant activities at high doses [40]. Therefore, future studies should be designed to explore this possibility. Another hypothesis, explored in the present study, may be that the reduced antiepileptogenic effect of 7,8-DHF at relatively higher doses may be due to unwanted actions on TrkB receptors. In fact, conflicting data are available on pro- or anti-epileptogenic implications of the BDNF/TrkB system [4]. A possible explanation of this conundrum has been recently found to lay in the different TrkB receptor

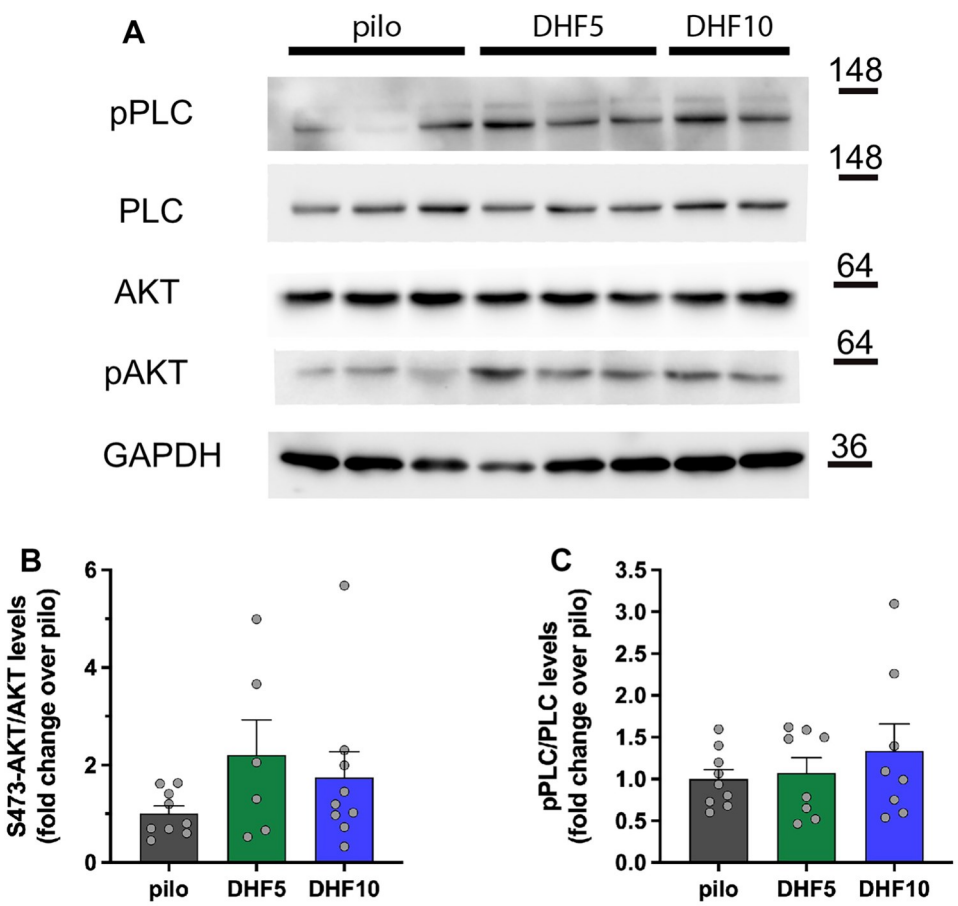

Fig. 6 Phosphorylation of AKT and PLCγ proteins in the hippocampi of epileptic rats treated with 7,8-DHF. (**A**) Representative western blot of the indicated proteins in extracts from hippocampi of 7,8-DHF-treated rats. (**B**–**C**) Quantification of S473-AKT (**B**) and Y783-PLCγ (**C**) phosphorylation. Protein levels are shown as fold change over control (lithium-pilocarpine-treated rats). Levels of phosphorylated proteins are normalized against the corresponding total protein, then for loading (GAPDH). Pilocarpine: $n = 9$; 7,8-DHF 5 mg/kg: $n = 9$; 7,8-DHF 10 mg/kg: $n = 9$

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 [17]. 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 [37].

We observed beneficial effects on epileptogenesis with the low dose (5 mg/kg) of 7,8-DHF, a dose regimen at which, in naïve rats, we found a selective phosphorylation of the Y515 residue of TrkB (i.e., no phosphorylation at Y816) and a 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 [16], 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.

However, we were unable to confirm these findings in lithium-pilocarpine rats. It should be kept in mind that this was a very dificult experiment, because of multiple confounding factors. First, lithium-pilocarpine SE per se increases TrkB activation, making changes induced by 7,8-DHF proportionally smaller. Second, recent seizures can further boost TrkB receptor activation for a relatively long time. Therefore, levels of phosphorylations are expected to undergo remarkable oscillations in time in epileptic animals. Seven days after SE, some of the animals treated with vehicle- or 10 mg/kg 7,8-DHF (but not those treated with 5 mg/kg — Fig. 2) may have already experienced EEG seizures. All these factors notwithstanding, our hypothesis remains undemonstrated at this time, and further studies will be required to clarify the reason of the paradoxically greater effects of low-dose 7,8-DHF.

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 [41]. Incidentally, these findings suggest that 7,8-DHF and BDNF activate TrkB with different mechanisms, indirectly 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 [16]. 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 [42–45]. In the chronic course of epilepsy, these behavioral alterations are generally maintained [43–45], 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 (good pharmacokinetics and tolerability, antioxidant effects, profile of actions on the TrkB receptor, and prospective short-term administration following an epileptogenic insult), 7,8-DHF may represent a candidate for a preventive, anti-epileptogenic therapy or, probably more realistically, a template for developing an effective and well-tolerated anti-epileptogenic drug. In the prospect of clinical translation of 7,8-DHF, the present data suggest that an accurate dose titration would be needed, and this may imply identification of an optimal window of plasma concentrations and a better understanding of the mechanism of 7,8-DHF bell-shaped anti-epileptogenic effect.

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Declarations

Ethic Statement All experiments with laboratory animals were conducted according to European guidelines (directive 2010/63/EU) and the Ethics Committee on Animal Experimentation at the University of Ferrara (Ferrara, Italy).

Conflict of Interest The authors declare no competing interests.

