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Adenosine augmentation therapies (AATs) for epilepsy: prospect of cell and gene therapies

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Abstract

Deficiencies in the brain's own adenosine-based seizure control system contribute to seizure generation. Consequently, reconstitution of adenosinergic neuromodulation constitutes a rational approach for seizure control. This review will critically discuss focal adenosine augmentation strategies and their potential for antiepileptic and disease modifying therapy. Due to systemic side effects of adenosine focal adenosine augmentation – ideally targeted to an epileptic focus – becomes a therapeutic necessity. This has experimentally been achieved in kindled seizure models as well as in post status epilepticus models of spontaneous recurrent seizures using three different therapeutic strategies that will be discussed here: (i) Polymer-based brain implants that were loaded with adenosine; (ii) Brain implants comprised of cells engineered to release adenosine and embedded in a cell-encapsulation device; (iii) Direct transplantation of stem cells engineered to release adenosine. To meet the therapeutic goal of focal adenosine augmentation, genetic disruption of the adenosine metabolizing enzyme adenosine kinase (ADK) in rodent and human cells was used as a molecular strategy to induce adenosine release from cellular brain implants, which demonstrated antiepileptic and neuroprotective properties. New developments and therapeutic challenges in using AATs for epilepsy therapy will critically be evaluated.

Keywords

adenosine; cell therapy; gene therapy; silk; epilepsy; kindling

1. Introduction

The key roles of purines in neurotransmission and neuromodulation were first recognized by Burnstock in 1972 with the identification of 5'-adenosine-triphosphate (ATP) as a novel neurotransmitter, a finding that led to the concept of purinergic neurotransmission (Burnstock, 1972). Subsequently, the release of the endogenous purine ribonucleoside adenosine, a degradation product of ATP, was shown to regulate hippocampal excitability *in vitro* (Dunwiddie, 1980; Dunwiddie and Hoffer, 1980). A few years later it was demonstrated that adenosine and its analogues modulated amygdala kindling in rats and adenosine was proposed to be the brain's endogenous anticonvulsant (Dragunow and Goddard, 1984; Dragunow et al., 1985; Dragunow, 1986). The crucial role of adenosinergic neuromodulation in the control of

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seizure activity is now well established and has recently been reviewed (Boison, 2005). In addition, adenosine is involved in one of several endogenous mechanisms of the brain that have evolved to terminate seizures (Lado and Moshe, 2008).

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 receptors (Fredholm et al., 2001; Fredholm et al., 2005; Fredholm et al., 2007). Binding of adenosine to the high affinity A_1R , which is prominently expressed at pre- and postsynaptic sites within the hippocampal formation, leads to decreased neuronal transmission and reduced excitability that are largely based on inhibition of presynaptic transmitter release and stabilization of the postsynaptic membrane potential through increased potassium efflux via G protein-coupled inwardly rectifying potassium (GIRK) channels (Sebastiao and Ribeiro, 2000). The A_1R -mediated functions are largely responsible for the anticonvulsant and neuroprotective activity of adenosine. Thus, A_1R knockout mice experience spontaneous hippocampal seizures (Li et al., 2007a) and are hypersensitive to status epilepticus- or trauma-induced brain injury (Fedele et al., 2006; Kochanek et al., 2006). While the A_1R is thought to set a global inhibitory environment within the brain and to provide heterosynaptic depression, the stimulatory $A_{2A}R$ on postsynaptic locations 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 (Cunha, 2008). In contrast to the well characterized role of the A_1R in epilepsy, A_{2A} receptor activation in epilepsy appears to have both proconvulsant as well as anticonvulsant characteristics depending on the context of activation (Boison, 2005; Boison, 2007b). Whereas A_1Rs and $A_{2A}Rs$ are primarily responsible for the central effects of adenosine (Ribeiro et al., 2003), the low affinity and low abundance $A_{2B}Rs$ and A_3Rs are currently not considered as therapeutic targets for epilepsy (Boison, 2005; Boison, 2007b). Functional receptor-receptor interactions of A_1Rs and different types of metabotropic and ionotropic receptors allow a further complexity in adenosinergic neuromodulation (Sichardt and Nieber, 2007).

