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Epilepsy and the inflammasome: Targeting inflammation as a novel therapeutic strategy for seizure disorders

Abstract: Epilepsy is the most common serious brain disorder worldwide. Recent evidence from experimental models of epilepsy and clinical brain tissue from epilepsy surgery suggests inflammation may play a pathological role in this disorder. Activation of a multi-molecular protein complex termed the 'inflammasome' occurs during inflammation to drive the innate immune response. Inflammasome activation, with release of inflammatory mediators including interleukin-1β and high-mobility group box-1, may play a crucial role in the development of epilepsy (epileptogenesis) after brain insult. Immunomodulatory drugs targeting the inflammasome pathway may represent a novel anti-epileptogenic treatment strategy for epilepsy. This review summarises the current literature surrounding inflammasome activation and epilepsy.

Keywords: Inflammasome, epilepsy, seizures, high mobility group box-1, HMGB1, interleukin-1β

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1 Introduction

Epilepsy affects people of all ages, sex and ethnicity and has been recognised since the earliest medical writings, yet despite more than 20 currently available antiepileptic drugs (AEDs) on the market, almost one third of sufferers are resistant to treatment (WHO 2012; Fact sheet 999; www.who.int). Epilepsy is defined as two or more spontaneous seizures (WHO 2012; Fact sheet 999; www.who.int) and the development of this disorder (epileptogenesis) involves processes such as increasing network excitability (by increasing glutamate levels and sensitivity and reducing GABA inhibition), blood brain barrier breakdown, neuronal loss and mossy fibre sprouting [1]. Classical AEDs primarily target ion channels to prevent the hyper-excitability that can lead to seizures, and ultimately exert their effect by raising the seizure threshold. Therefore, despite the nomenclature, AEDs are purely symptomatic and exhibit no disease-modifying potential. Recent studies using rodent models of seizures and epilepsy are uncovering other pathways, particularly involving inflammation, that may be implicated in both initiation and exacerbation of this disorder. Immunomodulatory AEDs represent a potentially novel avenue for drug development, not only to alleviate seizures but also to target the epileptogenic process [2].

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2 Inflammation in epilepsy

Inflammation is classically described as a beneficial process to remove pathogens and aid healing. However, it is now also known to contribute to a range of central nervous system (CNS) disorders including Alzheimer's disease, Parkinson's disease, stroke, and epilepsy [2–4]. Seizures themselves can induce inflammation through the up-regulation of cytokines, chemokines, prostaglandins, toll-like receptors (TLRs), complement and cell adhesion molecules [5]. Inflammation not triggered by a pathogen is termed 'sterile inflammation'. During a sterile insult, endogenous danger signals (damage associated molecular patterns; DAMPs) such as ATP, monosodium urate crystals, amyloid beta, or cholesterol crystals can induce the formation of a multi molecular protein complex termed an inflammasome. Recent evidence also suggests that cell swelling and the associated decrease in intracellular K^+ and Cl^- that can occur during seizures also activates

the inflammasome [6]. Activation of the inflammasome results in the release of pro-inflammatory cytokines that drive inflammation [3].

3 The inflammasomes

Inflammasomes are typically expressed in myeloid cells and form part of the innate immune system. They contain pattern recognition receptors (PRRs) that respond to danger signals through the detection of pathogen associated molecular patterns (PAMPs) or DAMPs. The PRRs contain a central nucleotide-binding and oligomerisation (NACHT) domain, a C-terminus leucine rich repeat (LRR) for ligand sensing and autoregulation, and an N-terminus caspase activation and recruitment domain (CARD) or a pyrin domain (PYD) for binding adaptor and effector proteins [7]. The type and combination of these components determines the inflammasome formed. The inflammasome that is activated by DAMPs under sterile conditions (such as may occur during epilepsy) is the NACHT, LRR and PYD-containing protein 3 (NLRP3) inflammasome. This comprises the PRR NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1. In the brain, the NLRP3 inflammasome is primarily expressed in microglial cells. There is also some suggestion of NLRP3 function in neurons but this has not been confirmed [8]. Formation of the NLRP3 inflammasome causes the activation of caspase-1 which can then cleave the precursor of the pro-inflammatory cytokine interleukin-1 beta (pro-IL-1β) into its biologically active form (IL-1β) [7]. Recently the NLRP3 inflammasome has also been shown to exert pro-inflammatory, yet IL-1-independent, effects including regulating the release of High Mobility Group Box-1 (HMGB1) [9].

