More than a drug target: Purinergic signalling as a source for diagnostic tools in epilepsy

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ARTICLE INFO

Keywords:
Epilepsy
Seizures
Diagnosis
Purinergic signalling
Purines
P2X7 receptor

ABSTRACT

Epilepsy is one of the most common and disabling chronic neurological diseases affecting people of all ages. Major challenges of epilepsy management include the persistently high percentage of drug-refractoriness among patients, the absence of disease-modifying treatments, and its diagnosis and prognosis. To date, long-term video-electroencephalogram (EEG) recordings remain the gold standard for an epilepsy diagnosis. However, this is very costly, has low throughput, and in some instances has very limited availability. Therefore, much effort is put into the search for non-invasive diagnostic tests. Purinergic signalling, via extracellularly released adenosine triphosphate (ATP), is gaining increasing traction as a therapeutic strategy for epilepsy treatment which is supported by evidence from both experimental models and patients. This includes in particular the ionotropic P2X7 receptor. Besides that, other components from the ATPergic signalling cascade such as the metabotropic P2Y receptors (e.g., P2Y6 receptor) and ATP-release channels (e.g., pannexin-1), have also been shown to contribute to seizures and epilepsy. In addition to the therapeutic potential of purinergic signalling, emerging evidence has also shown its potential as a diagnostic tool. Following seizures and epilepsy, the concentration of purines in the blood and the expression of different compounds of the purinergic signalling cascade are significantly altered. Herein, this review will provide a detailed discussion of recent findings on the diagnostic potential of purinergic signalling for epilepsy management and the prospect of translating it for clinical application. This article is part of the Special Issue on ‘Purinergic Signaling: 50 years’.

1. Introduction

Epilepsy comprises a heterogeneous group of brain diseases that have a common predisposition to recurrent spontaneous seizures. With over 50 million people affected worldwide, epilepsy is one of the most common and disabling chronic neurological diseases affecting people of all ages, more prominently in the young and elderly (Beghi, 2019; Moshe et al., 2015; Thijs, Surges, O’Brien and Sander, 2019). Along with the occurrence of uncontrolled seizures, the major challenges in the management and treatment of epilepsy include its diagnosis and prognosis, treatment complexity, social disadvantages (e.g., unemployment, stigma), and the increased risks of premature mortality (up to 3-fold) and co-morbidities (up to 8-fold) such as depression and anxiety (Loscher, 2020; Moshe et al., 2015; Shlobin and Sander, 2022; Thijs et al., 2019). The healthcare and societal costs of epilepsy are amongst the highest for neurological diseases (Allers et al., 2015). While the underlying causes of epilepsy remain elusive in the majority of cases, the known causes of epilepsy include genetic abnormalities such as de novo mutations and a precipitating injury (e.g., traumatic brain injury (TBI), infection, stroke, tumours, or an episode of status epilepticus) (Klein et al., 2018; Pitkanen and Engel, 2014). The most common form of epilepsy in adults is temporal lobe epilepsy (TLE), which involves different structures within the limbic system, including the amygdala and hippocampus. TLE is normally accompanied by hippocampal sclerosis, a condition characterized by selective neuronal loss and gliosis, and is particularly prone to drug-refractoriness (Chang and Lowenstein, 2003). Epileptogenesis is defined as the process of transforming a normal healthy brain into an epileptic brain and can be characterized by...
multiple pathological changes within the brain. This includes acute and ongoing cell death, synaptic reorganization, blood-brain barrier (BBB) disruption, and inflammation (Pitkanen et al., 2015), which have been of particular interest over the past few years (Vezzani et al., 2019). To date, the first-line treatment for epilepsy is with anti-seizure medications (ASMs), which are heavily focused on targeting synaptic transmissions and ion channels. However, these are only effective in 70% of patients, show no significant impact on disease progression, and may cause serious side effects (Bialer and White, 2010; Thijs et al., 2019). Other therapeutic strategies include resective surgery, neuromodulation devices, and therapeutic diets (Consales et al., 2021; Foutz and Wong, 2022; Rio and Boisson, 2022).

Purinergic signalling is increasingly recognized to contribute to brain hyperexcitability with mounting data demonstrating the therapeutic benefits for epilepsy, where the different components of the signalling system can be potential drug targets (Beamer et al., 2021a). This includes the well-known endogenous anticonvulsant, adenosine (Boison, 2016), and extracellularly released adenosine triphosphate (ATP) (Engel et al., 2016). While the therapeutic potential of targeting the purinergic signalling system during epilepsy has been extensively studied (Beamer et al., 2021a; Cieslak et al., 2017; Engel et al., 2021; Tescarollo et al., 2020), recent findings suggest a promising diagnostic potential in seizures and epilepsy. The present review will provide a brief overview of the current shortcomings in the diagnosis of epilepsy, introduce the purinergic signalling cascade in the brain, summarize its therapeutic benefits, and discuss its diagnostic potential for epilepsy from recent findings. While the role of ATP as a signalling molecule during seizures and epilepsy is a relatively newer area of research when compared to extracellular adenosine (Beamer et al., 2021a), this review will focus mainly on ATPergic signalling. We will, however, also include data on adenosine that are relevant to epilepsy diagnosis (e.g., adenosine kinase (ADK) (Boison, 2013)).

