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Commentary

Therapeutic epilepsy research: From pharmacological rationale to focal adenosine augmentation

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ABSTRACT

Epilepsy is a common seizure disorder affecting approximately 70 million people worldwide. Current pharmacotherapy is neuron-centered, frequently accompanied by intolerable side effects, and fails to be effective in about one third of patients. Therefore, new therapeutic concepts are needed. Recent research suggests an astrocytic basis of epilepsy, presenting the possibility of novel therapeutic targets. In particular, dysfunction of the astrocyte-controlled, endogenous, adenosine-based seizure control system of the brain is implicated in seizure generation. Thus, astrogliosis – a pathological hallmark of the epileptic brain – is associated with upregulation of the adenosine-removing enzyme adenosine kinase (ADK), resulting in focal adenosine deficiency. Both astrogliotic upregulation of ADK in epilepsy and transgenic overexpression of ADK are associated with seizures, and inhibition of ADK prevents seizures in a mouse model of pharmacoresistant epilepsy. These findings link adenosine deficiency with seizures and predict that adenosine augmentation therapies (AATs) will likely be effective in preventing seizures. Given the wide-spread systemic and central side effects of systemically administered AATs, focal AATs (i.e., limited to the astrogliotic lesion) are a necessity. This Commentary will discuss the pharmacological rationale for the development of focal AATs. Additionally, several AAT strategies will be discussed: (1) adenosine released from silk-based brain implants; (2) adenosine released from locally implanted encapsulated cells; (3) adenosine released from stem cell-derived brain implants; and (4) adenosine augmenting gene therapies. Finally, new developments and therapeutic challenges in using focal AATs for epilepsy therapy will critically be evaluated.

1. Introduction

Epilepsy is a heterogeneous syndrome characterized by excessive electrical discharges in neuronal clusters that result in spontaneous and recurrent seizures. Although it is one of the most prevalent neurological disorders, the cellular and molecular basis of epilepsy is still largely unknown. It is widely assumed that any imbalance between synaptic excitation and inhibition may cause intense neuronal discharges and hyper-synchronous activity in a large number of neurons. Regarding effective drug design, two mechanisms need to be distinguished: *ictogenesis* refers to the mechanisms that trigger an individual seizure, whereas *epileptogenesis* refers to the long-term mechanisms – usually involving a "latent period" of months to years – that transform a healthy brain into a hyperexcitable state with the propensity to develop recurrent spontaneous seizures (i.e., epilepsy). Currently, pharmacotherapy of epilepsy is largely limited to seizure suppression, i.e. to *anti-ictogenesis*. This symptomatic treatment approach has little prospect to affect the underlying causes of the disease. In fact, currently available antiepileptic drugs (AEDs) largely fail to prevent *epileptogenesis* or disease progression. A prerequisite for the prevention of epileptogenesis is a concise understanding of the molecular and histopathological changes that occur between an initial precipitating injury (IPI, i.e., the trigger for epileptogenesis) and the occurrence of the first epileptic seizure. This includes identification of markers for epileptogenesis, identification of subclinical ("silent") seizures that precede the expression of clinical ("convulsive") seizures, and identification of novel molecular targets that might interfere with epileptogenesis.

Despite the fact that most currently available AEDs are effective in suppressing ictogenesis, about a third of all epilepsies – mostly those of focal origin within the temporal lobe (temporal lobe epilepsy, TLE) – remain refractory to pharmacotherapy [1].

Abbreviations: AAT, adenosine augmentation therapy; AAV, adeno-associated virus; AED, antiepileptic drug; ADK, adenosine kinase; AR, adenosine receptor; BHK, baby hamster kidney; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EEG, electroencephalogram; GABA, gamma amino butyric acid; GAD, glutamic acid decarboxylase; hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell; HSV, herpes simplex virus; IPI, initial precipitating injury; KA, kainic acid; mESC, mouse embryonic stem cell; NMDA, *N*-methyl-D-aspartate; NP, neural precursor; NPY, neuropeptide Y; PES, polyethersulfone; RNAi, inhibitory RNA; SE, status epilepticus; SOD1, superoxide dismutase 1; TLE, temporal lobe epilepsy.

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In addition, many of the existing treatments cause drowsiness, sleepiness, adverse skin reactions, and scores of other side effects [1] that limit their optimal use or dosage. Brain-wide side effects are largely due to systemic drug formulations that unspecifically target neurotransmission, rather than specifically target hypersynchronous bursting. Thus, the requirements for future, more effective therapeutic strategies are: (i) efficacy in pharmacoresistant epilepsy; (ii) prevention of epileptogenesis and targeting of underlying disease processes; and (iii) limitation of side effects by focal mode of action.

2. Failure of neuron-centered pharmacotherapy

Why does current pharmacotherapy fail in over 30% of patients with epilepsy? Most AEDs are based on the *neuron*-centered concept that epilepsy is due to an imbalance between synaptic inhibition and excitation, which in turn is dependent on the equilibrium of gamma amino butyric acid (GABA)-ergic and glutamatergic neurotransmission. Consequently, AEDs directly influence the function of *neurons*: they target GABA-ergic neurotransmission (e.g. vigabatrin, tiagabin, phenobarbital, valproate), glutamatergic receptors (e.g. felbamate, phenobarbital, topiramate), neuronal sodium channels (e.g. phenytoin, carbamazepine, phenobarbital, valproate), neuronal calcium channels (e.g. gabapentin, pregabalin), or affect the presynaptic release of neurotransmitter vesicles (levetiracetam). In summary, all available AEDs directly affect neuron–neuron communication. They largely fail to influence glia–neuron interactions, gap-junction signaling, or inflammatory pathways, processes that are all now known to play an important role in ictogenesis as well as in epileptogenesis.

The majority of AEDs were developed based on conventional screening methods or modification of pre-existing AEDs, rather than on mechanism-directed drug design. Screening methods were largely based on *neuro*-centric epilepsy models and on seizure suppression paradigms. Thus, it is unsurprising that most AEDs are not effective in preventing epileptogenesis. Furthermore, the *neuro*-centric pharmacotherapy for epilepsy of the past decades has failed to provide a major breakthrough in overcoming refractoriness to currently available AEDs. Although some of the newer drugs (e.g. levetiracetam) provide benefit in individual patients with refractory epilepsy, drug refractoriness or intolerability is still a major problem. Several major antiepileptic drugs are substrates for multi-drug transporters [2], thus the failure of current AEDs in a significant portion of patients is aggravated by the development of pharmacoresistance due to increased activity of ABC multi-drug transporters, particularly P-glycoprotein, that become overexpressed during the course of epilepsy and facilitate the efflux and clearance of AEDs [2,3]. Thus, multi-drug transporters in endothelial endfeet of the blood brain barrier constitute an important non-neuronal target for epilepsy therapy. Tariquidar (XR9576) is likely to be a good candidate that appears to inhibit these proteins [3].