4 High Mobility Group Box-1

HMGB1 is a 25KDa highly conserved protein comprising three major protein domains; two DNA binding regions (termed the A and B boxes) and a 30 amino acid acidic carboxyl terminus [10]. Discovered 30 years ago [11], it has myriad intracellular and extracellular functions. Primarily nuclear, HMGB1 bends DNA and regulates transcription. In all cells, HMGB1 continuously shuttles between the nucleus and cytoplasm. Acetylation of HMGB1 near the nuclear localization sequences blocks communication with the nuclear importer and inhibits re-entry into the nucleus [12]. HMGB1 can be passively released during necrosis but active secretion also occurs from immune cells in response to injurious stimuli following inflammasome activation [13, 14]. Once outside the cell, HMGB1 acts as a potent DAMP to induce inflammation by signalling through TLR4 [15]. HMGB1 contains three conserved cysteine residues (C23, C45 and C106). Post-translational modifications of these residues critically regulate the protein's function. Fully reduced all-thiol HMGB1 acts as a potent chemo-attractant through complex formation with CXCL12 binding exclusively via CXCR4 [16], whereas the cytokine stimulating activity of HMGB1 depends upon disulphide linkage between cysteine 23 and cysteine 45, with cysteine 106 reduced as a thiol. Only in this configuration can HMGB1 trigger an inflammatory response via TLR4 [16, 17].

5 Interleukin-1β

IL-1β is the most widely studied of the IL-1 family and was initially described as the endogenous pyrogen for its role in fever [18]. Since then, a number of physiological and pathological roles for IL-1 have been described [4]. Background levels of IL-1β are low but following pathogenic insult or stimulation with tumour necrosis factor alpha (TNFα) or even IL-1 itself, synthesis of pro-IL-1β is induced [19]. Following activation of IL-1β via the NLRP3 inflammasome and caspase-1, mature IL-1β is released, by an as yet unconfirmed mechanism. Following release, IL-1β acts on its receptor IL-1R1 to induce gene transcription of cytokines, chemokines and adhesion molecules to further promote the pro-inflammatory cascade [4].

As described earlier, activation of the NLRP3 inflammasome induces release of both IL-1β and HMGB1 (Figure 1) [7, 9]. In addition, IL-1β itself can induce HMGB1 release [20], and together, IL-1β and HMGB1 have been shown to act synergistically to release IL-6 from synovial fibroblasts [21]. This suggests that targeting the NLRP3 inflammasome may provide a means to abolish this inflammatory circuit; and targeting upstream of both IL-1β and HMGB1 in this way may achieve a more diverse anti-inflammatory effect over targeting further down the IL-1 cascade (i.e. with IL-1R1 antagonists or IL-1 antibodies). For example, anti-IL-1 therapies are not completely effective against cryopyrin-associated periodic syndrome caused by mutations in the NLRP3 gene, whereas targeting higher up the pathway with caspase-1 knock-outs is [22]. However, NLRP3 activation is not the only means of stimulating IL-1β or HMGB1 release, so although targeting inflammasome assembly may help to prevent the downstream cascade of events that can lead to the development of an epileptic focus, it may prove necessary to also include a more targeted approach to individual inflammatory mediators (i.e. specifically blocking HMGB1 or IL-1β).