Synaptic levels of adenosine in adult brain are largely regulated by an astrocyte-based adenosine-cycle (Boison, 2008c), and conversely, adenosine plays important roles for astrocyte physiology (Bjorklund et al., 2008). Synaptic adenosine largely originates from extracellular breakdown of ATP (Dunwiddie et al., 1997; Ziganshin et al., 1994; Zimmermann, 2000), which in turn is derived from vesicular release from astrocytes or neurons (Fields and Burnstock, 2006; Halassa et al., 2007; Haydon and Carmignoto, 2006; Pascual et al., 2005). Alternatively, adenosine as such can directly be released from astrocytes (Frenguelli et al., 2007; Martin et al., 2007). Under physiological conditions, extra- and intracellular levels of adenosine are rapidly equilibrated via distinct families of nucleoside transporters (Baldwin et al., 2004; Gray et al., 2004). Intracellularly, adenosine is rapidly phosphorylated into 5'-adenosine-monophosphate (AMP) via adenosine kinase (ADK; EC 2.7.1.20), an evolutionary conserved member of the ribokinase family of proteins (Park and Gupta, 2008). Due to the high metabolic activity of ADK and the existence of equilibrative transport systems for adenosine, synaptic levels of adenosine are thought to be controlled by intracellular metabolism of adenosine via ADK that assumes the role of a metabolic reuptake system for adenosine; in contrast to classical neurotransmitters, which all have their specific re-uptake transporters, a comparable transporter-controlled re-uptake system for adenosine appears to be lacking (Boison, 2006). It is important to note that in adult brain ADK is almost exclusively expressed in astrocytes (Studer et al., 2006).

2. Adenosine augmentation: therapeutic rationale

Based on the failure of traditional neuron-centered pharmacotherapy in about one third of patients with epilepsy, the exploitation of non-neuronal and non-chemical synaptic signalling

pathways may offer alternatives for epilepsy therapy (Szente, 2008). Several lines of evidence suggest that astrocyte dysfunction and deficiencies in endogenous adenosinergic neuromodulation contribute to seizure generation. In healthy adult brain, physiological adenosine concentrations (25 – 250 nM) are kept in the range of the affinity of the A₁ receptor for adenosine (around 70 nM) (Dunwiddie and Masino, 2001) by a steady state expression of ADK in astrocytes (Boison, 2006). Consequently, small increases in ambient adenosine can augment inhibitory A₁R-mediated functions and adenosine receptor antagonists such as caffeine, have stimulatory effects on brain function (Fredholm et al., 1999). The density of A₁Rs was shown to change as a consequence of seizure activity. In human patients as well as in animal models of epilepsy both upregulation as well as downregulation of A₁Rs have been described; most of these studies were based on quantitative receptor autoradiography with insufficient resolution to discriminate between different cell types. Overall, it appears that acute seizures are associated with upregulation of A₁Rs, whereas chronic seizures are accompanied by downregulation of A₁Rs (Glass et al., 1996). In line with these findings, in the hippocampus of kindled rats deficits of the adenosine system were attributed to a combined decrease in the density of A₁Rs in hippocampal nerve terminal membranes and to metabolic changes that led to lower basal levels of adenosine (Rebola et al., 2003).

More recently, studies from our laboratory have identified the enzyme ADK in astrocytes as a molecular link between astrogliosis – a pathological hallmark of the epileptic brain – and neuronal dysfunction in epilepsy (Li et al., 2007a; Li et al., 2008). These findings led to the “ADK hypothesis of epileptogenesis” (Boison, 2007a; Boison, 2008a; Boison, 2008b), which is based on the following findings:

1. In a mouse model of CA3-selective epileptogenesis we demonstrated spatial and temporal colocalization of astrogliosis, upregulated ADK, and spontaneous electrographic seizures that were all restricted to the CA3. In this model we found a highly localized area of astrocyte/ADK dysfunction (Li et al., 2007a; Li et al., 2008). Indeed, increased levels of brain ADK are associated with a reduced tone in ambient adenosine (Fedele et al., 2005). Most importantly, the CA3-selectivity of this model allows to study adenosine dysfunction in a highly restricted brain area within a largely intact environment with normal adenosinergic neuromodulation that likely prevents spread of seizures from the CA3 to distant brain areas (Li et al., 2007a; Li et al., 2008).
2. Transgenic mice overexpressing ADK in brain express spontaneous recurrent seizures (Li et al., 2007a; Li et al., 2008) in the absence of astrogliosis or any other epilepsy-specific histopathology, indicating that overexpression/upregulation of ADK *per se* is sufficient to trigger seizures.
3. Conversely, engineered mice with reduced ADK expression in forebrain were resistant to both acute seizures as well as the development of spontaneous chronic seizures after an initial precipitating injury was delivered in the presence of the selective A₁R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Li et al., 2008). These findings indicate that both acute seizures, as well as the development of chronic seizures were inhibited by elevated levels of adenosine in hippocampus.