In summary, drug development based on the same *neuro*-centric principles as current AEDs is unlikely to provide a major breakthrough in epilepsy therapy. Thus, the search for novel therapeutic concepts and new targets outside classical chemical neurotransmission is currently an area of intensive investigation.

3. Neuron–glial interactions

Information processing in the brain depends on coordinated interplay between cellular circuits comprised of neurons and glia. Neuronal networks communicate via electrically excitable membranes and synaptic contacts embedded in a glial network. Formerly discredited as "glue of the brain," glial cells are now recognized as important contributors to intercellular ion-flux, neurotransmitter exchange, neuromodulation, and metabolite exchange. They function as a long-range communication route that integrates blood vessels and millions of synapses from different neurons into neuronal-glial-vascular units and then into more complex structures connected through a glial syncytium [4]. Thus, glial cells play important roles in coupling neuronal function to the cerebral microvasculature that controls cerebral blood flow (CBF) in the sense that increased neuronal activity requires corresponding increases in CBF. Apart from large stellar processes that stain for intermediate filaments, astrocytes have a multitude of fine processes that have little overlap with processes from other astrocytes and that define individual astrocytic domains, which in rodent hippocampus each contain 300–600 neuronal dendrites and 10^5 synapses [5,6]. Thereby, single astrocytes can sense the activity, and integrate the function, of hundreds of neurons within its domain. In addition, each astrocyte extends at least one process with endfeet surrounding blood vessels of the microvasculature. Therefore, astrocytes are uniquely located to adjust regional CBF to regional energy metabolism. Glia, in particular astrocytes, further serve as key regulators of neuronal function through a mechanism called gliotransmission [7]. Through the release of ATP and glutamate, frequently via regulated synaptic release, a single astrocyte has the unique capability to regulate the activity of hundreds of neuronal dendrites [5,6]. The loss of this astrocytic domain organization appears to play a major role in the pathogenesis of epilepsy [6]. It is therefore not surprising that glial cells are of utmost importance in determining pathological reactions of the brain and that glial function and dysfunction influences the outcome of a wide spectrum of neurological diseases.

4. An astrocytic basis for epilepsy

The central dogma that epilepsy is merely caused by dysfunctional neurons has recently been challenged. An "astrocytic basis of epilepsy" was first proposed by Maiken Nedergaard based on findings that seizures can be triggered by excessive release of astrocytic glutamate that directly targets *N*-methyl-D-aspartate (NMDA) receptors [8]. New studies from several laboratories suggest that non-neuronal (in particular glial) mechanisms comprised of self-reinforcing interplay between dysfunctional energy homeostasis, inflammation, and astrocytic signaling play a critical role in the development of epilepsy [9]. It was recently shown that specific gap-junction subunit proteins allow activity-dependent intercellular trafficking of glucose and its metabolites through astroglial networks [10]. In the absence of extracellular glucose, the delivery of glucose or lactate to astrocytes was able to sustain glutamatergic synaptic transmission and epileptiform activity when astrocytes were connected by gap-junctions [10]. These findings demonstrate that astroglial gap-junctions provide an activity-dependent intercellular pathway for the delivery of energetic metabolites from blood vessels to distal neurons [10]. Thus, excessive neuronal firing in epilepsy may be downstream from pathologically altered neuron–glia interactions.

In addition to mediating gliotransmission, astrocytes can synthesize numerous pro- and anti-inflammatory cytokines during seizures [11]. Astrocytic production of cytokines can lead to pro- or anticonvulsive outcomes, depending on the timing of expression and the receptors activated. Astrocytes also play important roles in modulating inflammatory pathways via purinergic mechanisms. Such findings underline novel functional neuron–glia interactions mediated by cytokines that can contribute to the neuropathology associated with inflammatory reactions in epilepsy [11].