2. Seizure and epilepsy diagnosis

With regard to the diagnosis of epilepsy, it should be noted that seizures and epilepsy are not the same. While seizures are defined as a transient occurrence of signs and/or symptoms due to abnormally excessive or simultaneous neuronal activity in the brain, the diagnosis of epilepsy requires at least two unprovoked seizures occurring more than 24 h apart. This includes cases with one seizure and a high likelihood of having additional seizures (more than 60%) or the diagnosis of an epilepsy syndrome. According to the International League Against Epilepsy (ILAE), epilepsy can be classified into three levels: seizure-type, epilepsy, and epilepsy syndrome, with a strong emphasis on etiology and comorbidities (Sarmast et al., 2020; Scheffer et al., 2017).

To date, an accurate diagnosis of epilepsy remains a clinical challenge and requires multiple criteria including patient history, seizure type, brain magnetic resonance imaging (MRI), and electroencephalogram (EEG) video recordings (Aaebeg et al., 2017; Moshe et al., 2015; Thijs et al., 2019). The most reliable method available to diagnose epilepsy and provide an accurate prognosis is with long-term EEG video recordings, where patients are admitted for long periods under video surveillance during continuous EEG monitoring. However, this is very costly, has low throughput, and is not always available as primary healthcare (e.g., in developing and resource-poor countries (Sarmast et al., 2020)). Thus, most patients thought to have epilepsy are treated based on the clinical features alone (Engel and Pitkanen, 2020). In addition, the complexity of diagnosis and technical difficulties in differentiating epileptic seizures from other similar conditions (i.e., convulsive syncope or psychogenic non-epileptic attacks) result in the misdiagnosis of up to 42% of patients, which potentially leads to wrong or unnecessary medications (Dickson et al., 2017).

Biomarkers are important diagnostic tools that reflect both health and disease conditions and can be objectively measured, preferably in a minimally invasive manner (Engel and Pitkanen, 2020). The uncertainty and complexity of seizure diagnosis, unpredictable timing of individual seizures, and the overall course of the disease with its associated co-morbidities stress the need to develop biomarkers that can accurately diagnose seizures, and assess the progression of epilepsy and treatment response. Epilepsy biomarkers can also support the monitoring of therapeutic effects, therapeutic trial designs, and decision-making when dispensing seizure-suppressive or anti-epileptogenic drugs. To facilitate their implementation, these biomarkers should be easily accessible (e.g., blood, urine), measurable using easy-to-use platforms, reliable, and long-lasting (i.e., detectable even hours after the last seizure activity). Biomarkers of epileptogenesis and ictogenesis (propensity to generate seizures) could predict the development of an epileptic condition or even a seizure episode and may help identify the underlying pathology and stages of disease severity. They could also be used for more cost-effective screening of potential anti-epileptogenic and anticonvulsive drugs in animal models. To date, the search for epilepsy biomarkers has resulted in the identification of numerous candidates. This includes several molecules in circulation such as inflammation markers (e.g., high-mobility group box 1 (HMGB1)) (Walker et al., 2022) and non-coding RNAs (e.g., microRNAs and transfer RNA fragments (tRNAs) (Henshall et al., 2016; Hogg et al., 2019)), as well as imaging biomarkers (e.g., translocator proteins (TSPOs) identified with positron emission tomography (PET) (Gershen et al., 2015; Koepf et al., 2017)). However, the possible disadvantages of these biomarkers include the lack of a rapid and cost-efficient analytical platform, difficult-to-access biofluids (e.g., cerebrospinal fluid (CSF)), large blood volume requirements, tedious blood processing procedures, and inter-hospital analytical variability (Hanin et al., 2020). Some of the TSPO radioligands have been reported to have a high level of non-specific binding. Moreover, binding affinities to TSPO may be influenced by a common polymorphism (rs6971) in the TSPO gene, which would require genotyping of patients before conclusive results could be drawn (Scott et al., 2017).