Together, these studies provide a neurochemical rationale for therapeutic intervention. The identification of upregulated ADK resulting in adenosine deficiency 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 (Anscher et al., 2004). Likewise, adenosine A₁R agonists are very effective in the inhibition of neuronal activity and in the suppression of seizures (Fredholm, 2003; Jacobson and Gao, 2006). However, despite activity in a variety of models and efficacy in pharmacoresistant epilepsy (Gouder et al., 2003), A₁R agonists, when given systemically are

not potential antiepileptic agents because of profound peripheral, mainly cardiovascular, effects (Monopoli et al., 1994). Since endogenous adenosine levels increase during times of stress (e.g. lack of oxygen, seizures), agents (e.g. the ADK inhibitor ABT-702) that amplify this site- and event-specific surge of adenosine could provide antiseizure activity similar to that of adenosine receptor agonists (Kowaluk and Jarvis, 2000; McGaraughty et al., 2005). Thus, pharmacological inhibition of ADK has been demonstrated to be an efficient tool for the inhibition of epileptic seizures (Gouder et al., 2004; Kowaluk and Jarvis, 2000) and chronic pain (McGaraughty and Jarvis, 2006); these successes were associated with an improved therapeutic window compared to A₁R agonists (Jarvis et al., 2002). However, systemic application of ADK inhibitors might not be a therapeutic option due to interference with methionine metabolism in liver (Boison et al., 2002b; Mato et al., 2008) and the risk of brain hemorrhage (Erion et al., 2000; McGaraughty and Jarvis, 2006).

Although adenosine, A₁R agonists, and ADK inhibitors are effective in seizure suppression (see above) their systemic application is precluded by peripheral side effects. Therefore, focal adenosine delivery becomes a necessity. Focal treatment approaches for refractory epilepsy have demonstrated that focal drug delivery to the brain is generally well tolerated and devoid of major side effects (Nilsen and Cock, 2004). Focal drug delivery can be achieved by devices such as synthetic slow-release polymers, pump systems, which can be coupled to integrated seizure prediction systems (Stein et al., 2000), or by cellular implants. However, even with the most advanced drug delivery or slow-release mechanisms, in the lifetime of an epilepsy patient, repeated implantation or refill procedures would become a necessity, with the attendant risks of complications. In this context, cell therapy or gene therapy approaches to locally augment the adenosine system (i.e. focal AATs) might eventually provide more long-term solutions.

3. Polymer-based drug delivery

The prolonged focal delivery of adenosine or other small molecule drugs can be achieved by including the drug within a biocompatible polymer. The usefulness of focal brain implants of biocompatible polymers in epilepsy therapy has been evaluated in only a few experimental paradigms. As an example, intracerebral implants of GABA-, but not of noradrenaline-releasing polymer matrices manufactured from ethylene vinyl acetate copolymers (EVAc) were shown to suppress seizures in kindled rats (Kokaia et al., 1994). Likewise, EVAc controlled-release polymers engineered to release phenytoin resulted in a significant reduction in seizures in a rat model of cobalt-induced epilepsy (Tamargo et al., 2002). These studies demonstrated that seizure suppression with focal polymer-based drug delivery is effective in seizure suppression, safe, and that this strategy is highly suited to avoid systemic side effects. In one study a polymer was used to release the drug for calculated 3.5 years (Tamargo et al., 2002). However, it remains to be demonstrated whether the focal release of GABA, or of conventional antiepileptic drugs would be effective in pharmacoresistant epilepsy. New developments explore a spray-drying technique in the bioengineering of phenytoin (PHT) containing poly(epsilon-caprolactone) (PCL) microcarriers (Li et al., 2007c) and the use of silk-fibroin based carriers for small drug delivery (Hofmann et al., 2006; Wang et al., 2006; Wong et al., 2006).

