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REVIEW ARTICLE

Molecular and cellular mechanisms of pharmacoresistance in epilepsy

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Epilepsy is a common and devastating neurological disorder. In many patients with epilepsy, seizures are well-controlled with currently available anti-epileptic drugs (AEDs), but a substantial (~30%) proportion of patients continue to have seizures despite carefully optimized drug treatment. Two concepts have been put forward to explain the development of pharmacoresistance. The transporter hypothesis contends that the expression or function of multidrug transporters in the brain is augmented, leading to impaired access of AEDs to CNS targets. The target hypothesis holds that epilepsy-related changes in the properties of the drug targets themselves may result in reduced drug sensitivity. Recent studies have started to dissect the molecular underpinnings of both transporter- and target-mediated mechanisms of pharmacoresistance in human and experimental epilepsy. An emerging understanding of these underlying molecular and cellular mechanisms is likely to provide important impetus for the development of new pharmacological treatment strategies.

Keywords: epilepsy; pharmacoresistance; anti-epileptic drugs; multidrug transporter; ion channel

Abbreviations: AED = anti-epileptic drug; ABC = adenosine triphosphate-binding cassette; PGP = P-glycoprotein

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Introduction to the clinical problem of pharmacoresistance in epilepsy

Epilepsy is a common and devastating neurological disorder. In many patients with epilepsy, seizures are well-controlled with currently available anti-epileptic drugs (AEDs). However, seizures persist in a considerable proportion of these patients. The exact fraction of epilepsy patients who are considered refractory varies in the literature, mostly because the criteria for classification as pharmacoresistant have varied. Nevertheless, a substantial proportion (~30%) of epilepsy patients do not respond to any of two to three first-line AEDs, despite administration in an optimally monitored regimen (Regesta and Tanganelli, 1999). The fraction of patients who are pharmacoresistant appears to correlate with certain features of the epileptic condition, such as a high seizure frequency or febrile seizures prior to treatment, early onset of seizures or the presence of certain types of structural brain lesions. In addition, pharmacoresistance occurs frequently in patients with partial seizures (Aicardi and Shorvon, 1997; Regesta and Tanganelli, 1999 for a more complete discussion of clinical aspects of pharmacoresistance). Despite the obvious clinical relevance of uncontrolled seizures in a large fraction of epilepsy patients, the cellular basis of pharmacoresistance has so far remained elusive. The availability of tissue from epilepsy patients undergoing surgery for focal epilepsies, primarily temporal lobe epilepsy, has allowed to address some of the mechanisms underlying pharmacoresistance of focal epilepsies. The mechanisms underlying the development of resistance in certain forms of generalized epilepsies are still enigmatic.

Introduction to the cellular candidate mechanisms of pharmacoresistance

Which key mechanisms govern efficacy of CNS drugs? Firstly, in the presence of adequate, carefully monitored serum AED levels, drugs have to traverse the blood–brain barrier (BBB). Subsequently, CNS activity of AEDs is determined by a

multitude of factors, including physical properties, such as lipophilicity, that affect their distribution in different compartments within the CNS. Consequently, one scenario to explain pharmacoresistance could be that sufficient intraparenchymal AED concentrations are not attained, even in the presence of adequate AED serum levels. Such a phenomenon could arise via an enhanced function of multidrug transporters that control intraparenchymal AED concentrations (transporter hypothesis of pharmacoresistance, Kwan and Brodie, 2005).

Following permeation into the CNS parenchyma, drugs have to bind to one or more target molecules to exert their desired action. Thus, pharmacoresistance may also be caused by a modification of one or more drug target molecules (*see* Table 1). These modifications would then cause a reduced efficacy of a given AED at the target. This concept has been collectively termed the target hypothesis of pharmacoresistance (Fig. 1).

Modification in drug targets as basis for pharmacoresistance

The cellular mechanisms of AEDs have been examined to some extent in normal brain tissue, or ion channels and receptors in expression systems. These data are summarized qualitatively in Table 2. Many of these drug targets are altered on a molecular level in epilepsy. In the following sections, we will attempt to summarize briefly the known mechanisms of AEDs on ion channels. We will then focus on emerging experimental evidence supporting a loss of AED efficacy at selected targets, and discuss the possible molecular basis of these findings.