3. Purinergic signalling - overview

The purinergic signalling system, collectively also called purinome, involves the synthesis and release of purine nucleosides (e.g., adenosine) and nucleotides (e.g., ATP, uridine triphosphate (UTP)), purinergic receptors, as well as the enzymatic machinery to eliminate purine molecules from the extracellular space (Burnstock, 2008; Zimmermann, 2006). Although the purinergic signalling system represents probably the most primitive and widespread chemical messenger system in animals (Verkhratsky and Burnstock, 2014), the idea of ATP acting as a signalling molecule in the brain was initially heavily disputed (Burnstock, 1972, 2006). Due to the groundbreaking work by Geoffrey Burnstock (Di Virgilio, Jacobson and Williams, 2021), ATP is now recognized to play major roles in the central nervous system (CNS) as a neuromodulator, neurotransmitter and gliotransmitter. Physiologically, extracellular ATP concentrations are relatively low (micromolar range) but can increase dramatically into the millimolar range during pathological conditions (Dale and Frenguelli, 2009). ATP can be released via exocytotic mechanisms, such as the activation of the Cl––dependent vesicular nucleotide transporter (VNUT) on secretory vesicles (Sawada et al., 2008), non-exocytotic mechanisms (i.e., voltage-dependent anion channels (Murphy et al., 2005)), ATP-binding cassette transporters (Johnson et al., 2006), purinergic P2 receptors (i.e., P2X7 receptor), hemichannels (i.e., connexins and pannexins (Bao et al., 2004; Kang et al., 2008; Pellegratti et al., 2005)), or passively through damaged cell membranes. Once released, ATP can activate specific receptors termed P2 receptors or can be broken down sequentially into adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine via different ectoenzymes (e.g., nucleoside triphosphate diphosphohydrolase-1 [NTPDase1, CD39] and ecto-5’nucleotidase [NT5E, CD73] (Allard et al., 2017; Perrot et al., 2019; Vijayan et al., 2017)). Extracellular adenosine concentrations are largely dependent on the action of equilibrative and/or concentrative nucleoside transporters.
Adenosine receptors (ARs), also called P1 receptors, are a family of G protein-coupled receptors (GPCRs) comprised of four subtypes: A₁R, A₂A₂R, A₂B₂R and A₂R (Fredholm et al., 1999; Benarroch, 2008; Dunwiddie et al., 1997). ARs are widely distributed throughout the body, including the CNS (Peleli et al., 2017). All ARs are activated by their endogenous ligand, adenosine, with each AR showing slight differences in their affinity for adenosine [A₁Rs (1–10 nM) > A₂A₂Rs (30 nM) > A₂B₂Rs (100 nM) > A₂Rs (1000 nM)] (Borea et al., 2018; Fredholm, AP, Jacobson, Linden and Muller, 2011). While A₁Rs and A₂A₂Rs are coupled to inhibitory Gₐᵣ proteins, A₂B₂Rs and A₂Rs are coupled to stimulatory G proteins (Borea et al., 2018).

ATP receptors are subdivided into two families. This includes P2X receptors (P2XRs) and P2Y receptors (P2YRs) (Burnstock and Kennedy, 1995; Kennedy, 2021). We now recognize eight different P2YR subtypes that have the typical seven transmembrane segments of GPCRs. Of these, P2Y₃ is activated best by ATP, whereas the others are selective for ADP (P2Y₁, P2Y₁₂ and P2Y₁₃), ATP/UTP (P2Y₂), UTP/uridine diphosphate (UDP) (P2Y₆, P2Y₉) and the ligand UDP-glucose (P2Y₁₄) (von Kugelgen, 2021). The P2XRs, comprising seven different subfamilies (P2X₁-7), form a trimeric ion channel that can be either homomeric or heteromeric, with significant Ca²⁺ permeability. The ATP is the main endogenous ligand for all P2XRs (Khakh, 2001). Among the P2XRs, the P2X₇R subtype has attracted the most attention as a possible drug target in epilepsy (Beamer et al., 2017), and also as a diagnostic target, which will be discussed in later sections. The P2X₇R is unique among the P2XRs, as it has a relatively low affinity for ATP (EC₅₀ half maximal effective concentration) ≥ 100 μM, activation threshold: 0.3–0.5 mM), thus suggesting that P2X₇R activation occurs mainly under pathological conditions of high ATP release. Consequently, P2X₇R-based treatments may lead to fewer unwanted side effects. It also has slow desensitization dynamics, the ability to permeabilize the cell membrane to molecules up to 900 Da in size, and is a key driver of inflammation (Jimenez-Mateos et al., 2019; Kopp et al., 2019). The P2X₇R mediates a wide array of pathological functions in the brain, particularly pro-inflammatory processes (e.g., interleukin-1β (IL-1β) release). In addition to neuro-inflammation, P2X₇Rs have also been associated with numerous processes that are altered during epileptogenesis, such as cell death, synaptic plasticity, disruption of the BBB, and neurotransmitter release (Sperlagh and Illés, 2014).

4. Purinergic signalling during epilepsy and its therapeutic potential

While the anticonvulsant actions of adenosine have been well-established for decades, more recent data from experimental models and patient tissues has provided compelling evidence of (1) the changes in the expression levels of several components of the ATPergic system during epilepsy, (2) its contributing to seizure and epilepsy development, and (3) its therapeutic potential (Beamer et al., 2021a). This section will briefly describe the findings that suggest a contribution of ATP-mediated signaling to seizures and epilepsy; for a more detailed description, please refer to other recent extensive reviews on this topic (e.g., Beamer et al., 2021a; Boison and Rojo, 2019; Engel et al., 2021; Murugan et al., 2021; Tescarollo et al., 2020; Weltha et al., 2019).

Several studies have shown increased release of adenosine during seizures, including studies in humans and mice (Dulla et al., 2009; During and Spencer, 1992; Ille et al., 2012; Lovat et al., 2012). This is most likely a protective feedback mechanism to limit the duration, intensity or spread of focal seizures. To date, it is well-established that A₁Rs mainly carry out an anticonvulsant function, while A₂A₂Rs seem to be mainly pro-convulsive. A₁R knock-out (KO) mice develop spontaneous seizures and lethal status epilepticus after an intrahippocampal kainic acid (KA) administration and display increased seizure and TBI-induced neurodegeneration (Pedele et al., 2006; Kochanek et al., 2006; Masino et al., 2011). Likewise, A₁R agonists show seizure-suppressive effects in several mouse models (e.g. (Gouder et al., 2003; Li and Zhang, 2011; Mares, 2010; Muzzi et al., 2013)), whereas A₁R antagonists increase seizure activity and decrease responsiveness to ASMs (Chwalisz et al., 2008; Fukuada et al., 2010).