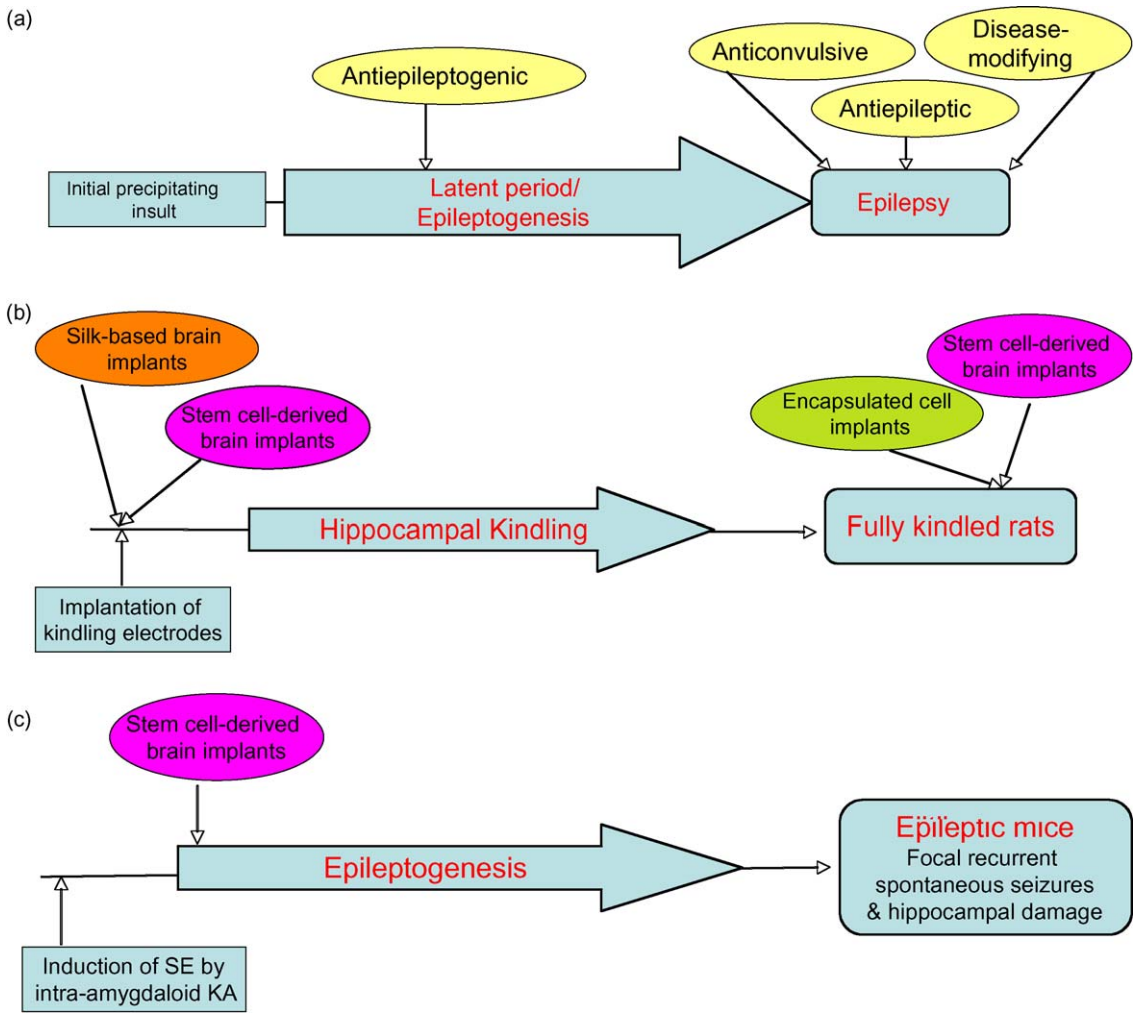

Fig. 1. (A) Steps in the development of acquired epilepsy and possible timepoints for therapeutic intervention. (B and C) Summary of animal models of temporal lobe epilepsy and experimental manipulations previously used to evaluate AATs.

5. Astrocytes: a novel target for antiepileptic therapy

Astrogliosis is a pathophysiological hallmark of the epileptic brain and a ubiquitous constituent of the epileptogenic cascade (Fig. 1). Several lines of evidence suggest that astrocyte dysfunction contributes to epileptogenesis and to seizure expression in epilepsy. Thus, failure of glia to buffer extracellular glutamate or dysfunctional release of glutamate by glia was shown to contribute to the maintenance of the paroxysmal depolarizing shift that characterizes neuronal dysfunction in epilepsy [8]. Astrocytes also play a key role in regulating the extracellular availability of the endogenous anticonvulsant adenosine [12,13] and an astrocyte-based adenosine-cycle has been proposed [13]. In addition to releasing adenosine directly, astrocytes can also release ATP – a precursor of adenosine – via a vesicular mechanism [12]. ATP is a wide-spread gliotransmitter, and regulated ATP release via vesicular exocytosis or via membrane channels has been demonstrated [14]. Glial release of ATP is implicated in the generation and maintenance of Ca^{2+} waves within the glial syncytium [15]. Additionally, ATP is an important regulator of pathophysiological glial reactions, including reactive gliosis and microglial activation [16]. In the extracellular space, ATP is rapidly degraded into the anticonvulsant adenosine by a series of extracellular nucleotidases. Thus, astrocytic release of ATP constitutes a major source of adenosine.

Geoffrey Burnstock was the first to recognize the key roles of purines in neurotransmission and neuromodulation in 1972. The identification of 5′-adenosine-triphosphate (ATP) as a novel neurotransmitter led to the concept of purinergic neurotransmission, which has been reviewed recently [17]. Adenosine, in particular, plays a prominent role in seizure regulation and has been found to be elevated in patients following seizures, leading to the conclusion that adenosine released during a seizure mediates seizure arrest and postictal refractoriness [18]. The crucial role of adenosinergic neuromodulation in the control of seizure activity is now well established and has recently been reviewed [19]. Key findings that contribute to our current understanding of the role of adenosine in epilepsy include the demonstration that the formation of adenosine is linked to energy metabolism [20], the demonstration that adenosine concentrations in brain rise as a consequence of seizures [18,21], and the demonstration of anticonvulsant effects of adenosine [22]. Given the prominent role of adenosine as a major modulator of a wide range of organ systems, the adenosinergic system has become a focus of drug development efforts; in particular the receptors for adenosine, and the enzyme adenosine kinase have become major targets for drug development (see below) [23–27].

As indicated above, adenosine is involved in one of several endogenous mechanisms of the brain that terminate seizures [28] and prevent seizure spread [29]. Adenosine exerts its neuromodulatory functions by binding to four known adenosine receptor subtypes (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) that all belong to the family of seven-membrane-spanning G-protein coupled recep-

tors [30]. The A_1R-mediated functions are largely responsible for the anticonvulsant and neuroprotective activity of adenosine. Thus, binding of adenosine to the high affinity A_1R, which is prominently expressed in the hippocampal formation, leads to decreased neuronal transmission and reduced excitability, primarily through inhibition of presynaptic transmitter release and stabilization of the postsynaptic membrane potential. Consequently, A_1R knockout mice experience spontaneous hippocampal seizures [31] and are hypersensitive to status epilepticus- or trauma-induced brain injury [29,32]. While the A_1R is thought to set a global inhibitory environment within the brain and to provide heterosynaptic depression, the stimulatory A_{2A}R is thought to be preferentially activated by high frequency stimulation and thus is ideally suited to potentiate selected synaptic transmission within a globally inhibited network [33]. In contrast to the well characterized anticonvulsant role of the A_1R in epilepsy, A_{2A} receptor activation appears to have both proconvulsant and anticonvulsant characteristics depending on the context of activation [19]. Whereas A_1Rs and A_{2A}Rs are primarily responsible for the central effects of adenosine, the low affinity and low abundance A_{2B}Rs and A_3Rs are currently not considered as therapeutic targets for epilepsy [19]. Functional receptor–receptor interactions of A_1Rs and different types of interactions with metabotropic and ionotropic receptors allow further complexity in adenosinergic neuromodulation. Due to their key role in regulating the availability of the endogenous anticonvulsant adenosine and due to their established dysregulation in epilepsy, astrocytes constitute an important, and hitherto neglected, target for antiepileptic therapeutic strategies.

6. Adenosine kinase: rational target for AATs

The astrocyte-specific enzyme adenosine kinase (ADK) constitutes a major metabolic reuptake system for adenosine and therefore largely regulates extracellular levels of adenosine [9,13]. A highly active substrate cycle between adenosine and AMP involving ADK and 5′-nucleotidase allows minor changes in ADK activity to rapidly translate into major changes in ambient adenosine [34]. Increased ADK levels are associated with seizures and increased susceptibility to brain injury, whereas decreased levels of ADK lead to seizure suppression and neuroprotection [35].