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3.3 Fibroblast growth factor-2

Fibroblast growth factor-2 (FGF-2) is a protein belonging to the family of fibroblast growth factors that play a significant role in tissue development and repair. FGF-2 is a single-chain polypeptide comprised of 146 amino acids, which primarily localizes in specified neuronal populations, e.g., CA2 pyramidal cells of the hippocampus. Structurally, FGF-2 exhibits a β tre-foil structure containing 4 β-sheets that are arranged in a triangular conformation. Two β stands (β10 and β11) have various amino acid residues constituting the primary binding site for heparin. There are four distinct isoforms of the FGF-2: 3 high molecular weight forms (22, 22.5, and 24 kDa) predominantly localized inside the nucleus and one low molecular weight form (18 kDa), which is localized in the cytosol (Simonato 2018). It executes its functions by binding to specific receptors called FGFR1-3, found on the cells' surface. FGF-2 preferably binds to certain forms of these receptors, called FGFR1c, FGFR2c, FGFR3c, and FGFR1b, which are generated through the alternative splicing of extracellular immunoglobulin domain-III. FGF-2 binds FGFR1 with the highest affinity. High-affinity receptors are tyrosine kinase receptors capable of phosphorylating protein substrates at the level of tyrosine residues. The binding of FGF-2 to FGFR1 results in the dimerization of FGFR1 and trans-phosphorylation of their tyrosine kinase domain. This process activates the receptor and triggers a series of intracellular signaling pathways, including PI3K-AKT, RAS-MAPK (ERK1/2), STAT, and PLCγ-PKC (Koledova et al., 2019; Robbins, 1999). On the other hand, low-affinity receptors consist of heparan sulfate (HS) proteoglycans that allow the receptor dimerization necessary to enhance its affinity and stabilization of the neurotrophic factor-receptor complex. FGF-2 exerts multiple pleiotropic biological activities that can influence multiple neuronal populations. It regulates the proliferation, survival, and differentiation of neural stem cells, modulates synaptic plasticity, and maintains the integrity of brain tissue (Simonato, 2018).

FGF-2 is also the most potent known mitogenic factor for neural stem cells. As a mitogenic factor, it stimulates the proliferation of the cellular progenitors and directs their differentiation towards the different phenotypes, while as a NTF, it endorses the survival and the differentiation of the neuronal

precursors in a glial or neuronal sense (Simonato, 2018). FGF-2 promotes neuroprotective effects against a variety of damages or injuries by reducing cellular damage and stimulating brain recovery in experimental models of epilepsy and ischemia as well as traumatic brain or spinal cord injury (Q. Li & Stephenson, 2002).

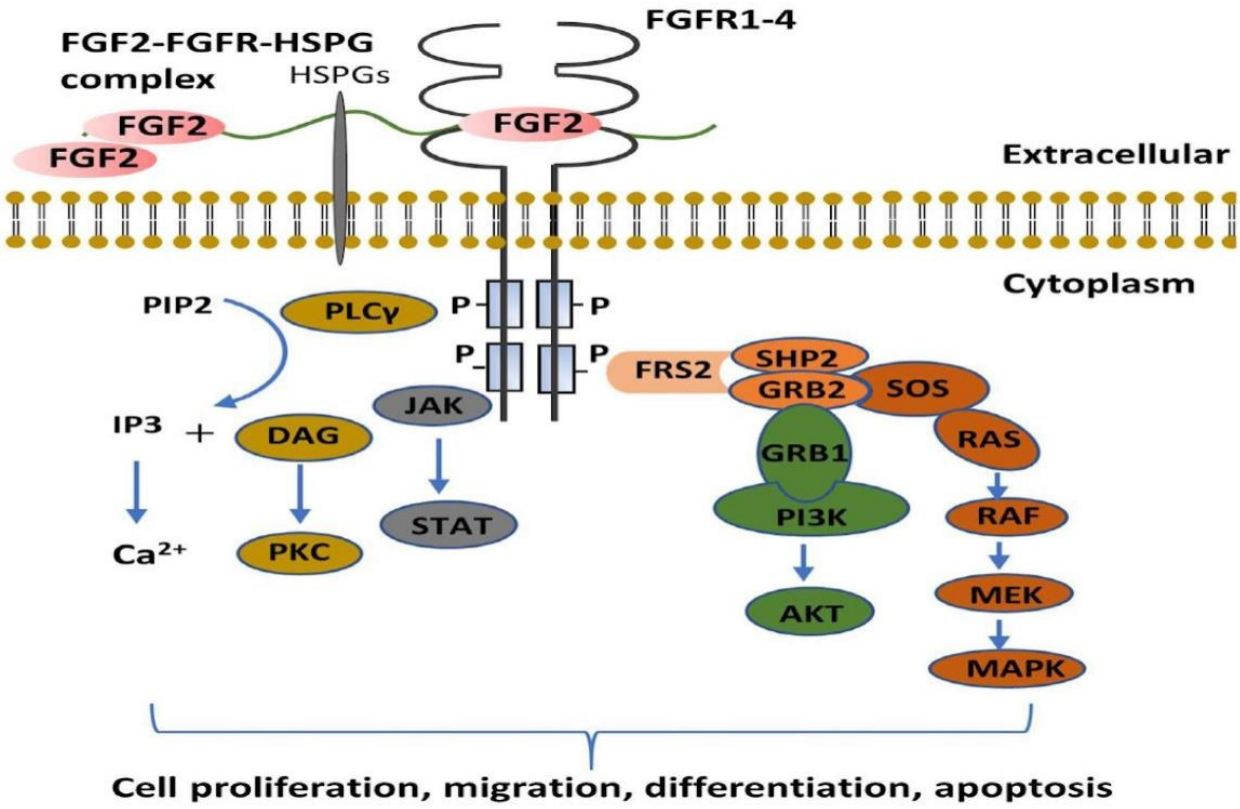

Figure 7. Activation of signaling pathways by the binding of FGF-2 with *FGFRs* (Tan et al., 2020)

FGF-heparin/heparan sulfate proteoglycan interaction stabilizes the peptide from thermal as well as enzymatic denaturation, increases the affinity and half-life of FGF-2-FGFR complex, and limits the diffusion of FGF-2 into the interstitial spaces.

3.4 BDNF and FGF2 in TLE models

Shreds of evidence suggest that mRNA and protein levels of FGF-2 are increased and FGFR1 receptors are upregulated after seizures. Acute FGF-2 intrahippocampal injection evokes seizures,

while low-dose FGF-2 chronic intracerebroventricular (i.c.v.) infusion improves the behavioral recovery and hippocampal damage but does not affect kainate-induced seizures. Hence, FGF-2 might favor the generation of seizures and reduce their consequences, like hippocampal damage (Simonato, 2018). A study was also carried out in transgenic FGF-2 (TgFGF2) mice to evaluate the effect of upregulated FGF-2 expression on seizure susceptibility. Higher expression of FGF-2 was observed in hippocampal pyramidal and granule neurons. In addition, TgFGF2 mice display an increase in the density of hippocampal glutamatergic synaptic vesicles and enhanced excitatory inputs in the pyramidal cells of CA1, suggesting the presence of a latent hyper-excitability. These mice are also more susceptible to SE induced by kainate when compared with wild type (WT), but they display a reduction in seizure-induced cellular death.