Changes in molecular drug targets for AEDs

Voltage-gated Na^+ channels

Voltage-gated Na^+ currents are ubiquitously expressed in excitable cells (Fig. 2A, Goldin, 1999; Goldin *et al.*, 2002), and appear to be targets for multiple first-line AEDs. Upon depolarization of the membrane, the channels activate and give rise to a fast 'transient' inward Na^+ current (I_{NaT}, Fig. 2B), responsible for the rising phase of the action potential, and—in some cells—a slowly-inactivating 'persistent' current (I_{NaP}, *see* Fig. 2C). Both current components represent major targets of several first-line AEDs including carbamazepine, phenytoin (PHT), lamotrigine and valproate (Ragsdale and Avoli, 1998; Catterall, 1999; Köhling, 2002, see also Table 2).

Most AEDs block Na^+ channels in their resting state (tonic block) at hyperpolarized membrane potentials (Ragsdale and Avoli, 1998), with a voltage-dependent enhancement of the block towards more depolarizing potentials. This voltage-dependent inhibition is associated with a shift of the steady-state inactivation curve in a hyperpolarized direction (Fig. 2D). Importantly, blocking effects are activity- or use-dependent, i.e. blocking effects are enhanced when neurons are repetitively depolarized at higher frequencies (Fig. 2E and F). This activity-dependence is expressed as a slowing of recovery from fast Na^+ channel inactivation (Ragsdale and Avoli, 1998; Catterall, 1999). It has been suggested that use-dependent blocking effects are important because they result in a preferential block of I_{NaT} during prolonged high-frequency neuronal activity, such as that occurring during seizures.

Several lines of evidence so far have indicated that reduced efficacy in inhibiting I_{NaT} may be a candidate mechanism of

Table 1 Changes in known AED targets or drug efflux transporters in experimental epilepsy models and human epileptic tissue

Target	Modification	Cell type	Human data (yes/no)
Voltage-gated sodium channels	Downregulation of accessory subunits	Dentate granule cells CA1 pyramidal neurons	Yes
	Altered alpha subunit expression, induction of neonatal isoforms	CA1 pyramidal neurons CA3 pyramidal neurons Dentate granule cells	Yes
Voltage-gated calcium channels	Increased expression of T-type channels	CA1 pyramidal neurons	No
Hyperpolarization-activated current (I_H)	Loss of dendritic I_H	Entorhinal cortex layer 3 neurons	No
GABA receptors	$GABA_A$ receptors: decrease of α_1 subunits increase of α_4 subunits	Dentate granule cells	Yes
P-Glycoprotein (MDR1)	Overexpression	Astrocytes Capillary endothelial cells Neurons	Yes
MRP1	Overexpression	Astrocytes Neurons	Yes
MRP2	Overexpression	Astrocytes Capillary endothelial cells	Yes
MVP (major vault protein)	Overexpression	Microglial cells	No