Recently, we characterized a novel mouse model of CA3-selective epileptogenesis triggered by unilateral intraamygdaloid injection of the excitotoxin kainic acid (KA) [35]. As a consequence of the KA-injection, status epilepticus (SE) develops and is experimentally terminated after 30 min with lorazepam. Within 24 h, CA3-selective neuronal cell loss develops and constitutes a trigger for subsequent epileptogenesis. After 3 weeks CA3-selective astrogliosis along with associated ADK upregulation and CA3-specific spontaneous seizures becomes prominent [35]. Using this model, we identified ADK in astrocytes as a molecular link between astrogliosis and neuronal dysfunction in epilepsy. To further assess the respective molecular roles of astrogliosis and ADK expression in seizure generation, we generated a line of transgenic mice (Adk-tg) that lacks the endogenous regulatable *Adk*-gene, but overexpresses a ubiquitously and constitutively expressed *Adk*-transgene on top of this *Adk*-knockout background. These mice are characterized by global ADK overexpression in the brain, including a novel ectopic expression of ADK in pyramidal neurons that results in spontaneous hippocampal seizures [35]. Together, these studies suggest that astrogliosis – a pathological hallmark of the epileptic brain – causes overexpression of ADK, which, – *per se* – was shown to be sufficient to trigger seizures. Thus, reconstitution or augmentation of adenosine and/or inhibition of ADK constitutes a pharmacological rationale for seizure suppression.

7. Pharmacological approaches

The identification of adenosine deficiency – as a consequence of upregulated ADK – as a major culprit for seizure generation implies that adenosine augmentation therapies (AATs) should be highly effective in preventing seizures. Indeed, focal intracranial injection of adenosine prevented seizures in rats [36]. Likewise, adenosine A_1R agonists are very effective in the inhibition of neuronal activity and in the suppression of seizures [25] and have been the subject of intense drug development efforts [37–39]. However, despite activity in a variety of models and efficacy in pharmacoresistant epilepsy [40], A_1R agonists, when given systemically are not potential antiepileptic agents because of profound peripheral, mainly cardiovascular, effects [41]. Since endogenous adenosine levels increase during times of stress [21] (e.g. lack of oxygen, seizures), agents (e.g. the ADK inhibitor ABT-702 [42–44]) that amplify this site- and event-specific surge of adenosine could provide antiseizure activity similar to that of adenosine receptor agonists [26,27]. Thus, pharmacological inhibition of ADK has been demonstrated to be an efficient tool for the inhibition of epileptic seizures [26,45] and chronic pain [46]; these successes were associated with an improved therapeutic window compared to A_1R agonists [47]. However, systemic application of ADK inhibitors might not be a therapeutic option for epilepsy due to interference with methionine metabolism in liver [48,49] and the risk of brain hemorrhage [46,50].

8. Need for focal intervention

Although adenosine as such, as well as A_1R agonists and ADK inhibitors are effective in seizure suppression, their systemic application is precluded by peripheral side effects. Therefore, focal adenosine delivery, – for example by devices such as synthetic slow-release polymers, pump systems that can be coupled to integrated seizure prediction systems, cellular implants, or gene therapy – constitutes an elegant strategy to avoid wide-spread side effects. Focal therapeutic approaches for refractory epilepsy have demonstrated that focal drug delivery to the brain is generally well tolerated and devoid of major side effects. The following arguments support focal AATs as new pharmacological tools for the treatment of pharmacoresistant epilepsy: (i) deficits in the adenosine-based neuromodulatory system have been associated with epileptogenesis and these deficits promote seizures [13]; thus, reconstitution of the inhibitory adenosinergic tone is a rational therapeutic strategy. (ii) Focal delivery of adenosine from encapsulated cells suppressed seizures in kindled rats without overt side effects [51]; thus, paracrine release of adenosine from therapeutic cells is sufficient to suppress seizures and functional integration of a graft is not necessary. (iii) The anticonvulsant activity of locally released adenosine is maintained in animal models of epilepsy that are resistant to major antiepileptic drugs [40]; thus, adenosine promises to be effective in drug resistant forms of epilepsy. In the following paragraphs four different AAT strategies are presented and discussed. These strategies are also summarized in Table 1.

9. AAT-strategy #1: adenosine-releasing silk-based brain implants

The slow and sustained release of small molecule drugs (e.g. adenosine) remains a major challenge in the field of controlled drug delivery. A number of biomaterials are available for drug delivery devices; however, purified silk fibroin protein is a unique option for several reasons. Silk fibroin is biocompatible and biodegrades slowly [52]. Degradation lifetimes of silk can be regulated, allowing control of degradation timeframes from weeks

Table 1
Summary of AAT studies.

AAT	Model	Intervention time point	Structural target	Effect on seizures	Interpretation	References
Silk-based brain implants	Hippocampal kindled rats	Prior to kindling	Infrahippocampal fissure	Dose-dependent retardation of kindling acquisition	Antiepileptogenic properties	[53,70]
Encapsulated cells	Hippocampal kindled rats	Fully Kindled	Lateral ventricle	Significant seizure suppression; transient due to life expectancy of encapsulated cells	Anticonvulsant properties	[51,56]
Stem cell-derived brain implants	Hippocampal kindled rats	Prior to kindling	Infrahippocampal fissure	Robust suppression of kindling epileptogenesis	Antiepileptogenic or disease-modifying properties	[61]
	Mouse model of focal epileptogenesis	24 h after KA-induced status	Infrahippocampal fissure	Complete lack of chronic spontaneous seizures, reduced astrogliosis, and normal ADK expression levels		[35,64]
	Hippocampal kindled rats	Fully kindled	Lateral ventricle	Complete seizure suppression, transient due to limited viability of implants		[57]
Gene therapy	N/A	N/A	N/A	N/A	N/A	N/A

AAT: adenosine augmentation therapies; N/A: not applicable.

to years [52]. The frequent use of silk sutures in brain confirms the feasibility of implanting silk biomaterials into brain.