In contrast, mice with genetic inactivation of A₂A₂Rs have reduced seizure susceptibility (El Yacoubi, Ledent, Parmentier, Costentin and Vauggeois, 2008, 2009). An anticonvulsant function of A₂A₂Rs was also suggested (Adami et al., 1995; Viana, Ferreira, Dona, Cavalheiro, & da Silva Fernandes, 2005). The role of A₂B₂Rs and A₂Rs remains to be established, although some studies suggest both receptors are involved in seizure generation (Dunwiddie et al., 1997; Roseti et al., 2008).

Several P2XRs have been shown to undergo expression changes after evoked seizures (i.e., mainly in models of status epilepticus) and during epilepsy in several brain structures, including the hippocampus and cortex. For example, the expressions of P2X2R, P2X4R and P2X7R are typically upregulated after status epilepticus and during epilepsy. Regarding the P2X7R, its expression has been reported to be increased in the brain following status epilepticus (Avignone et al., 2008; Dona et al., 2009; Engel et al., 2012; Morgan et al., 2020) and during epilepsy in mice and resected tissue from patients (Jimenez-Pacheco et al., 2013, 2016). Nonetheless, little is known about its cell type-specific expression. While broad consent exists concerning its microglial expression, the neuronal expression of P2X7R remains to be established (Dona et al., 2009; Jimenez-Pacheco et al., 2016; Kaczmarek-Hajek et al., 2018; Morgan et al., 2020; Viana et al., 2005).

Although less investigated, changes in the expression of the different P2YR subtypes have also been reported in both rodent models and patients (Alves et al., 2018). Interestingly, following status epilepticus, the P2Y expression pattern seemed to correlate with receptor subtype-specific substrates and downstream signalling (Alves et al., 2018). Similar to what has been observed for P2XRs, most P2YR subtypes have been found to be upregulated during epilepsy (Alves et al., 2018), possibly due to the sustained inflammation in the epileptic brain. In contrast to their expression, very little is known regarding their cell type-specific expression. Whilst P2Y2Rs have been shown to be increased in microglia post-intra-amygdala KA-induced status epilepticus (Alves et al., 2019), P2Y2 and P2Y4Rs have been detected on astrocytes in brain tissue from patients with intractable epilepsy (Sukigara et al., 2014).

In addition, we have evidence that shows the involvement of both P2XRs and P2YRs in seizures and epilepsy. Whilst P2X7R is the most studied receptor among the P2XRs family, the P2Y₁ and P2Y₁₂Rs are the most studied among the P2Y family (Engel et al., 2021). Despite these findings, several issues have yet to be resolved. P2X7R antagonism has been shown to reduce seizure severity during acutely evoked seizures, but this seems mainly to be restricted to models where status epilepticus was induced via KA (Engel et al., 2012; Jimenez-Pacheco et al., 2013) or Cortaria lactone (Huang et al., 2017). In contrast, P2X7R antagonisms exacerbated seizure severity when status epilepticus was induced via pilocarpine (Kim and Kang, 2011; Rozmer et al., 2017). No effect was observed in less severe seizure models, including models where seizures were induced via pentylenetetrazole (PTZ), or in the maximal electroshock seizure and 6 Hz psychomotor seizure threshold tests (Fischer et al., 2016; Nieczcym et al., 2017). Likewise, no effect was observed in a model of genetic absence epilepsy (Dogan et al., 2020). Of note, when given in conjunction with ASMs, P2X7R antagonism potentiated the anticonvulsant actions of ASMs in several models, which suggests P2X7R-based treatments as adjunctive therapy (Engel et al., 2012; Fischer et al., 2016). In line with this, a recent study by us has shown that increased P2X7R expression contributed to the unresponsiveness
towards ASMs during status epilepticus, possibly via pro-inflammatory processes (Beamer et al., 2022). More consistent results have been obtained during epilepsy development and once epilepsy was established, where it has been shown that P2X7R antagonisms reduce seizure severity and frequency in several models, including the PTZ kindling model (Amhaoul et al., 2016; Fischer et al., 2016; Jamali-Raeufy et al., 2020; Jimenez-Pacheco et al., 2016). The other P2XRs with a functional role included P2X3R and P2X4R. P2X3R antagonist treatment of neonatal mice with P2X7R antagonist prior to intra-amygdala KA led to a more severe clinical phenotype. In contrast to a pre-treatment regime, treatment of neonates with NE increased and reduced seizure severity. Treatment with P2X7R antagonist post-status epilepticus also delayed the onset of epilepsy, and when applied during epilepsy, depressed epileptic seizures (Alves et al., 2018; Engel et al., 2019).