3.1. Ethylene vinyl acetate copolymers

EVAc polymers have widely been used as an intracerebral delivery vehicle for a variety of neurological conditions (During et al., 1989; Freese et al., 1989; Hoffman et al., 1990; Saltzman et al., 1999; Sanberg et al., 1993). The first polymer-based AAT approach was performed in 1999 using EVAc polymers engineered to release adenosine (Boison et al., 1999). A 10% (w/v) solution of purified EVAc in methylene chloride was used to which 20% adenosine (w/w) was added. This suspension was frozen and the solvent was lyophilized out of the mixture. The remaining mixture was pressure extruded at 50°C into cylindrical adenosine-EVAc rods. Single

polymers (1×0.4 mm) released an amount of around 20 to 50 ng adenosine per day into Ringer solution. Individual polymers were implanted into the lateral brain ventricles of rats that had been kindled in the hippocampus. In these rats stimulus-induced epileptic seizures were drastically reduced after polymer implantation. This was demonstrated by a strong reduction of stage 5 seizures for at least 7 days and by suppression or reduction of epileptiform electrical afterdischarges up to 3 days as recorded bilaterally in the hippocampus. With the diminishing supply of adenosine, the antiseizure effects gradually decreased and had expired after 14 days. Control implants that were loaded with BSA failed to display any antiepileptic activity or changes in stimulus-evoked EEGs. This study was the first published demonstration that focal release of adenosine within the brain can suppress epileptic seizures. It was concluded that the local release of 20 to 50 ng adenosine per day is sufficient to exert a significant antiepileptic effect.

3.2. Silk-based polymers

As an alternative to synthetic drug delivery-systems, such as those described above, a novel generation of silk-based drug-delivery systems has recently been developed and offers several advantages: Silk fibroin is biocompatible (Altman et al., 2003) and biodegrades slowly (Horan et al., 2005). As an important property for sustained release applications, the degradation lifetimes of silk can be regulated by the extent of physical crosslinking for beta sheet formation, thus allowing control of degradation timeframes from weeks to years (Horan et al., 2005). Silk also can be processed under aqueous conditions, thus allowing for the incorporation of labile biomolecules without loss of biological activity (Jin and Kaplan, 2003; Li et al., 2006). In addition, the frequent use of silk sutures in brain and nerve tissue confirms the feasibility of implantation of silk biomaterials in the brain.

In a recent study, silk-based polymers were engineered to release target release rates of 0, 40, 200, and 1000 ng adenosine per day (Wilz et al., 2008). These polymers were implanted into the infrahippocampal fissure of rats prior to the onset of kindling. Using this approach it was demonstrated that focal adenosine release from silk-based polymers is effective in retarding kindling epileptogenesis in a dose-dependent manner. Combining polymers designed to release the specified target doses for a limited 14 day period with a kindling-epileptogenesis paradigm extended to 20 days after polymer implantation (i.e. a total of 48 kindling stimulations distributed over 20 days) it was possible to document kindling progression after expiration of the polymers. In this study there was a clear dose-dependency in the average number of stimulations needed to elicit the first stage 1 or 2 seizure. These findings suggested a linear dose-response relation. Remarkably, recipients of polymers releasing a target dose of 1000 ng adenosine per day did not display any behavioral seizure during the first 22 stimulations (i.e. stage 0) despite the presence of afterdischarges in EEG recordings, whereas control rats had reached stage 3 seizures at that time. When adenosine-release from the polymers began to wear off, seizures gradually developed with progressive intensity. This delayed seizure-development curve was parallel to the curve of control rats, but shifted to the right by 7 days. These findings suggested a potential antiepileptogenic effect of intracerebral adenosine release (Wilz et al., 2008).