BDNF is also implicated in the epileptogenesis, as seizures can highly increase the expression of BDNF and activate the TrkB receptors (Simonato, 2018). It has also been reported to augment excitatory synaptic transmissions and attenuate GABAergic inhibitory synaptic transmissions (H. E. Scharfman et al., 1999; Tanaka et al., 1997). The local delivery in the hippocampus of a viral vector expressing the combination of FGF-2 and BDNF can reduce damage, promote the growth, development, and differentiation of neural stem cells, and prevent seizures in an epilepsy model (Paradiso et al., 2009). These experiments were in an animal model of TLE, pilocarpine-induced status epilepticus (SE). This model closely resembles the damage pattern observed in the hippocampus of many epileptic patients (hippocampal sclerosis) who experiences an epileptogenic insult like SE. The vector was administered in the hippocampus during epileptogenesis, i.e., before the occurrence of spontaneous seizures. When the vector expressing FGF-2 and BDNF was administered, it slightly reduced ongoing cell loss, but this effect was not statistically significant. This suggests that the neuroprotective effect of the vector in vivo may be limited or may require higher levels of transgene expression over a longer period of time. However, the vector had a significant effect on neurogenesis, promoting the proliferation of early progenitors and the production of cells that developed into neurons. It also reduced the abnormal aspects of neurogenesis induced by SE.

One month after induction of SE, all of the animals that were not treated with TH-FGF2/0-BDNF (the therapeutic vector) developed hippocampal sclerosis and had SRSs. In contrast, the animals that were treated with TH-FGF2/0-BDNF had a significant reduction in cell loss in the hippocampus. The animals were also monitored with video-EEG for 20 days to see the effect of these NTFs on seizure frequency and severity of SRSs. All of the rats that were not treated with the vector developed SRSs, as expected. In contrast, the rats that were treated with the vector had a significant improvement in the frequency and severity of SRSs: some animals (2 out of 11) never developed SRSs during the observation period, the average number of seizures/day was significantly reduced, and the seizure severity was also significantly attenuated (Paradiso et al., 2009).

This epilepsy model also causes severe neuroinflammation, particularly in the phase preceding the occurrence of spontaneous seizures. Supplementing with FGF-2 and BDNF was able to attenuate various parameters of neuroinflammation, including microcytosis, astrocytosis, and the expression of IL-1β. The effect was most prominent on IL-1β expression, whose overexpression was almost completely prevented (Bovolenta et al., 2010).

The following study aimed to test if the attenuation of neuroinflammation obtained in vivo with BDNF and FGF-2 was direct or secondary to other effects, such as the reduction in SRS severity and frequency. To pursue an answer to this question, we used an in vitro model of neuroinflammation induced by lipopolysaccharide (LPS, 100 ng/ml) in a mouse primary mixed glial culture. The release of cytokines and NO was analysed. Moreover, the effect of LPS and neurotrophic factors on the cell viability were also determined. Our study suggests that the anti-inflammatory effect of BDNF and FGF-2 in vivo in epilepsy model is indirect and likely due to a reduction in seizure frequency and/or severity.

Lack of direct effects of neurotrophic factors in an in vitro model of neuroinflammation

Original article

Lack of direct effects of neurotrophic factors in an in vitro model of neuroinflammation

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Abstract

Neuroinflammation is associated with several neurological disorders, including temporal lobe epilepsy. Seizures themselves can induce neuroinflammation. In an in vivo model of epilepsy, the supplementation of Brain-derived neurotropic factor (BDNF) and Fibroblast growth factor-2 (FGF-2) using a Herpes-based vector reduced the epileptogenesis-associated neuroinflammation. The aim of this study was to test if the attenuation of the neuroinflammation obtained in vivo with BDNF and FGF-2, was direct or secondary to other effects, for example the reduction in spontaneous recurrent seizures severity and frequency. An in vitro model of neuroinflammation induced by lipopolysaccharide (LPS, 100 ng/ml) in a mouse primary mixed glial culture has been used. The release of cytokines and NO was analysed by ELISA and Griess assay respectively. The effect of LPS and neurotrophic factors on the cell viability was determined by performing MTT assay. BDNF and FGF-2 have been tested alone and co-administered. LPS induced a significant increase in pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and NO. BDNF, FGF-2 and their co-administration did not counteract these LPS effects. Our study suggests that the anti-inflammatory effect of BDNF and FGF-2 in vivo in epilepsy model was indirect and likely due to a reduction in seizure frequency and severity.

Keywords: Neuroinflammation; Brain derived neurotropic factor; Fibroblast growth factor-2; Cytokines; Lipopolysaccharide

1. Introduction

The Neuroinflammation is a well-orchestrated and complex process involving glial cells, particularly astrocytes and microglia. Many lines of evidence have revealed the association of neuroinflammation with neurological diseases, including epilepsy (Rana & Musto, 2018; Vezzani et al., 2019). Experimental data have demonstrated that acute seizures can induce neuroinflammation and that spontaneous recurrent seizures (SRSs) can cause chronic neuroinflammation (Vezzani et al., 2011). On the other hand, neuroinflammation can facilitate the occurrence of seizures (Pracucci et al., 2021).

Neurotropic factors (NTFs) are involved in the growth, survival, and differentiation of developing and mature neurons. In the adult central nervous system (CNS), not only they exert these neurotrophic effects, but also functional effects at the synaptic level (Schinder & Poo, 2000). These features led to the hypothesis that some NTFs, in particular the fibroblast growth factor-2 (FGF-2) and the brain-derived neurotrophic factor (BDNF), may positively impact on epileptogenesis-associated cell death and circuit rearrangements (Simonato et al., 2006). In fact, both FGF-2 and BDNF protect neurons from ongoing damage and, furthermore, FGF-2 is a potent proliferation factor for neural stem cells (Nakatomi et al., 2002), whereas BDNF favours their differentiation into neurons (H. Scharfman et al., 2005).

In fact, the local delivery (achieved using a non-replicating herpes virus–based vector) of a combination of FGF-2 and BDNF during epileptogenesis was found to limit damage, favour proliferation of hippocampal neural stem cells and their differentiation into neurons, and prevent SRSs (Paradiso et al., 2009). In addition, it was found that this treatment also produced robust anti-inflammatory effects (Bovolenta et al., 2010), arising the question if this latter effect was directly produced by the NTFs or it was secondary to other effects, for example the reduction in SRS severity and frequency. In this study, we aimed at addressing this question by directly applying FGF-2 and BDNF to CNS cells in an in vitro model of neuroinflammation.

2. Results

2.1. Characterization of MGC

Astrocytes and microglial cells are engaged constantly in a fine crosstalk in the frame of neuroinflammatory events (K. Li et al., 2019). As both microglia and astrocytes are involved in neuroinflammation, we decided to use primary mixed glial cultures for our experiments. Immunofluorescence was applied for the determination of percentage of astrocytes and microglia using GFAP (astrocytes specific) and Iba1 (microglia specific) antibodies (Figure 1A and 1B). MGC was composed of 70-75% astrocytes and 20-25% microglia (Figure 1C).