In addition to purinergic receptors, other components of the purinergic signalling pathway have also been shown to contribute to seizures and epilepsy and hence have been proposed as possible therapeutic targets. This includes ATP release mechanisms and adenosine removal enzymes. Dona et al. reported using a rat model of intraperitoneal pilocarpine that the extracellular levels of adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine increased shortly after status epilepticus, whereas the level of ATP was not changed. However, ATP levels were found to be increased in the same model following an epileptic seizure (Dona et al., 2016). As mentioned previously, ATP can be released via several mechanisms into the extracellular space including the pannexin-1 hemichannel. Of note, it is suggested that this channel is one of the main pathways regulating ATP release during seizures and epilepsy, as shown by Lapatar et al. in rat hippocampal slices treated with the glutamate agonist (S)-3,5-dihydroxyphenylglycine) (Lapatar et al., 2015), and Dossie et al. using resected tissue from patients with epilepsy (Dossie et al., 2018). Similar to ATP, adenosine-based epilepsy therapies are not limited to the targeting of ARs. In this regard, ADK, which is highly expressed on astrocytes and is increasingly recognized as the treatment target of epilepsy, whereby the inhibition of ADK suppresses acute, evoked and epileptic seizures (Boisson and Yegutkin, 2019; Sandau et al., 2019).

5. Purinergic signalling and the diagnosis of seizures and epilepsy

The complexity of purinergic signalling, which includes the release of different signalling molecules (e.g., ATP, adenosine) during seizures and the changes in the expression of different purinergic receptors and purine-metabolizing enzymes, makes this signalling pathway particularly apt as a source for diagnostics for epilepsy. More importantly, biomarkers based on purinergic signalling would not only provide novel approaches for seizure and epilepsy diagnosis, but also mechanistic biomarkers that can accelerate purinergic signaling-based therapies into clinical use. Although this is a new area of research, several studies are providing proof of principle of its diagnostic potential (Table 1). These
will be described in detail within this section.

5.1. Blood purines as biomarkers of seizures and epilepsy

As described, purines such as ATP and adenosine are released in the brain during seizures and epilepsy (Beamer et al., 2019). Purines can readily cross the BBB, and be detected in the circulation (i.e., whole blood) (Tian et al., 2017). This makes purines a potentially valuable and convenient biomarker that could be elevated in the blood of epilepsy patients.

The increased concentrations of adenosine and its metabolites (e.g., inosine, hypoxanthine, and xanthine) in the blood during neurological insults have been known for some time. This includes stroke, ischemic brain injuries (Dale et al., 2019; Laghi Pasini et al., 2006; Saugstad, 1975; Weigand et al., 1999), TBI in children (Robertson et al., 2001), and TBI in adults (along with concomitant increased levels of xanthine, hypoxanthine and cyclic AMP (cAMP) in the interstitial fluid) (Bell et al., 2001). Recently, the increased levels of adenosine and its metabolites were also seen along with elevated concentrations of ATP and ADP in patients with schizophrenia (Kristof et al., 2022). With regards to seizures and epilepsy, Bruno et al. showed increased hydrolysis of ATP, ADP and AMP in the blood of rats after PTZ-induced seizures (Bruno et al., 2002, 2003), suggesting increased adenosine concentrations in the blood. In line with these findings, Oses et al. reported increased levels of adenosine in the CSF of rats following PTZ-induced seizures (Oses et al., 2004).

Until recently, the short half-life, low concentration, and technical difficulties of purine detection and quantification in biofluids were the main reasons preventing the translation of blood purine measurements into a practical biomarker assay, particularly where a fast diagnosis is crucial (e.g., seizure diagnosis in the emergency department). This changed with the development of an enzymatic amperometric detection technology that allows adenosine and its metabolites (i.e., inosine, hypoxanthine, and xanthine) to be rapidly detected in blood (Martin et al., 2019; Tian et al., 2007). By using this technology, we showed in a recent study that the induction of intra-amygdala KA-induced status epilepticus in mice rapidly elevated whole blood purine levels by nearly 4-fold (Beamer et al., 2021b). More importantly, the extent of elevation correlated with the intensity of seizures during status epilepticus and the degree of status epilepticus-induced neurodegeneration in the hippocampus, suggesting that the magnitude of the purine signal could indicate the severity of the epileptic insult. The sensitivity and specificity of these measurements had excellent performance in the mouse model with an area under the receiver operating characteristic (ROC) curve of 0.95. In the same study, we analyzed blood purine levels in 26 epileptic patients and 13 controls. Despite the patients not having experienced a seizure within 24 h of the measurement, we found that the baseline level of whole blood purines of epileptic patients was elevated by 2-fold relative to healthy controls (Beamer et al., 2021b). Overall, the diagnostic performance (area under the ROC curve of 0.79) in this study was highly promising and suggested that whole blood purine measurements could provide a rapid biomarker test suitable for triaging suspected epilepsy patients for more detailed clinical assessment via EEG as discussed further below (Beamer et al., 2021b).