4. Encapsulated cell systems

In contrast to polymer-based drug delivery, encapsulated cell biodelivery (ECB), first developed by Aebischer and colleagues in 1991 (Aebischer et al., 1991; Winn et al., 1991) and reviewed recently (Hauser et al., 2004; Tseng and Aebischer, 2000), constitutes an *ex vivo* gene therapy approach. In ECB approaches a cell line is first genetically modified to produce and release a therapeutic molecule of interest (e.g. adenosine). In a second step the cells are then enclosed within an ECB device, which is made up of a semi-permeable membrane and may contain additional matrix material. The semipermeable membrane is designed to permit

exchange of extracellular metabolites and nutrients between cells and host tissue, to permit delivery of cell-based therapeutics to the surrounding host tissue, but to prevent direct graft-cell/host-cell interactions, and to prevent interactions with the host immune system. ECB approaches offer the following advantages: (i) physical isolation of cells to prevent immune response and graft rejection; (ii) avoidance of irreversible genetic modification of host cells; (iii) termination of treatment by withdrawal of device; and (iv) avoidance of connections between grafted cells and hippocampal circuitry; this might be of importance within the context of epilepsy, since direct interactions between neuronal grafts and host circuitry can either suppress or induce seizures depending on the context (Buzsaki et al., 1988; Buzsaki et al., 1991). These advantages constitute important safety features of this therapeutic strategy. In the CNS ECB approaches have extensively been explored for the treatment of Parkinson's disease, amyotrophic lateral sclerosis, and chronic pain, and some clinical trials have been reported (Aebischer et al., 1994; Aebischer et al., 1996). However, based on safety concerns associated with xenotransplantation used in all of these initial approaches, new ECB devices have been developed recently that make use of human fibroblasts embedded in a polyethersulfone (PES) matrix; using this second generation ECB approach and human fibroblasts engineered to release glial cell line derived neurotrophic factor (GDNF) Kokaia and colleagues recently demonstrated seizure reduction in the epileptic hippocampus of rats (Kanter-Schlifke et al., in press).

4.1. Encapsulated fibroblasts

To utilize ECB technology for the paracrine cell-based release of adenosine, baby hamster kidney (BHK) fibroblasts were subjected to chemical mutagenesis followed by selection for ADK deficiency with 9- β -D-arabinofuranoside (araA, vidarabin) (Huber et al., 2001). As a result of ADK deficiency in BHK-AK2 cells, 10^5 of these engineered cells released around 20 ng adenosine into fresh culture medium during the first hour of incubation. Adenosine-releasing ECB devices were prepared by encapsulating 2×10^5 BHK-AK2 cells into PES hollow fibers (7 mm long; 0.5 mm inner diameter). Control ECB devices contained unmodified BHK cells. Adenosine-releasing and control-devices were unilaterally implanted into the lateral brain ventricle of rats that had been kindled to reproducibly react with stage 5 seizures after stimulation. During the first 12 days after transplantation the adenosine releasing devices provided nearly complete suppression from behavioral seizures (seizure scores < 1) as well as reduction of afterdischarges recorded in hippocampal EEGs; thereafter, in line with limited life expectancy of these encapsulated cells, seizure activity gradually recurred, indicating that seizure suppression was due to adenosine-release (Huber et al., 2001). In contrast, recipients of wild-type cells continued to display their pre-implantation stage 5 seizure pattern. These experiments demonstrated a robust adenosine-dependent treatment effect that was independent of stimulation frequency (Boison et al., 2002a) and could be reversed by A₁R blockade (Huber et al., 2001). In contrast to ADK-deficient BHK cells, fibroblasts lacking adenosine deaminase released reduced amounts of adenosine and were less efficient in seizure suppression (Huber et al., 2001).

These ECB-based AAT approaches demonstrated for the first time that seizure control using cell-based brain implants engineered to release adenosine is feasible. These experiments also demonstrate that disruption of ADK in the to-be-grafted cells is an efficient strategy to induce cellular adenosine release. The efficacy of this ECB approach demonstrates that the paracrine release of adenosine from the implant is sufficient to achieve full seizure control and that graft-host, or network interactions between pre-existing neuronal networks and the implanted cells are not necessary for therapeutic effectiveness of adenosine. One caveat of this approach, however, was the limited life-expectancy of the implanted cells resulting in diminished long-term efficacy. Since these experiments were done without immunosuppression, and ECB devices do not constitute an absolute barrier to immune reactions (Rinsch et al., 2001), BHK

cells with an inherently high metabolic activity and increased antigen shedding might be prone to immunological interactions, even when encapsulated and located within the immuno-privileged location of brain.