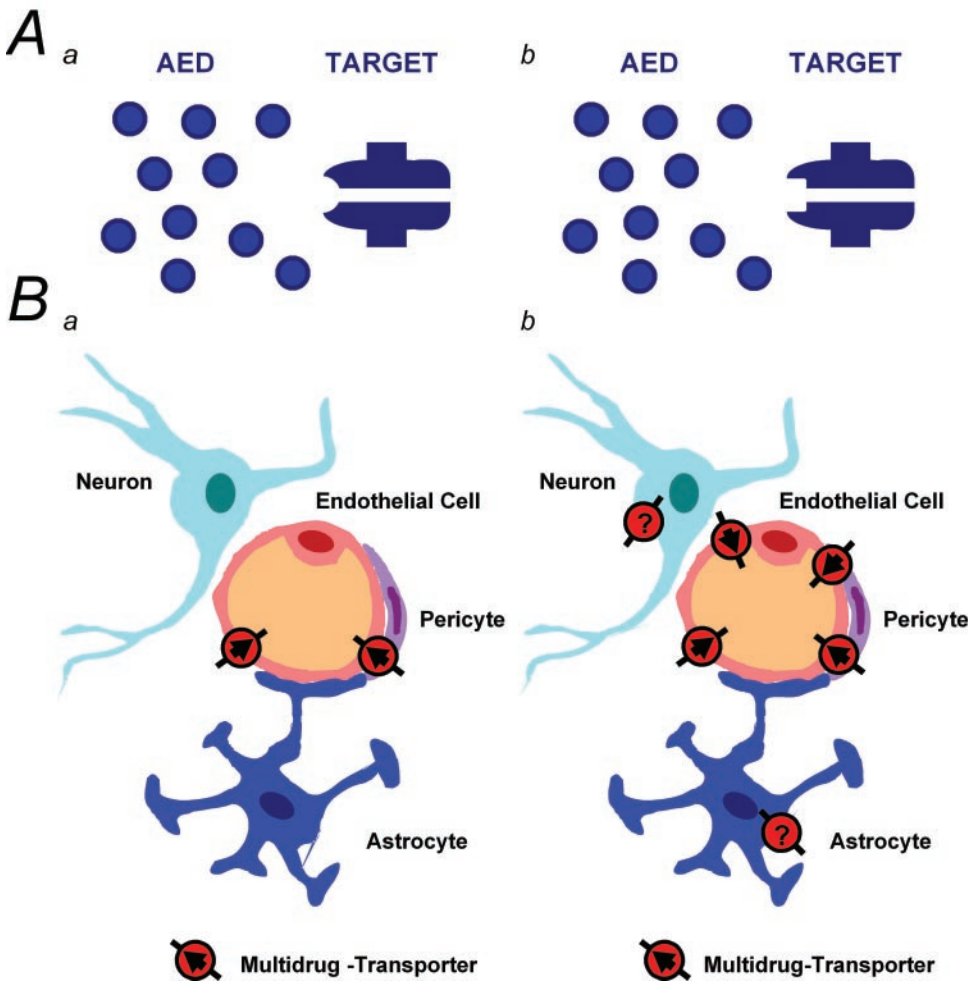

Fig. 1 In the pharmacoresistant patient the drug faces a modified target, with which it interacts less effectively (**A**, panel b). Putative candidate mechanisms resulting in target modifications are seizure-induced changes in transcription or alternative splicing of ion channel subunits, altered post-translational modification of the protein and/or phosphorylation by protein kinases. A second emerging concept to explain pharmacoresistance contends that increased expression or function of multidrug transporter proteins decreases availability of the AED at its target (**B**, panel b versus **B**, panel a). In the non-epileptic brain, drug transporter molecules are predominantly expressed in endothelial cells, and in some cases astrocytes (see text), and appear to regulate intraparenchymal AED concentrations by extruding certain AEDs over the blood–brain or blood–CSF barrier (indicated by arrows in **B**, panel a). An upregulation of various drug transporter molecules has been described in human epilepsy as well as in experimental epilepsy models (**B**, panel b). This upregulation may decrease the effective concentration of AEDs at their targets. In addition, in the setting of an epileptic brain, an ectopic expression of certain drug transporter genes has been observed in astrocytes and in neurons. It remains unresolved whether such transporters control access of AEDs to intracellular targets (indicated by the absence of an arrow in the neuron in **B**, panel b).

pharmacoresistance to some AEDs. Firstly, in CA1 neurons, the effects of carbamazepine on the steady-state inactivation properties of I_{NaT} were transiently reduced in the kindling model of epilepsy (Vreugdenhil and Wadman, 1999*b*). In contrast to these comparatively modest and transient effects, a complete and long-lasting loss of use-dependent blocking effects of carbamazepine was found in the pilocarpine model of epilepsy in hippocampal dentate granule cells, as well as in epilepsy patients with carbamazepine-resistant temporal lobe epilepsy (Remy *et al.*, 2003*a*). This dramatic loss of a major mechanism of action of carbamazepine did not extend to other AEDs known to affect I_{NaT}. Following pilocarpine-induced status epilepticus, the use-dependent effects of PHT were reduced, but not completely lost, while the effects of lamotrigine were completely unchanged (Remy *et al.*, 2003*b*). Although the mechanisms of I_{NaT} inhibition induced by valproate are still controversial (Xie *et al.*, 2001); but see (Vreugdenhil *et al.*, 1998; Vreugdenhil and Wadman, 1999*b*), this substance exhibits potent voltage-dependent blocking effects in various preparations (Fohlmeister *et al.*, 1984; Zona and Avoli, 1990; Vreugdenhil and Wadman, 1999*b*; Köhling, 2002). Notably, in tissue obtained from pharmacoresistant patients and in experimental epilepsy no differences regarding valproic acid effects on I_{NaT} could be observed (Vreugdenhil *et al.*, 1998; Vreugdenhil and Wadman, 1999*b*; Remy *et al.*, 2003*b*). Collectively, these results suggest that epileptogenesis causes changes in the properties of I_{NaT} that may differ depending on the cell type examined