Neonatal seizures are one of the most common neurological emergencies during the neonatal period, affecting 5 of 1000 infants (Pisani et al., 2018). Most neonatal seizures occur during the first 6 h after birth and are typically precipitated by an insult to the brain. The prognosis for infants with NE and seizures is particularly poor, with seizures leading to an increased rate of mortality and worsening of clinical outcomes (Pisani and Spagnoli, 2016). EEG is an excellent tool for detecting seizures but interpretation in neonates is an ongoing challenge that requires specialised personnel and equipment (Boylan et al., 2019). In a recent study, blood purines were measured, using the same technique as used for adults, in a mouse model of neonatal hypoxia-induced seizures and infants with neonatal encephalopathy (NE). Hypoxia was induced by exposing P7 mice, a developmental stage that roughly equates to the age of a term infant (Semple et al., 2013), to a 95% N2/5% O2 premixed gas for 15 min. In this model, seizures usually develop shortly after the onset of hypoxia and are also present post-hypoxia (Rodriguez-Alvarez et al., 2015). Blood purines were measured using a droplet of trunk blood that was taken immediately after 15 min of hypoxia. Whole blood sampling in infants was performed from the time of delivery until 4 days post-delivery via central and peripheral arterial lines and venous sampling at times of routine patient phlebotomy. The study included 21 infants with NE and five asymptomatic controls. Five infants with NE also experienced seizures. Here, our results showed that blood purine concentrations were elevated by 2-3-fold following hypoxia in mice. Similar to the hypoxia mouse model, infants with NE had a 2-3-fold elevation when compared to healthy controls. Of note, blood purine concentrations were higher in infants with NE and seizures as compared to infants with NE but without seizure activity (Beamer et al., 2021c). Therefore, our results suggest that analysing blood purine levels may not only support the detection of NE but also help in the identification of infants at risk of neonatal seizures. This is consistent with the increased blood purine concentrations in mice following status epilepticus, as described earlier (Beamer et al., 2021b).

5.2. Purinergic signalling components as diagnostic biomarkers for epilepsy

5.2.1. Circulating P2X7Rs as a biomarker for epilepsy

Increased inflammation within CNS tissues has been well-established as a contributing factor to epilepsy (Rana and Musto, 2018; Riazi et al., 2010). Several inflammatory molecules have been found to be altered in the blood of patients with epilepsy, which also highlight these bio-molecules as prospective diagnostic markers for epilepsy (Kobyłarek et al., 2019). This includes cytokines such as the known P2X7R down-stream pro-inflammatory cytokine IL-1β (Kamasak et al., 2020; Uludag et al., 2013; Uludag et al., 2015; Yu et al., 2012), HMGB1 (Zhu et al., 2018), and C-reactive protein (CRP) (Zhong et al., 2019).

P2X7Rs are highly expressed in innate and adaptive immune cells (Di Virgilio, Dal Ben, Sarti, GiulianI and Falzoni, 2017). Recent studies have shown P2X7R protein levels to be increased in the blood cells of patients with diabetes (Wu et al., 2015) and myasthenia gravis (Zhang et al., 2017), and in plasma of patients following sepsis (Martinez-Garcia et al., 2019). Notably, a recent study has shown that P2X7Rs can be shed into circulation under inflammatory conditions, most likely from monocytes (Giuliani et al., 2019). Suggesting increased P2X7R activation in the blood during epilepsy, the expression of several cytokines downstream of P2X7R activation (e.g., IL-1β, IL-18) has been found altered in the blood of patients with epilepsy (Kamasak et al., 2020; Mochol et al., 2020; Uludag et al., 2013, 2015; Yu et al., 2012).

To validate the change in P2X7R plasma levels during epilepsy, we recently analyzed plasma from healthy controls, patients with TLE and patients suffering from psychogenic non-epileptic seizures (PNES) by quantitative enzyme-linked immunosorbent assay (ELISA). Patients with PNES undergo events which resemble an epileptic seizure, but without the characteristic alterations on the EEG associated with epilepsy. Most patients with PNES will be on similar treatment regimens as patients with epilepsy and therefore represent a valuable control group (Lopez and LaFrance, 2022). Analyzed plasma samples included 34 healthy controls, 30 TLE patients, 11 patients with PNES and six patients with status epilepticus. Blood samples were taken via venipuncture at baseline (following a seizure-free period of at least 24 h) and 1 h following a seizure. P2X7R plasma levels were higher in TLE patients when compared to controls and patients with PNES. Of note, P2X7R plasma levels remained similarly elevated in samples collected 1 h following an EEG-detected seizure, suggesting no imminent effect of seizures on P2X7R plasma levels. P2X7R plasma levels also increased by 20% in patients with status epilepticus when compared to control. ROC analysis demonstrated a moderate level of sensitivity (60%) and good
P2X7R-dependent plasma biomarker following status epilepticus and attractant/human growth-regulated oncogene (KC/GRO) as a potential high sensitivity (90%) and specificity (63%) between TLE and PNES. Z.W. Wong and T. Engel identified white blood cells as the most likely cell type to overexpress P2X7Rs after status epilepticus, in line with a study showing that P2X7R was shed into the circulation from monocytes. Finally, to identify possible surrogate markers of P2X7R over-activation, blood samples from wild-type and P2X7R-deficient mice subjected to intra-amygdala KA-induced status epilepticus were analyzed using cytometry arrays. The analysis identified cytokine keratinocyte chemotactic/human growth-regulated oncogene (KC/GRO) as a potential P2X7R-dependent plasma biomarker following status epilepticus and during epilepsy (Conte et al., 2021). It is, however, important to bear in mind that the P2X7R is not the only purinergic receptor present in the blood. Other receptor subtypes, including P2 receptors (e.g., P2Y12 and P2Y1R (Dorsam and Kunapuli, 2004; Zerr et al., 2011)) and P1 adenosine receptors (Gessi et al., 2000; L. Zhong, Peng and Zeng, 2022), are also present in the blood and may change their expression according to disease progression, which may be presented as additional diagnostic targets.