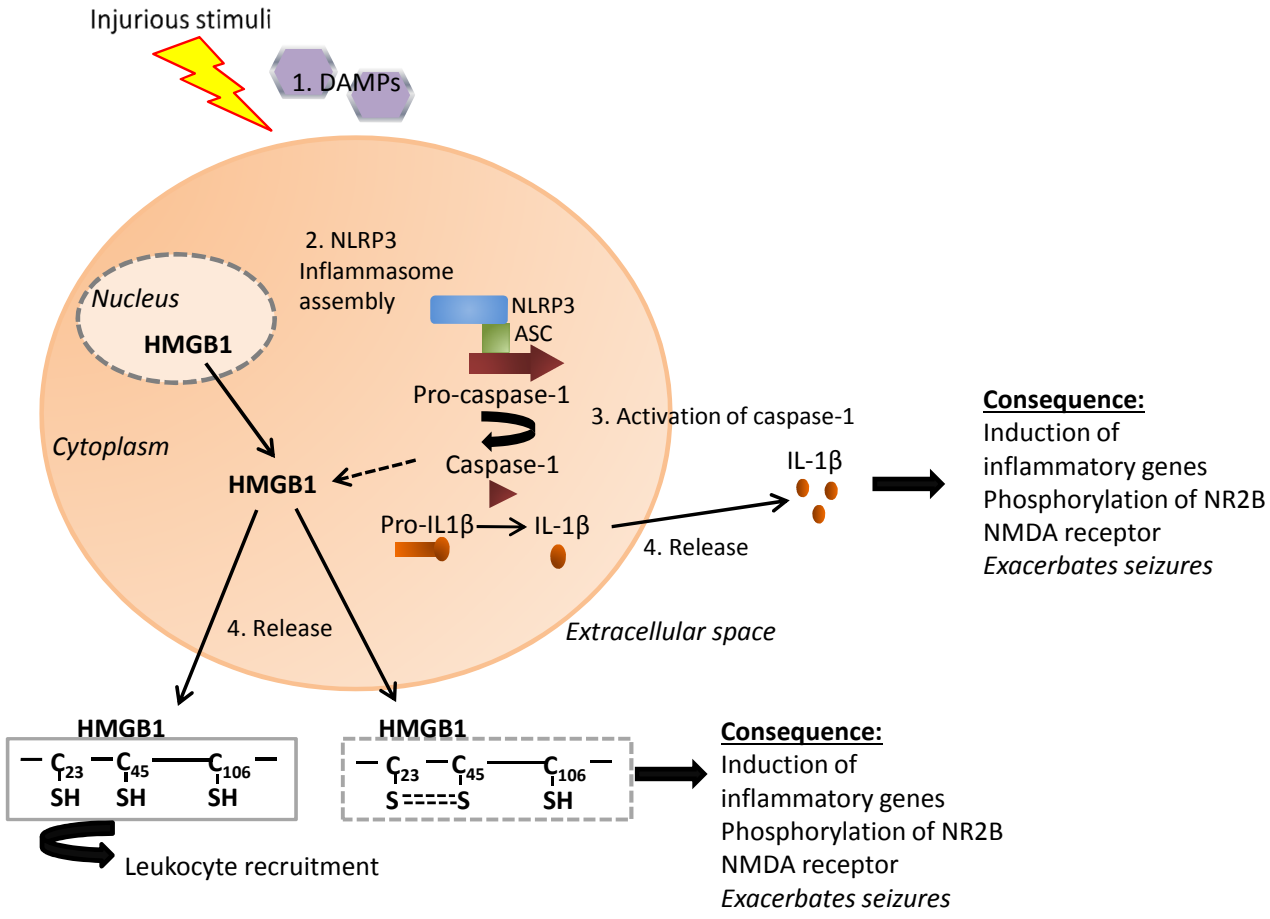

Figure 1: Inflammasome activation and release of HMGB1 and IL-1β. Injurious stimuli induce the release of damage associated molecular patterns (DAMPs; 1). DAMPs induce the formation of the NLRP3 inflammasome (2). Inflammasome formation induces caspase-1 activation (3). Caspase-1 cleaves pro-IL-1β into its active form and results in the release of IL-1β and HMGB1 (4). This causes transcription of inflammatory genes and phosphorylation of the NR2B subunit of NMDA receptors leading to an increase in neuronal Ca2+ entry. NLRP3: NACHT, LRR and PYD-containing protein 3; ASC: apoptosis-associated speck-like protein containing a CARD; HMGB1: high mobility group box-1; IL-1β: interleukin-1β; Pro-IL-1β: precursor inactive form of IL-1β; Pro-caspase-1: precursor inactive form of caspase-1; Fully-reduced HMGB1, dominant form in nucleus and cytoplasm, chemoattractant, no cytokine activity; Disulphide HMGB1, induces cytokine transcription and release via toll-like receptor 4.

6 IL-1β and HMGB1 expression in seizures

The involvement of IL-1β, and more recently HMGB1, in seizures has been well established using both genetic and pharmacological tools. Following seizures, IL-1β and HMGB1 are primarily expressed in microglia, although HMGB1 is also found in neurons [23, 24]. IL-1β and IL-1R1 are up-regulated as a result of seizures in a variety of experimental models, including those induced by electrical stimulation, kainate, bicuculline, and hyperthermia [23, 25–27]. Elevated IL-1β and IL-1R1 have also been observed in brain tissue from patients with temporal lobe epilepsy and hippocampal sclerosis, and elevated IL-1β has been reported in cerebrospinal fluid (CSF) from children with febrile seizures [28, 29]. In addition to increased IL-1 expression in patients with temporal lobe epilepsy, increases in IL-1β and inflammasome components have also been observed in other seizure disorders such as Rasmussen's encephalitis [30] and malformations of cortical development [31].