Table 2 Summary of AED targets

	Voltage-dependent ion channels						Neurotransmitter systems	
	I_{NaT}	I_{NaP}	I_{Ca}	I_K	I_K (I_M)	I_H	GABA	Glu
Phenytoin	(Willow *et al.*, 1985; Schwarz and Grigat, 1989; Ragsdale *et al.*, 1991; Kuo, 1998; Remy *et al.*, 2003*b*)	(Chao and Alzheimer, 1995; Segal and Douglas, 1997; Lampl *et al.*, 1998)	High-threshold: (Stefani *et al.*, 1997*a*, *c*; Schumacher *et al.*, 1998) Low-threshold (Todorovic *et al.*, 2000; Gomora *et al.*, 2001)	(Nobile and Lagostena, 1998)				
Carbamazepine	(Willow *et al.*, 1985; Schwarz and Grigat, 1989; Kuo, 1998; Reckziegel *et al.*, 1999; Vreugdenhil and Wadman, 1999*a*; Remy *et al.*, 2003*a*)		High-threshold (Schumacher *et al.*, 1998)					
Oxcarbazepine	(McLean *et al.*, 1994; Schmutz *et al.*, 1994)		High-threshold (Schmutz *et al.*, 1994; Stefani *et al.*, 1995; 1997*b*)					
Lamotrigine	(Xie *et al.*, 2001; Xie *et al.*, 1995; Zona and Avoli, 1997; Remy *et al.*, 2003*b*; Kuo, 1998)	(Spadoni *et al.*, 2002)	High-threshold (Stefani *et al.*, 1997*c*; Stefani *et al.*, 1997*a*; Stefani *et al.*, 1997*b*; Wang *et al.*, 1996)	(Huang *et al.*, 2004; Zona *et al.*, 2002)		(Poolos *et al.*, 2002)		
Valproic acid	(McLean and Macdonald, 1986; Zona and Avoli, 1990; Vreugdenhil *et al.*, 1998; Vreugdenhil and Wadman, 1999*a*; Remy *et al.*, 2003*b*)	(Taverna *et al.*, 1998), but see (Niespodziany *et al.*, 2004)	High-threshold (Taverna *et al.*, 1998) Low-threshold (Todorovic and Lingle, 1998)				(Löscher, 1989)	
Losigamone		(Gebhardt *et al.*, 2001)						
Retigabine					(Main *et al.*, 2000), (Tatulian *et al.*, 2001; Yue and Yaari, 2004)			
Zonisamide	(Schauf, 1987)		Low-threshold (Suzuki *et al.*, 1992)					

Felbamate	(Taglialatela *et al.*, 1996)		High-threshold I_{ca} (Stefani *et al.*, 1997*b*)			(Rho *et al.*, 1997)	(Subramaniam *et al.*, 1995; White *et al.*, 1995; Kuo *et al.*, 2004)
Topiramate	(Zona *et al.*, 1997; Taverna *et al.*, 1999; McLean *et al.*, 2000)	(Taverna *et al.*, 1999)	High-threshold I_{ca} (Zhang *et al.*, 2000)	(Herrero *et al.*, 2002)		(White *et al.*, 1997; Gordey *et al.*, 2000)	(Gibbs, III *et al.*, 2000; Gryder and Rogawski, 2003)
Ethosuximide	No effect: (McLean and Macdonald, 1986)	(Leresche *et al.*, 1998; Niespodziany *et al.*, 2004)	Low-threshold (Coulter *et al.*, 1989; Todorovic and Lingle, 1998; Gomora *et al.*, 2001), but see (Leresche *et al.*, 1998)	(Leresche *et al.*, 1998)			
Levetiracetam	No effect: (Zona *et al.*, 2001)	No effect: (Niespodziany *et al.*, 2004)	High-threshold (Lukyanetz *et al.*, 2002) Low-threshold, no effect: (Zona *et al.*, 2001)	(Madeja *et al.*, 2003)			
Gabapentin			High-threshold (Alden and Garcia, 2001), but see (Schumacher *et al.*, 1998)	(Freiman *et al.*, 2001)	(Surges *et al.*, 2003)		
Pregabalin			High-threshold (Ben Menachem, 2004), (McClelland *et al.*, 2004)				
Phenobarbital			High-threshold (Ffrench-Mullen *et al.*, 1993)			(Twyman *et al.*, 1989*b*)	
Benzodiazepines						(Study and Barker, 1981; Rogers *et al.*, 1994; Eghbali *et al.*, 1997; Rudolph *et al.*, 1999)	
Vigabatrin						(Jolkkonen *et al.*, 1992; Löscher and Horstermann, 1994; Wu *et al.*, 2003)	
Tiagabine						(Fink-Jensen *et al.*, 1992; Thompson and Gähwiler, 1992; Dalby, 2000)	