In a recent study, a scaled, hierarchically structured silk protein based delivery approach for adenosine with target release rates of 0, 40, 200, and 1000 ng adenosine per day was used [53]. These polymers were implanted into the infrahippocampal fissure of rats prior to the onset of electrical kindling, a widely used model of temporal lobe epilepsy (TLE) with high predictability for clinical applications. The rat kindling model used in this study is based on repetitive subconvulsive suprathreshold electrical stimulation of the hippocampus, a structure of the limbic system critical to epileptogenesis. After repeated stimulations, seizures develop gradually with progressive intensity, a convenient approach to monitor ongoing epileptogenesis. Using this model, it was demonstrated that focal adenosine release from silk-based polymers effectively retarded kindling epileptogenesis in a dose-dependent manner. Importantly, recipients of polymers releasing a target dose of 1000 ng adenosine per day did not display any behavioral seizures during the time window of active adenosine release, whereas control rats experienced convulsions during the same period. As soon as adenosine release from the polymers began to wear off, seizures gradually re-appeared with progressive intensity.

This study revealed a novel silk-based delivery system for adenosine that fulfills crucial requirements for future clinical application, such as: (i) biocompatibility, (ii) delivery of predetermined doses of adenosine, (iii) safety as opposed to xenotransplantation using animal cells, (iv) sustained function via slow degradation in vivo, and (v) therapeutic efficacy in a widely used preclinical model with a high predictive value for drug development. Although the polymer design used in this study [53] precluded long-term applications due to adenosine depletion over a period of 2 weeks, the system would nevertheless be suited for short-term clinical safety and feasibility trials, in which the implant would be placed into an epileptic focus prior to its scheduled surgical resection.

10. AAT-strategy #2: cell-encapsulation for focal adenosine delivery

First developed almost 20 years ago by Aebischer et al. [54], cell-encapsulation for localized drug delivery can be used in *ex vivo* gene therapy approaches. In this approach, a suitable cell line is first genetically engineered to release a therapeutic compound and then encapsulated in a semipermeable polymer membrane and transplanted locally into the host brain. Cell-encapsulation permits the exchange of oxygen, extracellular metabolites, and nutrients between the encapsulated cells and the host tissue, while simultaneously preventing direct graft-cell/host-cell interactions. This technology is therefore ideally suited to selectively study paracrine effects of transplanted cells without confounds that may arise through direct network interactions. This is of importance, since direct network interactions may either promote or prevent seizures, depending on the source of the grafted cells. An additional advantage of cell-encapsulation is the immunoisolation of the encapsulated cells, although this concept has been questioned [55].

To utilize cell-encapsulation technology for focal paracrine release of adenosine, baby hamster kidney (BHK) fibroblasts and mouse C_2C_{12}-myoblasts were subjected to chemical mutagenesis followed by selection for ADK-deficiency [51,56]. In both BHK fibroblasts and mouse C_2C_{12}-myoblasts, ADK-deficiency induced the release of adenosine in amounts up to 20 ng per 10^5 cells per hour. 2×10^5 cells of each cell type were encapsulated into PES hollow fibers. Cell capsules were implanted into the lateral brain ventricles of fully kindled epileptic rats. After recovery from surgery, all animals were subjected to electrical test stimulations delivered over several days and weeks following implantation. Initially, all adenosine-releasing devices afforded almost complete seizure suppression [51,56]. This was most evident during the first 12 days after implantation. In contrast, recipients of control cells continued to display their full spectrum of epileptic seizure activity. Seizure suppression by ADK-deficient implants depended on the paracrine release of adenosine, since the A_1R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Sigma–Aldrich Corp., St. Louis, MO, USA) transiently restored epileptic seizures in stimulated animals. Seizure suppression was limited by the life expectancy of the encapsulated cells, with BHK-cells losing their efficacy after 2 weeks of implantation. C_2C_{12}-myoblasts survived and suppressed seizures for up to 8 weeks. Most importantly, the focal use of cell-based adenosine delivery did not cause receptor desensitization, nor was it accompanied by sedative side effects [56].

In summary, the therapeutic use of adenosine-releasing encapsulated cells demonstrated that paracrine adenosine delivery to the brain effectively suppressed fully kindled seizures. While the lack of receptor desensitization and the lack of side effects appeared promising, the overall therapeutic success was limited by the poor long-term viability of the encapsulated cells and the use of xenografts. Future studies should therefore focus on improved cell-encapsulation technology and the use of human cells that are mandatory for future therapy development.

11. AAT-strategy #3: stem cell-derived brain implants to secrete adenosine

Stem cells, their derivatives, and fetal hippocampal neurons have recently received much attention as direct transplantation tools for epilepsy therapy [57–59]. While functional integration of graft-derived cells is needed for restorative approaches, stem cell-derived brain implants can also be engineered to release therapeutically active molecules with the aim to provide therapeutic benefit by paracrine mechanisms. Finally, a combination of functional integration and paracrine drug delivery may provide improved benefits.

The first generation of adenosine secreting stem cells was engineered by a bi-allelic targeted deletion of the endogenous *Adk*-gene in mouse embryonic stem cells (mESCs) [60]. Using established protocols to induce neural differentiation, neural progenitor (NP) cells were generated. In these cells ADK-deficiency induced the release of therapeutic doses of adenosine, whereas corresponding wild-type cells did not release significant amounts of adenosine [60]. Two studies were performed (in mice and rats, respectively) to investigate the therapeutic effectiveness of ADK-deficient mESCs [35,61]. In both studies, the therapeutic cells were grafted into the infrahippocampal fissure, where they survived for at least 4 weeks and formed a dense cell cluster, likely a source for the paracrine release of adenosine. In both studies, a subset of cells migrated into the ipsilateral CA1, where those cells assumed a neuronal morphology and expressed the neuronal marker NeuN [35,61]. In the first study [61] (performed in rats), ADK-deficient mESC-derived NPs were implanted prior to the onset of electrical hippocampal kindling. Thus, kindling stimulations were performed in the presence of the grafted cells. In contrast to sham-controls or recipients of wild-type cells, recipients of adenosine-releasing cells displayed sustained protection from generalized stage 4 or 5 seizures over 22 days following cell implantation. Overall, this study demonstrated a robust suppression of kindling epileptogenesis by adenosine-releasing mESC-derived infrahippocampal implants [61]. In the second study, either ADK-deficient mESC-derived NPs, corresponding wild-type cells, or a sham procedure was administered to mice 24-h after an epileptogenesis-triggering intraamygdaloid KA-injection [35]. In this model of epileptogenesis spontaneous recurrent electrographic seizures normally develop within 12 days following KA-injection. Three weeks after KA-injection all animals were subjected to extensive EEG-monitoring of seizure activity. While all control animals experienced recurrent electrographic seizures, none of the recipients of the adenosine-releasing cells developed any seizure [35]. In addition to the complete lack of seizures, recipients of adenosine-releasing cells were characterized by a significant reduction in astrogliosis and by normal levels of ADK expression [35]. These results suggest a potential antiepileptogenic effect of adenosine-releasing brain implants.