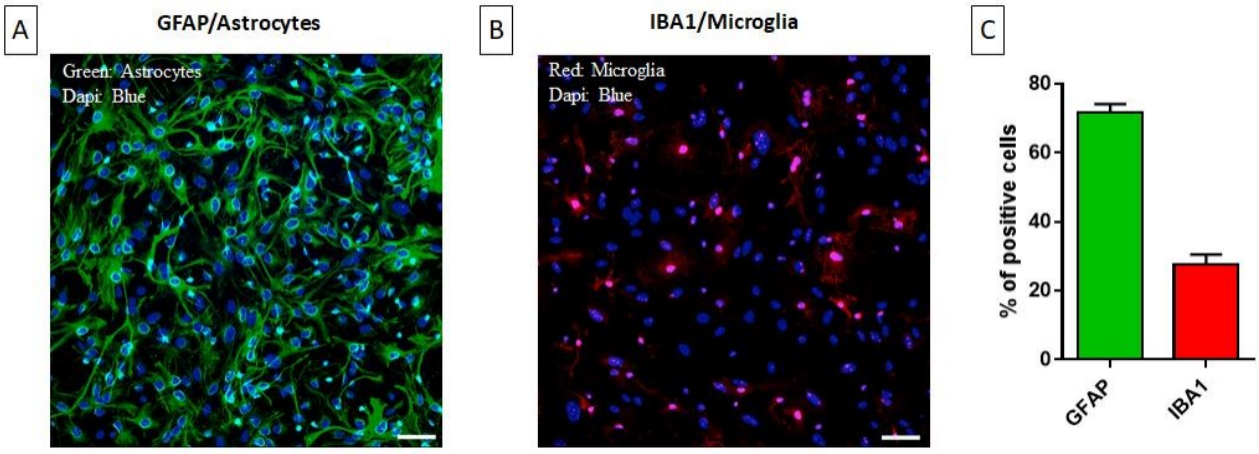

Figure 1. Astrocytes and microglia in MGC as measured by immunofluorescence using (A) GFAP (green; astrocytes), and (B) Iba1 (red; microglia) antibodies. Nuclei were stained using DAPI (blue). Scale bar = 200 µm. (C) Percentage of putative astrocytes (GFAP-positive cells) and putative microglial cells (IBA1-positive) cells in MGC. Data are reported as percent of DAPI positive cells. Quantification was performed using the ImageJ software. Results are the mean ± SEM of three independent experiments. Abbreviations: GFAP = Glial fibrillary acidic protein (GFAP); IBA1 = Ionized calcium binding adaptor molecule 1; DAPI= 4′,6-diamidino-2-phenylindole

2.2. Setting up a neuroinflammation in vitro model: concentration and time-dependent effect of LPS on cell viability, cytokine level, and NO production

Both astrocytes and microglial cells, when acquiring an activated state in response to potentially noxious insults, can release pro-inflammatory molecules like TNF-α, IL-6, IL-1β, and NO (Matejuk & Ransohoff, 2020). LPS has been used to mimic the inflammatory process, stimulating cells to release pro-inflammatory cytokines. In order to choose an

optimal LPS concentration, we first investigated cell viability after 72 h of exposure by using the MTT assay. We observed that only the highest concentration of LPS (10000 ng/ml) significantly reduced cell viability but a tendency of reduction can also be observed at 1000 ng/ml while no death was detected up to 100 ng/ml (Figure 2E).

We then measured the LPS-induced release of cytokines at different time points. Cells were incubated with different concentrations of LPS (1, 10, 100, 1000, 10000 ng/ml) their supernatant was collected at 24, 48, and 72 h, and the release of cytokines was measured using ELISA. Whereas we could not detect release of cytokines from primary neuronal cultures (data not shown), we observed a strong, LPS concentration-dependent increase in IL-1β, TNF-α and IL-6 levels (Figure 2A-C).

In addition to cytokines, microglial cells release reactive oxygen species (ROS) in response to noxious stimuli. ROS increase expression of the inducible nitric oxide synthase (iNOS) and thereby promote an excessive production of NO (Yuste et al., 2015). The production of NO from microglia stimulates the activation of astrocytes which then further enhance NO production (Solà et al., 2002). To determine the occurrence of these phenomena in our system, cells were treated with different LPS concentrations (1 – 10,000 ng/ml) for 72 h, and NO production was analysed indirectly by nitrite concentration measurement in the cell supernatant at different time points. As expected, we observed a significant, LPS concentration and time-dependent increase in NO production (Figure 2D). Based on the results obtained with the MTT assay, in the cytokines and NO release experiments, the LPS doses of 1000 and 10000 ng/ml were excluded from the statistical analysis, to avoid possible confounding effects due to cells death.

Taken together, these results suggest that 100 ng/ml is the lowest effective concentration of LPS able to increase production of cytokines and NO without affecting the viability of our MGC. Thus, we decided to use this concentration in all subsequent experiments.