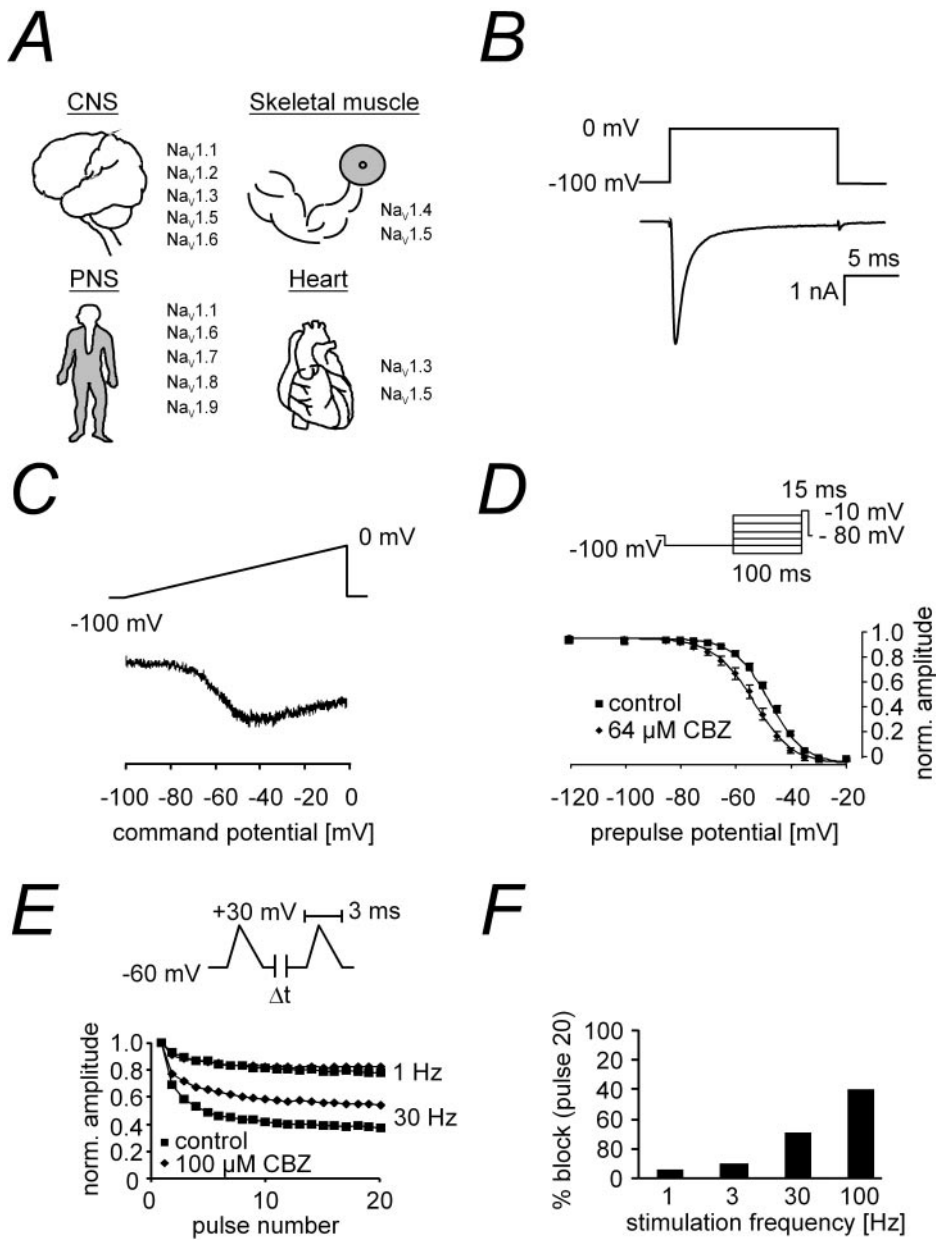

Fig. 2 Expression, functional properties and AED pharmacology of voltage-gated sodium channels. The Na^+ channel alpha subunits $Na_V1.1$–$Na_V1.9$ are widely distributed in excitable cells and exhibit a tissue-specific expression (**A**). Upon depolarization the channels activate and give rise to a rapidly inactivating 'transient' inward Na^+ current (I_{NaT}, **B**), responsible for the rising phase of the action potential. In addition, in some cells a slowly-inactivating 'persistent' current has been described (I_{NaP}, **C**). Slow voltage-ramp commands allow to describe the voltage-dependent properties of this current component in isolation. The persistent Na^+ current contributes to spike after-depolarizations in some neurons and plays a role in the generation and maintenance of subthreshold membrane oscillations. Na^+ channels are the main targets for a subset of AEDs including carbamazepine, PHT and lamotrigine. The drugs acting on I_{NaT} channels display two predominant mechanisms of action: 1. Voltage-dependent inhibition, associated with a shift of the steady-state inactivation curve in a hyperpolarized direction. This shift decreases the availability of the Na^+ channel during an action potential and, therefore, reduces the excitability of the cell (**D**). 2. Activity- or use-dependent blocking effects, i.e. blocking effects are enhanced when neurons are repetitively depolarized at higher frequencies (**E** and **F**). This results in a highly efficient block of I_{NaT} preferentially during prolonged high-frequency neuronal activity, such as that occurring during seizures.

(i.e. dentate granule cells versus CA1 neurons), and according to the epilepsy model studied. Changes in I_{NaT} may then dramatically affect sensitivity to some, but not all, AEDs.

Block of I_{NaP} may also be a crucially important mechanism of AED action. Pharmacological augmentation of I_{NaP} causes an increased propensity of individual neurons to generate burst discharges (Su *et al.*, 2001), and several mutations that give rise to increased I_{NaP} cause epilepsy in mice or humans (Kearney *et al.*, 2001; Lossin *et al.*, 2002; Rhodes *et al.*, 2004; Spampanato *et al.*, 2004). I_{NaP} is efficiently

blocked by many AEDs, frequently at a concentration range lower than that observed for I_{NaT} (Köhling, 2002). In native neurons, I_{NaP} is potently inhibited by lamotrigine and PHT, as well as losigamone (Chao and Alzheimer, 1995; Lampl *et al.*, 1998; Gebhardt *et al.*, 2001; S. Remy and H. Beck, unpublished data). Despite the potential importance of I_{NaP} in the regulation of neuronal firing, altered AED effects on I_{NaP} following epileptogenesis have so far not been described.