4.2. Encapsulated myoblasts

To develop an ECB-based system for extended adenosine-based seizure suppression mouse C₂C₁₂ myoblasts were engineered to release adenosine in an approach analogous to the production of adenosine releasing BHK cells. As an alternative source of cells with innate long-term viability mouse C₂C₁₂ myoblasts have the advantages of dividing cells when grown in the presence of serum (McMahon et al., 1994). However, they can be differentiated into postmitotic myotubes upon exposure to low-serum-containing medium. Encapsulated differentiated C₂C₁₂ cells can be maintained as myotubes with long-term survival and are able to provide sustained delivery of therapeutic compounds for up to several months (Déglon et al., 1996a; Déglon et al., 1996b). Encapsulated C₂C₁₂ cells have been used in a variety of approaches for the delivery of ciliary neurotrophic factor (CNTF), GDNF, and erythropoietin (Dalle et al., 1999; Déglon et al., 1996b; Kishima et al., 2004; Regulier et al., 1998).

In an approach similar to the one described above for encapsulated BHK cells, encapsulated ADK-deficient C₂C₁₂ cells were transplanted into the lateral brain ventricles of fully kindled rats (Güttinger et al., 2005b). These brain implants displayed enhanced viability compared to BHK cells and provided seizure suppression in most animals for at least two weeks ranging to at least eight weeks in one animal. Pharmacological control experiments with adenosine A₁R antagonists and agonists revealed no signs of receptor desensitization after long-term exposure to adenosine. In addition, encapsulated ADK-deficient C₂C₁₂ cells were capable of seizure suppression without any overt side effects. Specifically, no effects on locomotor activity were found as tested in an open-field paradigm. In contrast, the systemic administration of the A₁ receptor agonist CCPA led to sedation and reduction of locomotor activity. These studies therefore provide evidence for the feasibility of side-effect free long-term seizure suppression by local cell-mediated release of adenosine.

In summary, AATs based on ECB-technology demonstrated that paracrine cell-based adenosine delivery to brain effectively suppresses fully kindled seizures; most importantly this therapeutic success was not limited by receptor desensitization or side effects. However, limited long-term viability of encapsulated cells and the use of xenografts are not compatible with future clinical application. Therefore, improved ECB devices and the use of human cells are mandatory for future therapy development (Lindvall and Wahlberg, 2008).

5. Stem cells

Stem cells, stem cell-derived neural precursors, neuronal cell lines, and fetal hippocampal neurons have recently received much attention as direct transplantation tools for epilepsy therapy (Boison, 2007c; Loscher et al., 2008; Raedt et al., 2007; Shetty and Hattiangady, 2007; Suter and Krause, 2008). In general, two different therapeutic strategies are possible that may synergistically augment each other (Fig. 1): The first strategy is based on reconstitution of hippocampal circuitry by intrahippocampal transplantation of fetal hippocampal neurons. In an elegant set of experiments Shetty and coworkers have demonstrated that this approach is indeed feasible and can provide therapeutic benefit in post status epilepticus models of epilepsy. Most notably, fetal hippocampal cell grafts exhibited robust long-term survival (> six months) and integration in animal models of epilepsy (Rao et al., 2006). While functional integration of graft-derived cells is needed for these restorative approaches, stem cell- or neural progenitor-derived brain-implants can be engineered to release therapeutically active molecules with the aim to provide therapeutic benefit by paracrine mechanisms. Obviously, a

combination of functional integration and paracrine drug delivery may provide synergistic benefits.

5.1. Mouse embryonic stem cells

Mouse embryonic stem cells ESCs were engineered in our laboratory to release therapeutic amounts of adenosine based on bi-allelic disruption of the *Adk*-gene using a two-step gene targeting approach (Fedele et al., 2004). We used heterozygous *Adk*-knockout cells, obtained after the first targeting step, to generate ADK knockout mice (Boison et al., 2002b). A second gene targeting step, followed by chemical selection for ADK deficiency, was used to generate ESCs with a homozygous deficiency of ADK (Fedele et al., 2004). These cells provided transient seizure suppression, when encapsulated and transplanted into the lateral brain ventricles of fully kindled rats (Güttinger et al., 2005a).