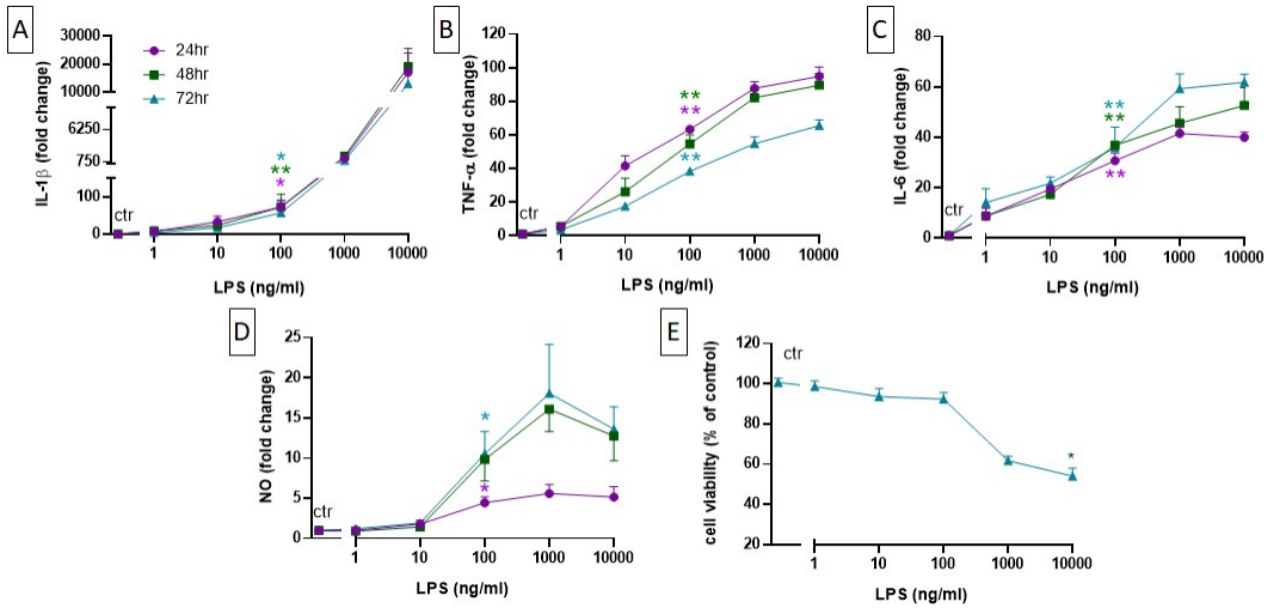

Figure 2. Concentration-dependent effect of LPS on the release of IL-1β (A), TNF-α (B), IL-6 (C), and NO (D) from MGC after 24 (purple), 48 (green), or 72 h (blue) of treatment. The release of cytokines was evaluated using a commercially available ELISA plate (Ella). NO release was determined using the Griess assay and readings of absorbance were made at 570 nm using a multimode plate reader. (E) Cell viability was assessed at 72 h using the MTT assay and reading absorbance at 550nm. Data are presented as the mean ± SEM of three independent experiments run in duplicate. Statistical analysis was performed using Kruskal-Wallis one-way ANOVA followed by the Dunn's test. *p<0.05, **p<0.01 against control. Abbreviations: LPS = lipopolysaccharide; IL-1β = interleukin-1 beta; TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6.

2.3. Effects of FGF-2 and BDNF on cell viability, cytokine level and NO production

Next, we examined the effect of BDNF and FGF-2 on cytokines and NO LPS-induced levels. MGC were incubated with different concentrations of FGF-2 and BDNF (0.1, 1, 10, 100, 1000 ng/ml) for 48 h following 24 h LPS (100 ng/ml) stimulation. Both neurotrophic factors had no effects on LPS-induced cytokines and NO levels (Figure 3A-D) as well as on cell viability (Figure 3E).

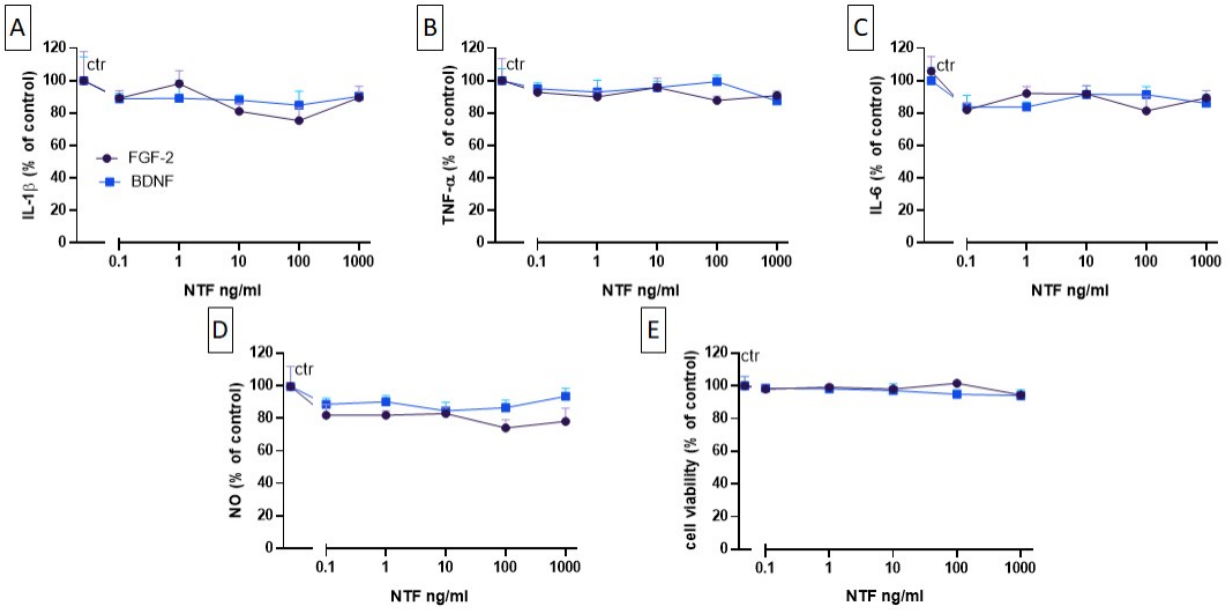

Figure 3. Effects of FGF-2 (purple) and BDNF (blue) on the release of IL-1β (A), TNF-α (B), IL-6 (C), and NO (D) from 100 ng/ml LPS stimulated MGC. Cells were treated with LPS for 24 h and then with FGF-2 or BDNF for 48 h. Release of cytokines and NO were evaluated as described in Fig. 2. (E) Effect of FGF-2 and BDNF on cell viability was measured using the MTT assay. Data are presented as the mean ± SEM of five independent experiments run in duplicate. Statistical analysis was performed using Kruskal-Wallis one-way ANOVA followed by Dunn's test against the LPS treated control. Abbreviations: FGF-2 = fibroblast growth factor-2; BDNF = brain-derived neurotrophic factor; LPS = lipopolysaccharide; IL-1β = interleukin-1 beta; TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6

2.4. Effects of FGF-2 and BDNF co-administration on cell viability, cytokine level and NO production

Although no reduction in cytokine levels and NO production could be observed after the administration of BDNF or FGF-2 alone, in line with data published by (Bovolenta et al., 2010), we decided to co-administer BDNF and FGF-2. The FGF-2 and BDNF co-administration effects were determined following two different concentration ratios: one 1:1 FGF-2:BDNF but also a 1:10 FGF-2:BDNF, chosen considering the fact that BDNF is about 10 times more potent than FGF2 (Baldelli et al., 2000; Dollé et al., 2005; Hollborn et al., 2004; Lin et al., 2010). However, neither co-administration ratios had any effect on LPS-induced cytokine and NO levels (Figure 4 A-D). No effect has been observed on cell viability (figure 4 E).