The use of human embryonic stem cells (hESCs) for research and therapy has spawned much controversy in recent years. Potential strategies and pitfalls concerning hESC-based treatment strategies have recently been reviewed [62]. hESCs isolated from the inner cell mass of human 4.5-day-old pre-implantation embryos have unlimited capacity for self-renewal in culture. Under defined culture conditions they can be directed to differentiate into any adult cell type. Apart from ethical concerns, significant scientific challenges need to be met before hESCs can safely be used in human patients: specific differentiation of the cells needs to be controlled by culture conditions, genetic modification, or selection procedures. Tumor formation needs to be excluded by depletion of tumorigenic cells or enrichment of non-tumorigenic cells and by the use of early passages and karyotyping to exclude genetic aberrations. Inflammation and graft rejection needs to be prevented by immunosuppression, the induction of immunotolerance, or somatic cell nuclear transfer. Although several protocols have been developed to direct hESCs into neuronal differentiation pathways *in vitro* these cells have not yet been used in experimental epilepsy paradigms. Due to the availability of ethically acceptable and safer alternatives that can be tailored to patient-compatible or autologous approaches, it remains to be seen whether hESCs will be developed further for antiepileptic therapy. Eventually, the use of adult germ line stem cells as a source for functional neurons might provide a more promising alternative.

Adult stem cells constitute a versatile source for regenerative medicine and have – if patient-derived – the potential for personalized therapies and the advantage of autologous grafting without the need for immunosuppression. Adult stem cells can easily be derived from bone marrow, skeletal muscle, skin, or lipoaspirate. They normally form progeny according to their tissue of origin (i.e. skin stem cells will form skin); however, if directed experimentally into specified differentiation pathways they can form a wide variety of differentiated progeny including neurons. This might be beneficial, if functional integration or network interactions are desired. However, if paracrine drug delivery is the major goal, then the differentiation state of the transplanted cells is not of crucial importance as long as the cells will survive long enough to provide therapeutic benefit.

In order to efficiently engineer human adult stem cells for therapeutic adenosine delivery, we developed a lentiviral expression vector that expresses an inhibitory micro-RNA directed against ADK. This vector was highly effective in downregulating ADK in human mesenchymal stem cells (hMSCs). This strategy yielded hMSCs with up to 80% ADK-knockdown and was sufficient to trigger adenosine release [63]. Using a mouse model of CA3-selective epileptogenesis, we transplanted ADK-knockdown hMSC, or respective control cells that expressed a lentivirus containing a scrambled RNA-sequence, into the infrahippocampal fissure at two different time points. When transplanted 1 week prior to KA-injection, the implanted ADK-knockdown cells provided robust neuroprotection and ameliorated acute KA-induced seizures [63]. When transplanted 24 h after the epileptogenesis-triggering KA-injection the ADK-knockdown cells reduced subsequent epileptogenesis [64]. Three weeks after KA-injection, recipients of ADK-knockdown cells had significantly reduced seizure activity, significantly reduced astrogliosis and significantly reduced ADK upregulation [64]. These findings suggest that hMSCs can survive at least 3 weeks within the infrahippocampal fissure. The implants exert therapeutic effects by paracrine adenosine release in acute seizure paradigms and in chronic epilepsy. A caveat of these studies was that these experiments involved a combination of immunosuppression and cross-species transplantation (human into mouse); nevertheless, these data suggest that hMSCs derived from a patient could be engineered for therapeutic adenosine delivery in a personalized treatment approach.

12. AAT-strategy #4: gene therapy

Gene therapy provides an intriguing strategy for treating seizures and epilepsy, especially focal epilepsies, where the site of

seizure genesis is well defined. Since the first successful demonstration of gene transfer and expression in the brain [65], investigators have utilized a number of viral vectors to deliver genes targeted for a variety of neurological disorders. Viral vectors, in particular lentivirus, herpes simplex virus (HSV) and adeno-associated virus (AAV) are currently the most promising tools to directly introduce genes into the brain. These viruses are able to transduce both mitotic and non-mitotic cells, and the use of cell type-specific promoters allows targeted gene transfer to specific cell types. Thus, it is possible to restrict viral expression and gene therapy effects to appropriate regions, minimizing the possibility of adverse side effects and maximizing therapeutic efficacy.

Experimental studies aimed at developing gene therapy for epilepsy to date have been restricted to the use of AAV vectors. AAV vectors are particularly attractive candidates as delivery vehicles for gene therapy applications due to their ability to efficiently transduce and maintain prolonged expression in brain cells, their general lack of pathogenicity, their lack of induction of a cellular immune response, and the large number of available serotypes. Although no clinical trials have yet been commenced for AAV-mediated gene therapy in epilepsy, a pilot study using brain slices obtained from patients undergoing temporal lobe resection provides promising data [66]. In this study, slices injected with an AAV vector containing a lacZ marker gene and incubated for up to 24 h showed preferential neuronal transgene expression within 5 h of incubation, with sustained expression for as long as the slices were viable. Furthermore, there was no evidence of cytotoxicity. Future studies substituting the lacZ marker gene with a functional gene to modulate hippocampal physiology may allow localized therapeutic intervention for focal seizures.

What gene targets should be utilized in gene therapy approaches? Presumably, a number of genetic targets may potentially be able to restore the compromised balance between excitatory and inhibitory transmission in epilepsy. Thus far, approaches have primarily been focused on neuronal targets, selectively modulating neurotransmitters such as NMDA and GABA, or neuroactive peptides such as galanin and NPY [67]. Together, these studies demonstrate the ability to transduce specific cell populations based on precise and cell targeted gene therapy approaches. Furthermore, they provide a foundation for the idea that strategies targeting abnormal gene expression in epilepsy (such as upregulated ADK in astrocytes) hold promising therapeutic potential.