Similarly, increased immunostaining for HMGB1 and its receptors, TLR4 and Receptor for Advanced Glycation Endproducts (RAGE), has been demonstrated in the brain tissue of mice following induction of both kainate- and bicuculline-induced acute seizures and kainate-induced chronic epilepsy [24, 32]. Increased staining for HMGB1, TLR4 and RAGE, is detectable in an analogous pattern to that observed in mouse models of epilepsy in human hippocampal tissue obtained at surgery from patients with

temporal lobe epilepsy and hippocampal sclerosis, and also in malformations of cortical development associated with intractable seizures [20, 24, 32, 33].

7 IL-1β and HMGB1 exacerbate seizures

Pre-treatment with intra-hippocampal IL-1β or HMGB1 prior to treatment with bicuculline or kainate exacerbates seizures [25, 33, 34]. In contrast, intra-cerebral infusion of the endogenous IL-1R1 antagonist IL-1Ra or its over-expression in astrocytes delays seizure onset and reduces duration following kainate [35] or reduces seizure behaviour following bicuculline treatment or electrically-induced status epilepticus (SE) [34]. Seizure onset is also delayed in mice lacking IL-1R1 [35]. Similarly, selective inhibition of HMGB1 or TLR4 delays seizure onset and decreases seizure number and duration in both kainate- and bicuculline-induced acute seizure models and reduces the number of spontaneous epileptic seizures in the kainate model of chronic epilepsy [33]. Knock-out of TLR4 or RAGE is also anticonvulsant in kainate models of acute and chronic seizures [32]. Increased expression of IL-1β and HMGB1 in a variety of experimental models and clinical seizure disorders, in addition to their established pro-convulsant effects, provides evidence that targeting IL-1β and HMGB1 may prove successful in the treatment of epilepsy.

8 Pro-convulsant mechanisms of IL-1β and HMGB1

A fast signalling pathway has been characterised showing that IL-1R1 co-localises with the NR2A/B subunit of the NMDA receptor and IL-1β activation of this receptor results in phosphorylation of the NR2B subunit via Src kinases, resulting in increased neuronal Ca^{2+} influx [36]. Activation of this pathway *in vivo* using ceramide to activate Src kinases mimics the IL-1β-mediated exacerbation of kainate-induced seizures, whereas, inhibition of Src kinases has the opposite effect [37]. This fast signalling pathway has more recently also been described for HMGB1 [24]. The disulphide inflammatory form of HMGB1, but not its fully-reduced form, acts via TLR4 receptors to exacerbate kainate-induced seizures in a similar manner to that of IL-1β [38]. TLR4 receptors co-localise with NR1 and NR2B subunits of the NMDA receptor and disulphide HMGB1 enhances NMDA-mediated Ca^{2+} influx via neutral sphingomyelinase and Src kinases mediating phosphorylation of NR2B [38]. Thus activation of this fast signalling pathway has been proposed as one mechanism for the pro-convulsant effects of both IL-1β and HMGB1. Other potential excitotoxic mechanisms include increasing astrocytic glutamate release, reduction of glutamate uptake and increasing the translocation of AMPA receptors into neuronal membranes, [36, 39, 40].

9 IL-1β and HMGB1 as pharmacological targets

Pharmacological interventions targeting IL-1 and HMGB1 are already in development for a number of disorders and could therefore also be potential treatments for seizures and epileptogenesis. The IL-1 receptor antagonist IL-1Ra, the anti-IL-1β neutralising antibody canakinumab, and the soluble decoy receptor rilonacept are already approved as treatments for rheumatoid arthritis and cryopyrin-associated periodic syndrome [19]. A number of successful strategies have also been shown to inhibit HMGB1 in experimental models, including polyclonal and monoclonal antibodies [41], competitive inhibition with the truncated HMGB1 A-Box [24], recombinant soluble thrombomodulin [42], and selective alpha7-nicotinic acetylcholine receptor agonists [43]. Indeed, nicotine has been shown to inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and to suppress HMGB1 release from human macrophages [43]. Antagonists that neutralize HMGB1 have also demonstrated considerable success in pre-clinical models of various diseases, including severe sepsis, arthritis, colitis, trauma and cancer. For example, administration of anti-HMGB1 antibodies, even where delayed by up to 24 hours following cecal perforation in rodents, rescues the animals from otherwise lethal septicaemia [41, 44].