Subsequent experiments used a different approach to transplant adenosine releasing *Adk*^{-/-}-derived neural precursor cells (NPs) directly into the epileptic hippocampus. In these approaches, *Adk*^{-/-} ESCs were subjected to an established neural differentiation protocol (Okabe et al., 1996) to obtain adenosine releasing NPs. In the first approach these cells were injected into the infrahippocampal fissure of rats prior to the onset of kindling. 26 days after grafting, dense clusters of graft derived cells were located within the infrahippocampal fissure, presumably forming a cellular reservoir for paracrine adenosine release. In addition, individual graft-derived cells were found within the ipsilateral CA1, assumed a neuronal morphology, and expressed the neuronal marker NeuN (Li et al., 2007b). In contrast to sham controls or recipients of wild-type cells, recipients of adenosine releasing cells were characterized by sustained protection from generalized stage 4 or 5 seizures over 48 kindling stimulations delivered over 22 days following cell implantation. Remarkably, recipients of adenosine releasing NPs were almost completely protected from any seizure activity during the first 18 kindling stimuli (or 12 days after cell implantation). These studies demonstrated a robust suppression of kindling epileptogenesis by adenosine-releasing ESC-derived infrahippocampal brain implants.

In the second treatment approach, the same type of cells was implanted into a mouse model of CA3-selective epileptogenesis that is characterized by astrogliosis, upregulated ADK, and spontaneous recurrent seizures, all restricted to CA3 (Li et al., 2008). This type of epilepsy develops within 3 weeks after intraamygdaloid injection of the excitotoxin kainic acid (KA), which constitutes an initial epileptogenesis precipitating injury (IPI) including status epilepticus and CA3-selective neuronal cell death. 24 hours after the IPI animals received infrahippocampal implants of *Adk*^{-/-} ESC-derived NPs, corresponding wild-type cells, or a sham treatment. While all control animals developed chronic recurrent CA3-seizures 3 weeks after the IPI (~4 seizures per hour), none of the recipients of adenosine releasing cells developed any seizures (Li et al., 2008). In addition to complete lack of seizures, *Adk*^{-/-} graft recipients were characterized by a significant reduction in astrogliosis and by normal levels of ADK (Li et al., 2008).

5.2. Human mesenchymal stem cells

Adult stem cells have been isolated from several easily accessible tissue sources (Korbling et al., 2003), including bone marrow (Barry and Murphy, 2004; Kassem, 2004; Mezey et al., 2003), skeletal muscle (Gussoni et al., 1999; Jackson et al., 1999), skin (Fernandes et al., 2004; Fernandes et al., 2006; Toma et al., 2001), and lipoaspirate (Fatar et al., 2008). All of these stem cells preferentially generate differentiated cells of the same lineage as the tissue of origin. However, transplant studies indicate that adult stem cells can also generate cells of a different embryonic lineage *in vivo*. Protocols have been established to differentiate bone marrow derived mesenchymal stem cells into neurons (Black and Woodbury, 2001; Munoz-

Elias et al., 2004; Woodbury et al., 2000). These findings have therapeutic implications, since neural stem cells can promote functional recovery upon transplantation into the injured nervous system (Chu et al., 2003; Chu et al., 2004; Jeong et al., 2003; Pluchino et al., 2003).

In an effort to generate human stem cells for therapeutic adenosine delivery we developed a lentiviral expression system for inhibitory micro-RNA (miRNA) directed against ADK. This vector was highly effective in downregulating ADK (i.e. ADK-knockdown) in human mesenchymal stem cells (hMSCs) [up to 80% ADK-knockdown (Ren et al., 2007)]. The therapeutic efficacy of these hMSCs was tested in two different experimental paradigms.

In the first study (Ren et al., 2007) hMSCs with a knockdown of ADK were grafted into the infrahippocampal fissure of mice one week prior to the intraamygdaloid injection of KA. While control animals were characterized by KA-induced status epilepticus and subsequent neuronal cell loss, animals with therapeutic ADK knockdown-implants displayed a 35% reduction in seizure duration and 65% reduction in neuronal cell loss, when analyzed 24h after KA. This study demonstrates that hMSC-derived brain implants with a lentiviral knockdown of ADK provide potent anticonvulsant and neuroprotective effects in an acute seizure and cell-death model.