What mechanisms can account for an altered sensitivity of Na^+ channels in epileptic tissue? One possibility may be that the subunit composition of these channels is altered, such that the expression of AED-insensitive subunits or subunit combinations is promoted. Indeed, numerous changes in Na^+ channel subunit expression have been observed in both human and experimental epilepsy (Bartolomei *et al.*, 1997; Aronica *et al.*, 2001; Whitaker *et al.*, 2001; Ellerkmann *et al.*, 2003). In this respect, the downregulation of accessory Na^+ channel β_1 and β_2 subunits following experimentally induced status epilepticus appears to be a consistent finding (Gastaldi *et al.*, 1998; Ellerkmann *et al.*, 2003). In addition to changes in mRNA levels, altered alternative splicing of pore-forming subunit mRNAs has also been observed (Gastaldi *et al.*, 1997; Aronica *et al.*, 2001). A recent, very interesting study underscores the potential importance of the β_1 subunit in the development of pharmacoresistance. In the paper by Lucas *et al.* (2005), the pharmacology of Na^+ channels containing a mutant β_1 subunit causing the epilepsy syndrome generalized epilepsy with febrile seizures plus was examined. Surprisingly, Na^+ channels containing mutant β_1 subunits displayed a dramatic and selective loss of use-dependent block by the AED PHT, that was very similar to the effects observed in chronic experimental epilepsy for PHT and carbamazepine (Remy *et al.*, 2003*a*, *b*). These results collectively suggest that changes in accessory subunits might be promising candidates for further investigation as a molecular correlate of the AED-insensitive sodium channel. They are also intriguing because they suggest that use-dependent effects on Na^+ channels require some form of interaction with β_1 subunits, whereas this may not be the case for tonic block of Na^+ channels.

It is at present unclear why use-dependent block by carbamazepine and PHT is lost or reduced, whereas use-dependent block by lamotrigine remains intact in experimental epilepsy. This is an intriguing question because it has been suggested that all three drugs bind to the same site on Na^+ channels in CA1 neurons based on coapplication experiments (Kuo *et al.*, 1998). The mutual exclusivity among the binding of drugs simultaneously applied to a channel may however result from either an allosteric mechanism or from direct competition of the drugs at a single binding site. Even though a single binding site appears to provide the most parsimonious explanation for these results, it is quite conceivable that allosteric interactions between different binding sites may also exist. This would provide a basis for the AED-selective loss of sensitivity observed in chronic experimental epilepsy.