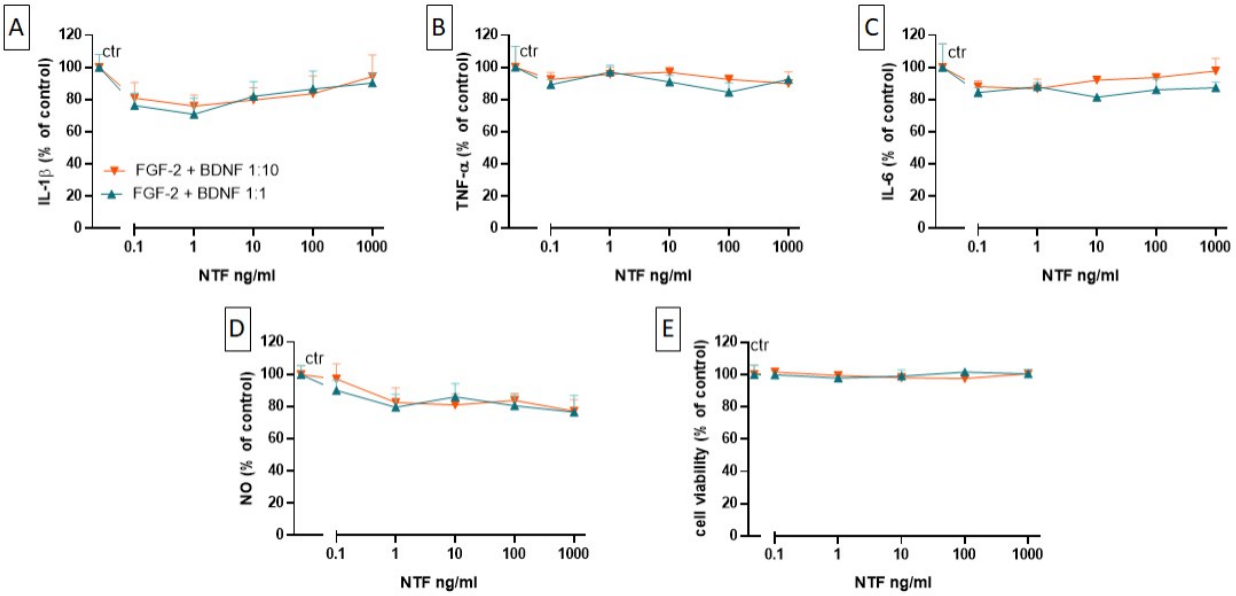

Figure 4. Effects of co-administration of FGF-2 and BDNF at 1:1 (blue) and 1:10 ratio (orange) on the release of IL-1β (A), TNF-α (B), IL-6 (C), and NO (D) from 100 ng/ml LPS stimulated MGC. Cells were treated with LPS (100 ng/ml) for 24 h and then with FGF-2/BDNF for 48 h. Release of cytokines and NO were evaluated as described in Fig. 2. (E) Effect of FGF-2 and BDNF on cell viability was measured using the MTT assay. Data are presented as the mean ± SEM of five independent experiments run in duplicate. Statistical analysis was performed using Kruskal-Wallis one-way ANOVA followed by Dunn's test against the LPS treated control. Abbreviations: FGF-2 = fibroblast growth factor-2; BDNF = brain-derived neurotrophic factor; LPS = lipopolysaccharide; IL-1β = interleukin-1 beta; TNF-α = tumor necrosis factor-alpha; IL-6 = interleukin-6

3. Discussion

In this study, we found that FGF-2 and BDNF, both individually and in combination, are unable to modulate the release of neuroinflammatory factors, such as pro-inflammatory cytokines and nitric oxide (NO), by glia in vitro, indicating that their effects in intact animals is likely indirect.

Here, we used a model of LPS-induced release of inflammation mediators in cell cultures. LPS initiates the process of neuroinflammation by binding to the toll-like receptor-4 (TLR4) and activating downstream signaling pathways resulting in the production of cytokines and chemokines, including TNF-α, IL-6, IL-1β, and NO (Marinelli et al., 2015; Solà et al., 2002). In our cultures, we observed that these events occur in astrocytes and microglia but not in

neurons. Astrocytes and microglial cells are engaged constantly in a fine crosstalk during the neuroinflammation process: reactive microglial cells release pro-inflammatory cytokines and NO activating astrocytes, that switch to an astrogliosis state and ultimately secrete more pro-inflammatory molecules, initiating a feed-forward loop (L. R. Liu et al., 2020; Matejuk & Ransohoff, 2020). Hence, we decided to optimize our model using a mixed astrocytes and microglia culture.

Both BDNF and FGF-2 have been reported to reduce neuroinflammation in epilepsy (Bovolenta et al., 2010) and in other disease models (Shi et al., 2018; Yin et al., 2020). However, the interpretation of these findings is still unclear. Initial in vitro studies on cell cultures suggested that these NTFs may exert direct effects on the secretion of pro-inflammatory cytokines and, therefore, on neuroinflammation, but data in this respect are inconclusive: (1) BDNF has been reported to decrease LPS-induced mRNA levels of TNF-α, IL-1β, and iNOS in astrocytes (Yin et al., 2020), but the question remains as to the actual levels of translation into proteins and secretion (in the case of TNF-α and IL-1β); (2) FGF-2 has been reported to decrease the release of TNF-α and IL-1β from astrocytes exposed to infrasound stimulation (Shi et al., 2018), but no concentration-response study was performed, and it should be taken into account that infrasound is a very specific type of stimulation, from which it is difficult to extrapolate general conclusions. In addition, the effect of combining the NTFs in vitro has never been studies. In the present study, we could not detect any effect at any concentration of FGF-2 and BDNF, either alone or in combination, on the LPS-induced release of cytokines from astrocyte-microglia mixed cultures.

4. Materials and Methods

2.1. Chemicals and antibodies

High glucose Dulbecco's Modified Eagle Medium (DMEM) w/L-Glutamine and sodium pyruvate (sterile filtered) were purchased from Carlo Erba, Italy. Streptomycin/penicillin cocktail (P0781), Lipopolysaccharide (LPS; Escherichia coli strain O111:B4; L4391), Brain

derived neurotropic factor (BDNF; SRP3014), and Fibroblast growth factor 2 (FGF-2; F0291), as well as Glial fibrillary acidic protein (GFAP; G9269), Triton-X100 (T8787), 4',6-diamidino-2-phenylindole (DAPI; MBD0015), FluoromountTM aqueous mounting medium (F4680), Bovine serum albumin (BSA; A3059), Sulphanilamide (S9251), Hank's balanced salt solution (HBSS; H-9394), L-Glutamine 200mM (G7513), and paraformaldehyde (PFA; 158127) were obtained from Sigma-Aldrich (Merk Life Science S.r.l, Milan). Heat-inactivated foetal bovine serum (FBS; ECS0180L) and Trypsin-EDTA in phosphate buffered saline (PBS; ECB3052D) were supplied by EuroClone (Euroclone S.p.A., Pero, Milan). PBS (pH 7.4; 10010-023), B-27™ supplement (17504-044l), Na+-pyruvate (11360-039), and neurobasal-A medium (21103) were from Gibco (Thermo fisher scientific, US). The microglial marker Ionized calcium binding adaptor molecule 1 (Iba1; 234003) were purchased from Synaptic Systems GmbH (Goettingen, Germany). Cross-adsorbed secondary antibody, Alexa Fluor™ 594 and 488 were acquired from Invitrogen (Watham, MA, USA) while naphthyl-ethylendiamine dihydrochloride (1062370025) was from Merck (Roma, Italy).

2.2. Primary mixed glial culture (MGC) and neuronal culture

The experiments were approved by the Italian Ministry of Health (authorization number: CBCC2.N.NUJ). Murine-derived primary mixed glial cultures (MGC) were prepared from cerebral cortices of C57BL/6J mice (P0-1) according to (Mecha, 2011) with slight modifications. Briefly, pups were decapitated, meninges and blood vessels were removed carefully under sterile conditions, and then cortices were dissected, mechanically dissociated by repeated pipetting in chilled high glucose DMEM supplemented with 1% penicillin and 10 mg/ml streptomycin and centrifuged at 1000 rpm for 10 min. The supernatant was removed, and the pellet resuspended in pre-warmed high glucose DMEM supplemented with the above-mentioned antibiotics and 10% heat-inactivated FBS.