Although the current studies modulating gene expression in epilepsy provide interesting data, they highlight a potential limitation: all approaches rely on the assumption that neuronal dysfunction is the underlying cause for seizure genesis, without addressing astrocytic dysfunction. As previously discussed, astrogliosis with accompanying upregulation of ADK in reactive astrocytes is a pathological feature in sclerotic epileptic tissue. This provides a readily available gene target for viral vector-mediated adenosine regulation. We suggest that it might be possible to address this limitation through precise manipulations (e.g., promoter design) to preferentially target ADK-knockdown vectors (e.g. expressing inhibitory RNAi directed against ADK) to astrocytes, with the aim to knockdown overexpressed ADK in the pathologic hippocampus. To date, most of the AAV serotypes have predominantly a neuronal tropism; however, given the large number of available serotypes, future studies may be able to utilize some with glial tropism.

Overall, available clinical and preclinical data suggest that epilepsy is an attractive candidate for which gene therapy is likely to have positive outcomes, and further emphasizes the need for novel strategies aimed at exploring the adenosine system as a gene therapy target. To date, over a thousand clinical trials using gene therapy have been designed, with several of those trials targeting neurological diseases. However, there are still ethical and technical issues that require further study, and the potential for adverse effects is still relatively unknown. One of the benefits of gene therapy compared to other current drug regimens is the persistent expression of the therapeutic gene; however, the sustained vector-mediated-transgene expression may itself cause adverse side effects. The potential for de-inactivation of the viral vector is also a significant concern, but results from human clinical trials appear promising regarding safety profiles [68,69].

13. Anti-ictogenic potential of focal AATs

The anti-ictogenic potential of AATs is well documented. Prophylactic focal adenosine infusion reduced induced seizures in rats [36], whereas polymers engineered to release adenosine effectively prevented established kindled seizures and kindling development when implanted either into the lateral ventricle or into the infrahippocampal fissure [53]. Likewise, local implants of cells engineered to release adenosine prevented established kindled seizures and the expression of seizures in a post status epilepticus model [35,51]. In these studies, seizures could be restored after the injection of the A_1R antagonist DPCPX, which is not proconvulsant in nonepileptic animals at the doses used [51]. These pharmacological control experiments demonstrated that focal AATs effectively suppress seizures via activation of A_1Rs. A dose response study performed in kindled rats suggests minimally effective doses of adenosine in the range of 150–600 ng per kg per day [53]. Doses of up to 3000 ng per kg per day did not cause any overt side effect. Therefore adenosine augmentation appears to have a fairly wide therapeutic window ranging from 150 to 3000 ng per kg per day. This wide therapeutic window can best be explained by effective metabolism (e.g. via ADK) of the endogenous substrate adenosine. Due to effective clearance of adenosine, it is unlikely that toxic concentrations of adenosine can accumulate within this wide therapeutic window.

14. Antiepileptogenic potential of focal AATs

The antiepileptogenic potential of focal AATs is more difficult to assess. The inherent problem in those studies is that adenosine will also suppress evolving seizures during the epileptogenetic process. Thus, epileptogenesis might be masked by continuous seizure suppression. Several studies suggest antiepileptogenic effects of focal AAT:

(i) Infrahippocampal implants of silk-based polymers or of stem cells engineered to release adenosine suppressed kindling epileptogenesis in rats [53,61,70].
(ii) During the suppression of kindling epileptogenesis, the adenosine A_1R antagonist DPCPX was not able to increase seizure scores [70], whereas DPCPX restored generalized stage 5 seizures in fully kindled rats that had received adenosine-releasing brain implants post kindling [51]. These findings demonstrate that the suppression of fully kindled seizures by adenosine is fully reversible by inhibition of the A_1R; however, the finding that DPCPX could not increase seizure scores during kindling epileptogenesis suggests that seizures were not masked by adenosine and that epileptogenesis was indeed suppressed.
(iii) Possible antiepileptogenic effects of implant-derived adenosine were recently studied in more detail using a paradigm in which rats were halfway kindled in the presence of a silk-polymer that was designed to release 1000 ng adenosine for a period of only 10 days [70]. After a delay of 9 days (to allow expiration of adenosine release) kindling was resumed. This

experimental paradigm is suited to quantify the degree of antiepileptogenesis [71]. Drugs that do not have any anticonvulsant effects (e.g. carbamazepine in [71]) result in matching kindling curves between control and treatment groups, both during and after the drug phase. Drugs that have partial antiepileptogenic effects (e.g. phenobarbital in [71]) suppresses kindling development during the drug phase and resume kindling development at the same stage at which kindling was discontinued. Drugs that exert complete antiepileptogenic effects (e.g. valproate in [71]) suppress kindling development during the drug phase and kindling is resumed after discontinuation of the drug at the same stage as before the drug was discontinued; however, the numbers of drug-free afterdischarges required to elicit corresponding seizures in drug-treated animals is significantly more than that required for control animals. According to these considerations, silk-based adenosine-releasing polymers demonstrated almost complete suppression of kindling development during the first set of kindling stimulations (which was sufficient to kindle recipients of control implants completely) [70]. When kindling was resumed after expiration of the polymer, recipients of adenosine-releasing implants were still protected and in the absence of implant-derived adenosine gradually developed kindled seizures. These findings demonstrate that the transient release of adenosine during the first kindling sessions provided at least partial prevention of epileptogenesis. If lack of seizures during that time were due to adenosine-based seizure suppression (masking epileptogenesis), then animals should have resumed kindling with stage 5 seizures after expiration of the polymer.

(iv) In contrast to animals receiving control implants or a sham procedure, mice implanted with adenosine-releasing stem cells in the infrahippocampal fissure immediately after acute ipsilateral CA3 injury displayed reduced astrogliosis, failed to upregulate ADK, and failed to develop spontaneous seizures [35]. These findings demonstrate that focal AATs cannot only prevent the expression of spontaneous seizures, but most importantly can also suppress the development of two histopathological hallmarks of the epileptic brain: astrogliosis and upregulation of ADK. These histopathological findings further support an antiepileptogenic role of focal AATs.

These examples support a novel role of focal adenosine release to provide at least partial antiepileptogenic and disease-modifying effects.

15. Therapeutic prospects and limitations

As discussed, the results of several studies in both acute and chronic models of epilepsy strongly suggest that focal AAT's have anticonvulsive, possibly antiepileptogenic, and possibly neuroprotective and/or neuro-reparative properties. The therapeutic conceptual framework of focal AAT development differs from classical AED development in several essential ways: (i) as pointed out initially, current AED development follows a *neuro-centric* concept. It is unlikely, that the development of new AEDs acting on

Table 2
Overview of benefits and limitations of alternative epilepsy therapies.