Other types of voltage-gated channels

Other types of voltage-gated channels have also been screened as potential drug targets. In many cases, effects of AEDs on specific ion channel subunits or ion channels in native neurons or expression systems have been described (*see* Table 2).

Ca^{2+} channels can be subdivided into two groups: high-threshold Ca^{2+} currents, and a group of low-threshold currents (also termed T-type Ca^{2+} currents, Ertel *et al.*, 2000). A number of AEDs has been shown to inhibit high threshold Ca^{2+} channels in native neurons at high therapeutic concentrations (Stefani *et al.*, 1997*b*, 1998; Schumacher *et al.*, 1998; *see* Table 2). Additionally, the AED gabapentin has been shown to exhibit strong and specific binding to the accessory $\alpha_2\delta$ subunit (Gee *et al.*, 1996). It has been proposed that this effect underlies inhibition of presynaptically expressed high-threshold Ca^{2+} channels by gabapentin, which causes a reduction in neurotransmitter release (Fink *et al.*, 2000; *see* Table 2). Some AEDs potently inhibit low-threshold T-type Ca^{2+} channels, which are not expressed presynaptically (Yaari *et al.*, 1987; Coulter *et al.*, 1989; Gomora *et al.*, 2001; *see* Table 2). The effects of AEDs on the three T-type Ca^{2+} channel subunits, as well as in native neurons, are diverse (cf. Todorovic and Lingle, 1998; Todorovic *et al.*, 2000; Lacinova, 2004). T-type channels are critically important in controlling the excitability of the postsynaptic compartment of neurons (Huguenard, 1996), both in normal and epileptic neurons. For instance, aberrant bursting is seen in CA1 hippocampal neurons from epileptic animals (Sanabria *et al.*, 2001) that is mediated by increased expression of T-type Ca^{2+} channels (Su *et al.*, 2002). Additionally, T-type Ca^{2+} channels in thalamic neurons have been implicated in the generation of spike-wave discharges in absence epilepsy (Huguenard, 2002 and references therein). Consequently, inhibition of burst discharges in thalamic neurons is thought to contribute to the anti-epileptic effects of antiabsence AEDs. It is so far unknown if the sensitivity of either presynaptic or post-synaptic Ca^{2+} channels to AEDs changes during epileptogenesis. The same applies to other voltage-gated ion channels such as K^+ channels (*see* Table 2).

H-currents (I_H) are mixed cationic currents that are activated by hyperpolarization and deactivated following repolarization of the membrane. I_H has multiple functional roles; for instance, it mediates some forms of pacemaker activity in heart and brain, it regulates membrane resistance and dendritic integration and stabilizes the level of the resting potential (reviewed, Robinson and Siegelbaum, 2003). An interesting feature of I_H appears to be that the corresponding channels are predominantly located in dendrites, rather than the soma of neurons (Poolos *et al.*, 2002). Interestingly, dendritic H-currents are potently enhanced by the AEDs lamotrigine and gabapentin at clinically relevant concentrations (Poolos *et al.*, 2002; Surges *et al.*, 2003, *see* Table 2), resulting in I_H-mediated inhibitory effects on action potential firing by selectively reducing the excitability of the apical dendrites (Poolos *et al.*, 2002). Cell-type specific changes in I_H have

been described in models of epilepsy (Chen *et al.*, 2001), including a dramatic loss of dendritic I_H in entorhinal cortex neurons (Shah *et al.*, 2004). The importance of this change for pharmacoresistance to lamotrigine has not been directly addressed, but it is conceivable that a sufficiently large reduction of these channels could constitute a *de facto* loss of a major drug target for lamotrigine in this subregion.

Neurotransmitter systems: GABA

GABA is the predominant inhibitory neurotransmitter in the adult brain and plays a critical role in the regulation of excitability of neuronal networks (Mody and Pearce, 2004). GABA binding to ionotropic $GABA_A$ receptors causes opening of the receptor ionophore, which is permeable to Cl^- and—to a lesser extent—to HCO_3. In the presence of a normal adult transmembraneous Cl^- gradient, this results in expression of an inhibitory post-synaptic current that hyperpolarizes the post-synaptic neuronal membrane. Direct modulators of $GABA_A$ receptors include benzodiazepines and barbiturates. Benzodiazepines increase GABA affinity of the receptor complex and may augment their Cl^- conductance via allosteric modulation (Twyman *et al.*, 1989*a*; Rudolph *et al.*, 1999, 2001). Substances that interact with the GABA system in a more indirect way affect the handling and metabolism of synaptically released GABA. Vigabatrin (gamma-vinyl GABA) is a GABA analogue that inhibits one of the main enzymes controlling GABA concentrations in the brain, GABA transaminase. Consequently, application of vigabatrin causes large elevations in brain GABA levels. The AED tiagabine inhibits the high-affinity GABA transporter GAT1 that normally terminates synaptic action of GABA via rapid uptake. So far, available evidence indicates that neither the efficacy of GABA uptake, nor its sensitivity to tiagabine is altered in chronic experimental epilepsy (Frahm *et al.*, 2003).