Cells were cultured in 75-cm2 flask (VWR®) and maintained at 37°C with humidified atmosphere of 95% air/5% CO2. The medium was changed after 1 day in vitro (DIV) and then every 3 days. Once confluency was reached, attached cells were removed by

trypsinization. The culture was characterized by immunocytochemistry using GFAP as specific astrocyte marker and Iba1 as microglial marker.

The 8 DIV cells were seeded in multi-well plates. To evaluate cell viability (MTT assay) cells were seeded in a 96-wells plate, 30,000 cells / wells / 100 µl. To evaluate NO and cytokines production, cells were seeded in a 48-wells plate, 60,000 cells / wells / 200 µl.

For neuronal culture, pups (P0-1) were decapitated, meninges and blood vessels were removed carefully under sterile conditions, and then cortices were dissected. The tissue was washed with HBSS and then incubated in 10% of trypsin in HBSS at 37oC for 8-10 minutes for tissue digestion. The action of trypsin was blocked by adding 1 ml of Neurobasal medium containing 10% of FBS, 10 µl/ml Hepes, 10 µl/ml Na+-pyruvate, 1% penicillin, 10 mg/ml streptomycin and 2.5 µl/ml L-glutamine. Following this, the tissue was homogenised by repeated pipetting using a Pasteur pipette and centrifuged at 500 rpm for 10 min. Then, the supernatant was removed and the pellet was re-suspended in Neurobasal medium. Finally, the cells were filtered using a 70 µm nylon cell strainer. Cells were cultured in 75-cm2 flask (VWR®) and maintained at 37°C with humidified atmosphere of 95% air/5% CO2. The medium was changed next day with fresh Neurobasal medium supplemented with 2% B-27™, 1% penicillin, 10 mg/ml streptomycin, 2.5 µl/ml L-glutamine in the absence of FBS. Cells were allowed to reach confluency.

2.3. Treatments

At 8 DIV, cells were stimulated with different concentrations (1 to 10000 ng/ml) of LPS for 24 h, 48 h, and 72 h, in order to determine the concentration- and time-dependent effect of LPS on NO production and release of cytokines (IL-1β, TNF-α, IL-6).

The effects of LPS on cell viability were measured 72 h post LPS treatment. To study the effect of BDNF and FGF-2 on cell viability, cytokine release and NO production, cells were first treated with LPS 100 ng/ml for 24 h and then supplemented with BDNF (0.1 to 1000 ng/ml) or FGF-2 (0.1 to 1000 ng/ml) or its combination in concentration ratio of 1:10 and 1:1

(FGF-2: BDNF) for 48 h. The incubation time and LPS concentration were chosen on the basis of the aforementioned experiments.

2.4. Immunocytochemistry

The cell phenotype was analysed performing immunocytochemistry experiments according to (Li et al., 2016) with slight modifications. The cells were seeded in 24-well culture plate with poly-L-lysine coated coverslip and allowed 24 h incubation. They were then washed twice with PBS 1X and fixed with 4% PFA for 20 min at room temperature (RT). Cells were washed with PBS 1X to remove the residual fixative and incubated with 0.1% triton-X100 (T8787-100ML; Sigma-Aldrich) for 10 min. After two rapid washes with PBS 1X, aspecific binding sites were blocked by using a blocking solution composed of 5% normal goat serum and 5% bovine serum albumin in PBS 1X for 60 min at RT. Overnight incubation with primary antibodies anti-GFAP (1:200) or anti-Iba1 (1:500) was performed at 4°C. In the following day, cells were washed again with PBS 1X and incubated with the respective cross-absorbed secondary antibodies (1:500) for 1 h at RT. Counterstaining with DAPI (1 µg/ml) was performed to visualize nuclei. Finally, cells were mounted with FluoromountTM Aqueous Mounting Medium and images were acquired using a Leica DMRA2/RXA2 microscope.

2.5. MTT assay

Cell viability was assessed according to the instructions of manufacturer using a commercially available kit (Cell proliferation kit I MTT; Roche; 11465007001). Briefly, 10 µl MTT labelling reagent 1X were added in each well and incubated at 37°C for 4 h. 100 µl of solubilisation buffer were then added in each well and incubated overnight at 37°C. On the next day, the results were evaluated by measuring the absorbance at 550 nm using the multimode plate reader EnSight (PerkinElmer).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The cell supernatant was collected and IL-1β, TNF-α, and IL-6 levels were measured using a commercially available simple plex 5th generation cartridge (Bio-techne; SPCKC-MP-005977) and the readings were taken using Ella automated immunoassay system (Bio-Techne), according to the manufacturer's instructions.

2.7. Nitric oxide measurement

The production of NO was evaluated as the accumulated nitrate concentration in culture medium by using a Griess reaction based colorimetric reaction (Bettegazzi et al., 2021). Briefly, 100 µl of a colour reagent (0.1% naphthyl-ethylendiamine dihydrochloride in bidistilled water and 1% sulphanilamide in 5% orthophosphoric acid) was incubated with an equal volume of sample supernatant. After 5-10 min of incubation at RT, the absorbance was determined at 570 nm using the multimode plate reader EnSight (PerkinElmer). The standard calibration curve was generated by sodium nitrite standards (0-100 µM).

2.8. Statistical analysis

Statistical analysis of data was performed using the Prism 6 software (GraphPadInc.). Data are shown as fold-change from the control, where control is vehicle treated cells in LPS concentration-response curve experiments, and LPS treated cells in neurotrophic factor experiments. All results are the mean ± SEM (standard error mean) of at least three independent experiments performed in duplicate. Data were analysed using Kruskal-Wallis one-way ANOVA and post-hoc Dunn's test. Data were considered statistically significant when $p < 0.05$.

5. Conclusions

The most plausible interpretation of these negative data is that FGF-2 and BDNF do act directly on the secretion of inflammatory factors by neural cells and, therefore, that their anti-inflammatory effects observed in vivo are indirect. For example, the attenuation of epilepsy-induced neuroinflammation (Bovolenta et al., 2010) may be due to the reduced number of spontaneous seizures that (as described in the Introduction) can cause chronic

neuroinflammation. Needless to say, however, the reductionist approach taken in this study cannot provide conclusive evidence in this respect, and further studies will be required in the future to strengthen this hypothesis.