Therapy	Benefits	Limitations	Selected references
Antiepileptic drugs	Satisfactory seizure control and favorable risk benefit balance for most patients Noninvasive	Refractoriness in about one third of patients Seizure aggravation Loss of effect (tolerance) Adverse effects Teratogenicity	[1]
Resective surgery	Can be highly efficacious in patients with refractory focal epilepsies	Invasive Seizures must be focal with clearly identifiable epileptic zone Seizure focus must be in a region suitable for surgical resection	[1]
Neurostimulation	Seizure freedom possible for some Individualized therapy (may be tailored to patient and/or specific form of epilepsy) Programmable restimulations in cases of persistent seizure activity Can be reversed and/or adjusted	Risks of brain hemorrhage and infection No consensus on what regions to stimulate and in what seizure types treatment may be effective Palliative	[72]
Polymeric implants	Paracrine release of AEDs Limitation to focal area Avoidance of side effects Safety	Invasive procedure Local effectiveness/limitation to partial epilepsies with defined foci	[53,70]
Cell therapy	Possibility for repair and network reconstruction Release of paracrine factors Local restriction to epileptogenic focus	Long-term effectiveness/viability Control of network interactions Immunosuppression if not autologous cell source.	[57–59]
Gene therapy	Possibility for permanent cure of epilepsy Rational therapeutic design possible Potential to prevent epileptogenesis Minimal immune responses and non-pathogenic nature of AAV	Gene inactivation over time Size-limitations for therapeutic gene inserts (e.g. AAV) Permanent integration/alteration of host genome	[65–69]
Dietary therapies	Effectiveness in children with epilepsy Effectiveness in many pharmacoresistant epilepsies Augmentation of adenosine	Insufficient controlled evidence to support efficacy Long-term safety profiles unknown Compliance	[73,74]

the same (neuronal) targets will lead to any striking breakthroughs in antiepileptic therapy. In contrast, adenosine augmentation constitutes a new pharmacological principle that has not yet been exploited in clinical epilepsy therapy. (ii) In contrast to AEDs adenosine is an *endogenous* anticonvulsant and therefore subject to physiological regulatory mechanisms. Due to rapid clearance through metabolism it is unlikely that toxic concentrations would accumulate; rather, adenosine augmentation is expected to restore the adenosinergic equilibrium. In addition, as pointed out earlier, the wide therapeutic window of adenosine, in particular when administered locally, offers the potential to avoid undue side effects. (iii) Augmentation of adenosinergic signaling was demonstrated to be effective in a mouse model of pharmacoresistant epilepsy; thus, adenosine – as an endogenous metabolite – does not seem to be a substrate for multi-drug transporters. These arguments, in combination with local (invasive) delivery strategies, make focal AATs a promising approach for future antiepileptic therapy in the clinic.

Preliminary assessment suggests that focal AATs are not associated with overt side effects; in particular, open field studies have demonstrated that focal AATs are not associated with sedation in contrast to systemic AATs [56]. To date however, potential central side effects of focal AATs have not been evaluated systematically, since a stable dose of adenosine delivery over several days is necessary for those studies. This latter goal has only been achieved recently [70]. Future studies are needed to address in detail potential CNS-side effects of focal AAT including psychomotor and cognitive function.

Several crucial issues regarding safety and efficacy need to be resolved pre-clinically, before progressing to the clinical realm. These concerns include, but are not limited to: (i) clinical relevance of animal models utilized; (ii) efficacy, safety, and general suitability of viral vectors or therapeutic cell sources; (iii) determination of appropriate time points for therapeutic intervention; (iv) determination of appropriate therapeutic targets; (v) viability of cell grafts and implants, or longevity of viral expression; and (vi) assessment of whether alterations in epileptic tissue (such as molecular and synaptic plasticity, neuronal cell loss, or astrogliosis) may affect therapeutic targets.

Current alternative options for patients with refractory epilepsy (Table 2) include temporal lobe resection, neuromodulation through neurostimulation (such as vagus nerve stimulation, transcranial magnetic stimulation, and deep brain stimulation [72]), and the ketogenic diet [73,74]. While temporal lobe resection is frequently effective, this procedure is restricted to certain patient populations. Interestingly, both neurostimulation as well as the ketogenic diet appear to work via an adenosine-dependent mechanism [74], which might be an explanation for their effectiveness in pharmacoresistant epilepsy. Eventually, focal AATs combine adenosine's therapeutic potential with a local mode of action, thus limiting the extent of surgical intervention and the spread of potential side effects.

16. Concluding remarks and outlook

AATs rationally utilize the brain's endogenous adenosine-based antiseizure system, thereby presenting significant therapeutic potential for epilepsy. Adenosine is already FDA-approved for the treatment of supraventricular tachycardia and has been used in intrathecal infusions in phase I clinical trials for the treatment of chronic pain [75]. Based on these considerations and based on its relative safety profile, focal AATs could rapidly be translated from animal studies to clinical trials. A time frame of 5 years for the initiation of first clinical trials with focal AATs appears to be reasonable. A first possible step could be the infusion of adenosine into an epileptic temporal lobe during its surgical removal. When coupled to synchronous EEG recordings, a proof-of-principle could be established that adenosine is effective in pharmacoresistant human epilepsy. Focal implantation of adenosine-releasing silk-based polymers appears to be the safest and most straightforward route for clinical implementation of focal AATs to demonstrate the safety and efficacy of intrafocal adenosine release for the suppression of pharmacoresistant seizures in patients. Clinical trials can be envisioned for patients with MTLE. This form of epilepsy is selected for three reasons: (i) it is one of the most common forms of refractory epilepsy, (ii) it has a relatively homogeneous pathophysiology, and (iii) it displays rather uniform clinical characteristics. The patients afflicted with this disease are frequently drug resistant and 70–80% of these are candidates for amygdalo-hippocampectomy. Patients who are referred for amygdalo-hippocampectomy might be selected for future implantation therapy. The adenosine-releasing devices could be implanted stereotactically into the epileptic focus. It is understood that patients who undergo such a treatment will require sophisticated follow-up studies to document the clinical efficacy of the treatment on the epilepsy of the patient (epileptological and EEG controls), the structural characteristics of the target area (MRI), to detect and measure functional/metabolic changes after treatment, and neuropsychological and cognitive evaluations. In the case that patients with an implant do not show seizure suppression, the primary epileptogenic zone would be excised using a conventional surgical approach. The resected brain region including the implant would then be carefully studied using advanced histological and biochemical methods to gain further insight into the behavior of reactions to the implant within the human brain.

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