5.2.2. Expression changes of the purinome in the brain as a diagnostic marker for epilepsy

The expression of several purine receptors (e.g., P2X7R) and purine metabolizing enzymes (e.g., ADK) has been shown to be increased in the brains of patients with drug-refractory epilepsy (Jimenez-Pacheco et al., 2016; T. Li et al., 2008). This suggests that their detection may support the diagnosis of epilepsy and inform on the choice of treatment and the risk of drug-refractoriness. Of note, we have previously shown that increased P2X7R expression led to decreased responsiveness to ASMs during status epilepticus in mice (Beamer et al., 2022). Functional neuroimaging using PET is a powerful, non-invasive tool for the identification of disease-specific biomarkers and is recommended in pre-surgical evaluation by the International League Against Epilepsy (ILAE) (Neuroimaging Subcommission of the International League Against Epilepsy (ILAE), 2000). In clinical practice, PET is primarily used to image glucose metabolism by the use of radiotracer 18F-fluoro-2-deoxy-D-glucose (18F-FDG), particularly in TLE (Lotan et al., 2020). In recent years, radiotracers have advanced beyond the identification of altered glucose metabolism, and ligand-specific radiotracers are currently being investigated. In particular, radioligands recognizing inflammatory molecules such as TSPO, a marker of activated glia and expressed in the outer membrane of mitochondria from microglia, astrocytes and macrophages, have shown promising results as prognostic markers of TLE and drug-refractory epilepsy (Gershen et al., 2015; Koepf et al., 2017). Of note, P2X7R radiotracers have been successfully developed and tested in animal models and humans (Berdyyeva et al., 2019; Kolb et al., 2019). Future studies should test these radiotracers in animal models and patients with epilepsy.

5.2.3. Purinome-associated polymorphisms as a risk factor for epilepsy and treatment success

With the advancement and development of new genomic technologies and high-throughput screenings, genetic alterations including de novo mutations and genetic polymorphism are increasingly recognized to contribute to seizure threshold and epilepsy development, and even to ASM resistance. This includes genes involved in neuronal excitability such as voltage-dependent sodium and potassium channels and the metabolism of endogenous and xenobiotic substances (Cardenas-Rodríguez et al., 2020; Myers and Melford, 2015). The identification of these genetic alterations may not only inform the risk of the patient developing epilepsy but also the choice of treatment (i.e., precision medicine (Demarest and Brooks-Kayal, 2018)). The gene encoding human P2rx7 is highly polymorphic with several single-nucleotide polymorphisms (SNPs) known to change the receptor function into either loss- or gain-of-function variants (Jimenez-Mateos et al., 2019; Slyuter, 2017). More importantly, several of these P2rx7 SNPs have been associated with common comorbidities associated with epilepsy, including major depression, mood disorders and sleep disorders (Gil et al., 2022; Lucae et al., 2006; Metzger et al., 2017). To date, several studies have linked SNPs of genes involved in purinergic signalling to seizures and epilepsy. This includes the P2rx7, the P2Y12 and ADK genes. In the case of the P2X7R, a study published by Emself et al. (2014) analyzed several inflammation-related genes in childhood-onset febrile seizures. Here, the authors found a strong association for the gain-of-function missense SNP rs208294 in P2rx7, suggesting this SNP to be involved in the susceptibility to childhood-onset febrile seizures (Emself et al., 2014). Interestingly, this P2rx7 SNP has been associated with several other pathologies including familiar mood disorders (Soronen et al., 2011). In the case of the P2Y12R, Wang et al. reported that two P2Y12 single-nucleotide SNPs (rs1491974 and rs6798347) may be associated with an increased risk of epilepsy. The authors analyzed 176 patients with epilepsy and 50 controls, however, without mentioning what type of epilepsy was analyzed (Wang et al., 2022). Regarding ADK, a recent study was carried out by Zhang et al. (2022), where certain ADK SNPs were analyzed for their possibility to predict treatment responses towards vagal nerve stimulation (VNS). The authors found a significant association between ADK SNPs (e.g., rs11001109, rs7899674, and rs94618S) and seizure reduction with VNS, thus suggesting that these SNPs may serve as biomarkers for the prediction of treatment success.

6. Potential applications and future directions

Owing to the strong connection of the purinergic signalling pathway with seizure and epilepsy, and the easily operated detection techniques (e.g., purine sensors, ELISA for P2X7R protein) (Beamer et al., 2021b), numerous potential purinergic signalling-based biomarkers could be employed in the diagnosis of seizures and epilepsy. This could enable a rapid screening of patients presenting seizure-like symptoms in the emergency department, support the stratification of patients with suspected epilepsy, and facilitate drug trials for the testing of new anti-convulsive and anti-epileptic treatments.