In the second study (Li et al., 2009) hMSCs with a knockdown of ADK were grafted into the infrahippocampal fissure of mice 24 hours after KA-induced acute CA3-injury. Seizure recordings performed three weeks after KA-injection revealed typical spontaneous seizures in the CA3 of all sham-treated animals at a frequency of 4.2 ± 1.2 seizures per hour and an average duration of 17.2 ± 5.1 seconds, data in agreement with the seizure characteristics of this model (Li et al., 2008). In contrast, seizure intensity was significantly reduced in recipients of hMSCs with a knockdown of ADK (2.7 ± 1.1 seizures per hour at 9.4 ± 4.1 seconds; $P < 0.001$). Seizure protection could be reversed after the injection of the selective A_1R antagonist DPCPX (4.3 ± 1.5 seizures per hour at 22.2 ± 13.0 seconds, $P > 0.05$), indicating that the reduction of seizure-intensity by ADK-knockdown cells was due to paracrine augmentation of adenosine.

Together these results suggest that hMSCs can survive in the infrahippocampal fissure for at least three weeks and exert therapeutic effects in acute seizure paradigms as well as in the chronic state of epilepsy, by paracrine release of adenosine. Although these experiments involved cross-species transplantation (human to mouse) and were done under immunosuppression, these data suggest that hMSCs derived from a patient could be used in autologous clinical transplantation approaches.

6. Conclusions and outlook

Based on the demonstrated anticonvulsant efficacy of intrafocal polymer and/or cell mediated adenosine release and the fact that adenosine is able to suppress pharmacoresistant seizures, the foundation is laid to move this novel therapeutic approach forwards towards clinical applications. The clinical target is pharmacoresistant temporal lobe epilepsy (TLE). Prior to clinical application of adenosine-releasing devices the following requirements must be considered: (a) Refined dose- seizure-response curves have to be determined; (b) A dose-dependent side effect profile has to be established; (c) A system for the sustained and controlled long-term delivery of adenosine has to be developed with a target of at least 6 months; (d) A proof of principle has to be established in patients with pharmacoresistant epilepsy, that intrafocal delivery of adenosine is safe and capable to suppress pharmacoresistant seizures. These four requirements can be addressed by combining polymer-based adenosine delivery with cell-mediated adenosine-release. Silk-based-polymers engineered to release adenosine combine the two FDA-approved natural compounds silk and adenosine. These can be engineered for the controlled release of different doses of adenosine. In kindled rats, an animal

model of TLE, the effective dose range for seizure suppression has already been established (Wilz et al., 2008) although a dose dependent side effect profile still needs to be worked out. Based on such findings silk-based polymers engineered to release adenosine might directly be used in short term clinical trials to assess the safety and efficacy of intrafocal adenosine release. The use of adenosine releasing polymers is likely the most straightforward approach to study short-term intrafocal adenosine release in clinical trials. To provide a more long-term solution for therapeutic approaches, it will become necessary to engineer a polymer/cell based system for the sustained long-term delivery of adenosine. To achieve this goal, silk scaffolds might be coated with human mesenchymal stem cells (hMSCs). These stem cells can be engineered to release adenosine by using a lentiviral expression system for anti-ADK miRNA (Ren et al., 2007). The use of hMSCs has the advantage that hMSCs can easily be obtained from a patient, thus providing the opportunity to use autologous patient-identical cells for therapy to avoid immune reactions. The therapeutic efficacy of such future brain implants needs to be tested in long-term studies in animal models of epilepsy. Thus, the combination of polymer-based adenosine release with cell-based delivery approaches for adenosine may constitute the foundation to develop intracerebral adenosine releasing implants for clinical applications. Alternatively – based on the therapeutic rationale that upregulation of ADK during epileptogenesis contributes to seizure generation – more direct *in vivo* gene therapies might be considered by designing viral vectors that downregulate (the pathologically upregulated) ADK within an epileptic focus.

In summary, polymer-, cell-, or gene-based AATs for refractory epilepsy hold substantial therapeutic promise, however hurdles (Fig. 1) such as proof of long-term therapeutic efficacy, proof of efficacy in clinically relevant models of epilepsy, demonstration of long-term safety, and demonstration of efficacy in resected human epileptic hippocampi, need to be overcome before clinical trials can be planned. Thus, the development of focal AATs for pharmacoresistant epilepsies will continue to progress further into an area of intense scientific and therapeutic interest.

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Figure 1.
Goals and challenges of cell therapies for epilepsy.