10 NLRP3 inflammasome, caspase-1 and HMGB1 as anticonvulsant targets

Consistent with the pro-convulsant actions of IL-1β and HMGB1, and the involvement of NLRP3 and caspase-1 in the activity of both of these molecules, caspase-1 inhibitors are anticonvulsant and knockout (KO) of the caspase-1 gene protects against seizure-inducing stimuli [45]. In the rat intrahippocampal kainate seizure model, pre-treatment with the caspase-1 inhibitors pralnacasan or VX-765 delayed seizure onset and decreased seizure number and time in seizures [45] (Table 1). In organotypic hippocampal slice cultures, both treatments also reduced IL-1β release [45]. These observations are supported by

Table 1: Anti-inflammatory therapies in experimental seizure models.

Model	Anti-inflammatory therapy	Target	Timing (relative to brain insult)	Outcome	Ref
Kainate seizure model; rat	IL-1Ra	IL-1R1	Pre- and post-	Reduction in seizure number and time in EEG seizure activity.	[34]
Electrical self-sustained SE; rat	IL-1Ra	IL-1R1	Pre- and post-	Reduction in seizure behaviour score.	
Kainate seizure model; rat	Pralnacasan	Caspase-1	Pre-	Reduction in seizure-induced IL-1β. Delay in seizure onset Reduction in seizure number and time in EEG seizure activity.	[45]
	VX-765	Caspase-1	Pre-	Delay in seizure onset. Reduction in seizure number and time in EEG seizure activity.	
Kainate and bicuculline seizure models; mouse	Box A LPS-Rs Cyp	HMGB1 TLR4	Pre-	Delay in seizure onset. Reduction in seizure number and time in EEG seizure activity.	[24]
Kainate epilepsy model; mouse	Box A LPS-Rs	HMGB1 TLR4	Post-	Transient (2 hour) reduction in number and frequency of spontaneous seizures.	
Pilocarpine and electrical model of epilepsy; rat	IL-1Ra and VX-765	IL-1R1 and caspase-1	Post-	Reduction in IL-1β expression in astrocytes and cell loss in rat forebrain. Frequency and duration of spontaneous seizures unaffected.	[46]
Lithium-pilocarpine-induced SE; rat	IL-1Ra and CAY 10404	IL-1R1 and COX-2	Concomitant	Acute delay seizure onset, decreased neuronal death. Reduction spontaneous seizure frequency, mossy fibre sprouting.	[47]
KA-induced SE; mouse	A438079	P2X7R	Pre- and post-	Reduction in EEG seizure activity and seizure behaviour score.	[49]
KA-induced SE; mouse	A438079	P2X7R	Pre	Reduction in time in EEG seizure activity. Reduction in cell death.	[50]
	Brilliant blue G	P2X7R	Pre	Reduction in time in EEG seizure activity. Reduction in SE-induced IL-1β. Reduction in reactive microglia. Reduction in cell death.	
	anti P2X7R Ab	P2X7R	Pre	Reduction in time in EEG seizure activity.	
	A438079	P2X7R	Post- (15 mins)	Reduction in time in EEG seizure activity and total power. Reduction in cell death.	
	A438079 and lorazepam	P2X7R	Post- (1 h)	Reduction in time in EEG seizure activity and total power. No effect on time in seizure activity when administered alone.	
Pilocarpine seizure model; mouse	Carbenoxolone or probenecid	Pannexin1	Pre-	Increase in seizure behaviour score and power.	[51]
	OxATP, A438079, or A740003	P2X7R	Pre-	Increase in seizure behaviour score and power.	
KA-induced SE; mouse	MFQ	Pannexin1	Pre-	Reduction in seizure behaviour score.	[52]