Purine biosensors are ideally suited for the rapid diagnosis of seizures (Beamer et al., 2021b), which is particularly critical in the emergency department. In the case of status epilepticus, a rapid diagnosis of seizure activity is vital to initiate fast and efficient treatments, thereby preventing/ameliorating the development of adverse clinical outcomes, or even death. Of note, patients with status epilepticus have to be treated as soon as possible due to an increased risk of drug refractoriness according to time spent seizing (Al-Faraj et al., 2021). While patients showing convulsions during status epilepticus may be diagnosed based on clinical manifestations, not all seizures come along with obvious behavioural changes. This includes patients with non-convulsive status epilepticus or neonatal seizures where behavioral changes are almost absent/non-detectable, and only identifiable with the use of EEG (Lee, 2022; Pisani and Spagnoli, 2016). Seizure-specific biomarkers would allow for their identification even in the absence of EEG or, at least, inform on the possible presence of underlying and ongoing seizure activity. Because purines can be measured in unprocessed whole blood immediately after sampling with minimal equipment, this technology
Purinergic signalling as a major convergence pathway in epilepsy linking diagnostics and therapeutics. Increasing evidence shows not only that the purinergic signalling system is an attractive target for the treatment of epilepsy driving numerous pathological pathways during epilepsy (e.g., inflammation, cell death, and hyperexcitability) but that this signalling system also offers several opportunities for diagnostic approaches (e.g., point-of-care devices and medical imaging).

While probably not suited for a fast diagnosis, the measurement of P2X7R in plasma may be used for an early stratification to identify patients with epilepsy and differentiate these from patients experiencing similar conditions such as PNES (Conte et al., 2021). This is particularly relevant as it is critical to distinguish patients experiencing real epileptic seizures from patients affected by psychogenic ones. This is because misdiagnosed cases between these conditions remain high, which may lead to unnecessary treatments, subsequent ASM-induced side effects, and possible delays in giving psychological therapy to PNES patients (Dickson et al., 2017; Rizzo et al., 2009). P2X7R measurements in plasma may also be used to detect underlying inflammatory conditions, thereby informing on the choice of treatment (i.e., adjunctive anti-inflammatory treatment). While not yet tested in experimental models or patients, PET imaging based on the purinergic signalling system may inform the choice of treatment (e.g., a patient with P2X7R overexpression may benefit from P2X7R blockers), drug-refractoriness (both P2X7R and ADK have been linked to a reduced response to ASMs (Beamer et al., 2022; Boison and Shen, 2010), or even identify people who are at risk of developing epilepsy after, for example, a brain injury such as TBI. Finally, genetic markers (e.g., P2rx7 SNPs) may inform the likelihood of developing epilepsy, and the choice of treatment. This would, however, require extensive research in much larger patient groups and at different hospitals/centres.

While we now have compelling evidence that demonstrates the diagnostic potential of the purinergic signalling system for epilepsy, there are several aspects which should be addressed before translating purinergic signalling-based diagnostic approaches into clinical practice. Diagnostic tests (e.g., purine biosensors, P2X7R levels in plasma), should be validated in increased patient cohorts. This would test their capability to detect and differentiate between different types, severity and frequency of seizures, underlying pathologies, and treatment responses. To date, biomarkers have been analyzed at one single time-point per patient, future studies should analyze these at various time points and whether biomarker levels change according to disease progression/treatment (e.g., after epilepsy surgery). While biomarkers detecting the occurrence of a recent seizure has been presented as a valuable tool, markers which would predict the next seizure event, as shown recently with tRNAs (Hogg et al., 2019), could be considered a game changer in epilepsy diagnosis. Being able to predict an imminent seizure would allow patients to receive seizure-suppressive treatment before its occurrence. Patients would be able to seek help if a seizure is about to occur. Sudden unexpected death in epilepsy (SUDEP), which refers to patient deaths from epilepsy that is not induced by injury, drowning or other known causes, is a major concern for patients and caregivers (Friedman, 2022). Being able to predict the next seizure attack may reduce the risk of patients having a seizure when unattended, and death as a consequence. Epilepsy comes along with numerous co-morbidities (Shlobin and Sander, 2022). Biomarkers supporting the diagnosis/identification of these comorbidities may help to design optimal treatment for patients. Another important aspect to consider is whether signalling-based biomarkers show circadian rhythmicity. The purinergic signalling system has not only been shown to impact circadian rhythms, but the expression of its components has also been shown to change their expressions according to the time of day (Ali et al., 2020).

While promising, changes in purinergic signalling (e.g., blood purines, receptor expression) are not unique to seizures and epilepsy and have been previously reported for different pathological conditions (Giuliani et al., 2019; Y. Li et al., 2022; Martinez-Garcia et al., 2019; Tian et al., 2017; Wu et al., 2015; Zhang et al., 2017). Nevertheless, single biomarkers are unlikely to be used as a stand-alone test and will be evaluated within a clinical context in combination with other measures. Notably, we have an array of potential new biomarkers for epilepsy including markers of inflammation or non-coding RNAs, but each of these is probably not specific towards a single disease. By combining these biomarkers into an “epilepsy biomarker panel”, the combined expression of these markers may be directed specifically towards effective epilepsy diagnoses.

7. Conclusions

Although the study of purinergic signalling as a diagnostic target is a relatively new area of research, in particular for the detection of seizures and the diagnosis of epilepsy, we now have evidence supporting its proof-of-principle. We have demonstrated that this signalling system is a rich source of both therapeutic targets and disease-specific biomarkers that can be used for the development of effective treatment regimens and diagnostic tests (Fig. 1). Future research should focus on validating the existing results in larger patient cohorts and advance these techniques into clinical practice.

Declaration of competing interest

The authors have no conflict of interest.

Data availability

No data was used for the research described in the article.

Acknowledgements

This work was supported by funding from Science Foundation Ireland (17/CDA/4708, and co-funded under the European Regional
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