Author Contributions Conceptualization, M.S., S.Z., C.R., N.A. and C.F.; methodology, N.A., S.Z., C.R. and B.B.; software, N.A. and C.R.; validation, M.S., S.Z., C.R. and N.A.; formal analysis, N.A. L.A., M.S. and B.B.; investigation, N.A. and A.G. ; resources, M.S.; data curation, S.Z., C.R. and N.A; writing—original draft preparation, N.A. and S.Z; writing—review and editing, M.S. and C.R.; visualization, M.S.; supervision, C.R. and S.Z.; project administration, C.R. and S.Z.; funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement:

"The study was conducted in accordance with the Declaration of animal authority, and approved by the Italian Ministry of Health (authorization number: CBCC2.N.NUJ)."

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, [C.R], upon request.

Conflicts of Interest: The authors declare that they have no competing interests.

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GENERAL CONCLUSION

The quest to find better ways to cure epilepsy is of utmost importance, as this neurological disorder affects millions of people worldwide, significantly impacting their quality of life. Epilepsy is characterized by recurrent seizures caused by abnormal electrical activity in the brain, and despite advancements in treatment options, a substantial portion of patients still struggle to achieve full control of their seizures. As we acknowledge the significance of this challenge, it becomes essential to harness all available resources, knowledge, and expertise to drive forward the research and development efforts. By acknowledging the gravity of epilepsy's impact on individuals, families, and society as a whole, we can foster a sense of urgency and purpose in advancing research and improving treatment options. TLE often originates from a precipitating event followed by epileptogenesis, in which multiple pathophysiological phenomena occur, including neuroinflammation. Neuroinflammation is one of the significant hallmarks contributing to the development or aggravation of epilepsy (Pracucci et al., 2021; Rana & Musto, 2018), and targeting neuroinflammation has become a major goal for the treatment of different neurological and psychiatric conditions, including TLE (a most common form of epilepsy). It is a well-orchestrated and complex process involving glial cells, particularly astrocytes and microglia. Sustained and prolonged neuroinflammation acts as a pivotal driver of various brain disorders. In fact, several experimental evidences support the association of neuroinflammation with neurological diseases, particularly with TLE. At the CNS level, neuroinflammatory processes could participate in common pathological mechanisms critical for the onset of seizures and epilepsy (French et al., 2017).

The association between neuroinflammation and epilepsy is quite complicated, which raises the question; at which stage targeting neuroinflammation in epilepsy could be clinically relevant? Several factors can be considered while addressing this issue. The presence of pre-existing neuroinflammation, such as after a brain injury or infection, can enhance seizure susceptibility, linked with alterations in neuronal excitability and increased seizure-induced neuropathology (Espinosa-

garcia et al., 2021; V. L. L. Lee & Shaikh, 2019; Vezzani et al., 2011). Addressing neuroinflammation at an early stage might help prevent the transition from the initial insult to epilepsy. SE can induce neuroinflammation and SRSs can perpetuate chronic neuroinflammation and brain damage. Targeting neuroinflammation at an early phase after status epilepticus can help mitigate its long-term consequences and improve outcomes. During epileptogenesis, inflammatory processes can contribute to the rewiring of neural circuits and promote hyperexcitability, ultimately favouring to the development of epilepsy. So, targeting neuroinflammation at this stage might help prevent the transition from a normal brain to an epileptic one. For individuals who already have epilepsy, ongoing neuroinflammation may contribute to the persistence and worsening of the condition. Modulating neuroinflammatory responses could potentially modify the disease course, reducing the frequency and severity of seizures. Finally, neuroinflammation may contribute to drug resistance in individuals with epilepsy do not respond adequately to available antiepileptic drugs. Targeting inflammatory pathways in drug-resistant epilepsy, either through new therapies or in combination with existing treatments, might ameliorate treatment outcomes. Initial pre-clinical studies have made this concept evident, displaying the antiseizure activity of anti-inflammatory therapies in some drug-resistant epilepsies. VX-765 is the only known anti-inflammatory agent that has been used for controlled efficacy trials in chronic focal epilepsy, although these trials were only preliminary (French et al., 2017).

The neurotropic factors (NTFs), BDNF and FGF-2, play a significant role in both the development and maturation of the nervous system, where they promote synaptic plasticity, neuronal survival, and long-lasting memory formation. Activation of the BDNF high affinity receptor TrkB can attenuate astrogliosis, microgliosis, proinflammatory cytokines and, ultimately, neuroinflammation. Moreover, in an in vivo model of TLE associated with severe neuroinflammation, supplemention of FGF-2 and BDNF in combination attenuated various parameters of neuroinflammation, including microcytosis, astrocytosis, and the expression of IL-1β (Bovolenta et al., 2010). The effect was most prominent on

IL-1β expression, whose overexpression was almost completely prevented . In this frame, the present study aimed to study the efficacy of the TrkB agonist 7,8 dihydroxyflavone (7,8-DHF) in an in vivo model of TLE. In our study, we found that low 7,8- DHF doses (5 mg/kg) display a clear reduction in epileptogenesis and (even if barely significant) tendency to reduce the neuroinflammation.

The local delivery (achieved using a non-replicating herpes virus–based vector) of a combination of FGF-2 and BDNF during epileptogenesis was also found to limit damage, favour the proliferation of hippocampal neural stem cells and their differentiation into neurons, and prevent SRSs (Paradiso et al., 2009). Since this treatment also produced robust anti-inflammatory effects, the question arises if this latter effect was directly produced by the NTFs or it was secondary to other effects, for example the reduction in SRS severity and frequency. It is important to note that targeting neuroinflammation as a potential therapeutic approach for epilepsy requires a better understanding of the specific mechanisms involved. Therefore, the second study in my thesis aimed at addressing this question by directly applying FGF-2 and BDNF to CNS cells in an in vitro model of neuroinflammation. Our results suggest that the anti-inflammatory effect of BDNF and FGF-2 in vivo in the epilepsy model was indirect and likely due to a reduction in seizure frequency and severity. This study has some limitations; epileptogenesis-associated neuroinflammation is a complex phenomenon and LPS-induced inflammation can partially but not fully mimic it. The timeframe of the study is limited, which might not allow the NTFs to exert robust effects. Further studies will be therefore required to strengthen this hypothesis.

In conclusion, this work further supports the activation of TrkB receptors as a new pharmacological strategy to prevent epileptogenesis. We demonstrated that this pharmacological activity may be achieved with small organic molecules, like 7,8-DHF, thus overcoming the pharmacokinetic limitations characterizing NTFs. Of note, some cautions should be still taken into account before considering DHF for a clinical trial, especially regarding the dose range and some selectivity concerns. Additionally, the present work provides new information on the mechanism of action of

NTFs as anti-epileptogenic agents. In the view of the promising therapeutic potential of growth factor receptor agonists, we decided to further investigate their biological action, particularly by testing if BDNF and FGF-2 directly modulate inflammation in vitro. The negative results of this study do not support this hypothesis and suggest that the protective effect of NTFs on epileptogenesis are not directly due to the modulation of neuroinflammation. This notwithstanding, the therapeutic potential of anti-neuroinflammatory drugs in epilepsy remains worthy of further investigation.

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