Key: IL-1β: Interleukin-1β; IL-1Ra: Interleukin-1 receptor antagonist; IL-1R1: Interleukin-1 receptor 1; LPS-Rs: *Rhodobacter sphaeroides* lipopolysaccharide; Cyp: cyanobacterial lipopolysaccharide; HMGB1: High mobility group Box-1; TLR4: toll-like receptor 4; SE: status epilepticus; P2X7R: P2X7 receptor

data from the caspase-1 KO mouse model, which showed a delay in seizure onset and a decrease in seizure number and time in seizures [45]. Similarly, in mouse models of kainate- or bicuculline-induced seizures, targeting HMGB1 with the antagonist fragment Box A has been shown to postpone the onset and limit the duration of seizures in both acute and chronic phases of the response to convulsant challenge [24] (Table 1). It is possible, however, that these pre-insult therapies that have been shown to be effective at reducing acute seizures, may merely reflect a reduction in the extent of the initial insult caused by the chemoconvulsant, and may not be effective in targeting epileptogenesis. Interestingly, when caspase-1 inhibitors and IL-1Ra treatment is given to rats after, rather than before, the epileptogenic insult (electrical stimulation or lithium-pilocarpine), the onset, frequency and duration of seizures in the chronic epileptic phase is unchanged. However, the combined post-injury treatment does reduce forebrain IL-1β levels and ameliorates the degree of neuronal cell loss [46] (Table 1). Post-insult challenges such as this are the gold-standard method to define therapeutic anti-epileptogenic efficacy, as pre-insult therapies are not clinically realistic. These data therefore suggest that the timing of administration of immunomodulatory AEDs may be an important factor to consider; earlier post-injury intervention may be necessary to block the rapid inflammatory cascade. Alternatively, combinations of anti-inflammatory therapy, with or without standard AEDs, may be required [46]. Indeed, IL-1Ra with the COX-2 inhibitor CAY 10404 was effective at delaying seizure onset and reducing neuronal injury, whilst neither was effective alone [47]. When these treatments were given immediately prior to pilocarpine and for ten days post lithium/pilocarpine-induced SE, the frequency of spontaneous seizures was also reduced, but again a post-seizure effect of this anti-inflammatory combination has not been studied [47].

Directly targeting the NLRP3 inflammasome has yet to be explored but its inhibition may reduce caspase-1/IL-1β- and HMGB1/TLR4-mediated exacerbation of seizures. ATP-mediated activation of the P2X7 receptor is well known to activate the NLRP3 inflammasome and recently this target has been investigated for its role in seizures [48]. P2X7 receptor antagonists reduce kainate-induced seizure behaviour [49] and seizure activity [50] and this effect is maintained even when administered 15 minutes after SE induction [50]. P2X7 receptor antagonists in combination with lorazepam halt SE when given 1 hour post SE, a time when neither is fully effective alone [50]. However, P2X7 antagonists are pro-convulsant in the pilocarpine seizure model [51]. Activation of P2X7 receptors opens a Pannexin1 pore but blocking this has again shown both pro- and anticonvulsant effects [51, 52]. Further work is required to explore the mechanisms of this P2X7 receptor-mediated effect on seizures but as P2X7 receptor antagonists reduce SE-induced mature IL-1β production, the reduction in seizure activity may, at least in part, be due to inhibiting NLRP3 actions on IL-1β/HMGB1 [50]. Thus combinations of anti-inflammatory and standard AEDs may be therapeutically effective. Additionally, neutralisation of the inflammasome adapter protein ASC has been shown to be protective in mouse models of acute sterile brain injury (traumatic brain injury and spinal cord injury) [8] and therefore may provide an additional target for anti-inflammatory AEDs.

11 Conclusion

The cycle of seizures inducing inflammation and inflammation exacerbating seizures highlights the potential benefit of targeting constituents of the inflammatory cascade to halt this cycle and to treat seizures and epilepsy. The effectiveness in a variety of seizure models of targeting IL-1β and HMGB1 signalling suggests that the NLRP3 inflammasome could be a successful future target for the treatment of seizures and epilepsy. The caspase-1 inhibitor VX-765 has already undergone Phase II trials for partial epilepsy (http://clinicaltrials.gov/ct2/show/NCT01048255), so the precedent has been set for safely targeting this pathway. As yet, no studies have specifically looked at inhibition of the NLRP3 inflammasome as an alternative means of targeting inflammation associated with seizure disorders. However, with small molecule inhibitors now being synthesised, targeting the NLRP3 inflammasome as a common point in both IL-1β and HMGB1 pathways could result in improved treatments over more selective approaches and could potentially provide relief for patients with treatment resistant epilepsy.

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