Regarding $GABA_A$ receptor agonists, reduced activity of such substances has been described in a chronic model of epilepsy. In the pilocarpine model of epilepsy, $GABA_A$ receptors of dentate granule cells show a reduced sensitivity to drugs acting on the benzodiazepine receptor site 1. While augmentation of GABA-evoked currents by the broad-spectrum benzodiazepine clonazepam was slightly enhanced in epileptic animals, augmentation by the benzodiazepine site 1-selective agonist zolpidem was strongly decreased (Gibbs *et al.*, 1997; Brooks-Kayal *et al.*, 1998; Cohen *et al.*, 2003). In CA1 pyramidal cells, the effects of clonazepam were dramatically reduced in chronically epileptic animals (81% reduction relative to control, (Gibbs *et al.*, 1997). This suggests that the same might also apply to clinically employed benzodiazepines.

What is the molecular mechanism of this change in $GABA_A$ receptor pharmacosensitivity? An enormous diversity of $GABA_A$ receptors has been reported in the CNS, reflecting the fact that in each receptor at least three different subunits are present, which derive from one of eight structurally distinct and genetically distinct families (Costa, 1998; Sperk *et al.*, 2004). Combined molecular and functional studies indicate that a transcriptionally mediated switch in the alpha subunit composition of $GABA_A$ receptors occurs in epileptic animals, in particular a decrease of α_1 subunits and an increase of α_4 subunits (Brooks-Kayal *et al.*, 1998). These findings correlate well with the observed changes in benzodiazepine receptor pharmacology.

Neurotransmitter systems: glutamate

Despite the undoubted importance of altered glutamate-mediated excitatory neurotransmission in chronic experimental (Mody and Heinemann, 1987; Martin *et al.*, 1992; Kohr *et al.*, 1993) and human epilepsy (Isokawa and Levesque, 1991), few substances acting on this system have been developed to clinical use so far. Felbamate exerts complex effects on the NMDA receptor (Kuo *et al.*, 2004), some of which may be mediated via the modulatory glycine binding site (White *et al.*, 1995). Some of the effects of felbamate have been shown to be affected by NMDA receptor subunit composition (Kleckner *et al.*, 1999; Harty and Rogawski, 2000). AEDs acting on AMPA receptors are also scarce, some drugs currently in clinical trials inhibit AMPA receptors (talampanel, *see* Chappell *et al.*, 2002). Likewise, topiramate has been shown to reduce excitatory synaptic transmission via an inhibition of AMPA receptors (Qian and Noebels, 2003). Altered cellular expression of glutamate receptors in epilepsy should be considered in future development of compounds acting on these receptors. Given the paucity of established AEDs acting on individual glutamate receptors, however, we will abstain from an in depth discussion of these changes here.

Role of changes in drug targets in the setting of a chronically epileptic brain

The specific changes in drug targets described above are an attractive concept to explain pharmacoresistance. It is important to realize, however, that not only changes in drug targets themselves, but also changes in other molecules that affect their function may have important consequences for AED efficacy. This idea is exemplified by recent findings regarding the role of GABA in epilepsy. GABA may on occasion act as an excitatory neurotransmitter in the immature brain. A depolarizing action of $GABA_A$ receptor activation arises because of an altered chloride homeostasis, resulting in a changed chloride gradient across the neuronal membrane. The altered chloride reversal potential then results in a net outward flux of Cl^- through the $GABA_A$ receptor ionophore, causing depolarization of the neuron (Mody and Pearce, 2004). Interestingly, in addition to the developing brain, depolarizing GABA responses appear to be a feature of some neurons in the epileptic brain (Cohen *et al.*, 2002; Wozny *et al.*, 2003). Augmenting such depolarizing GABA-mediated potentials by application of GABA agonists is likely to facilitate action potential generation to excitatory

input (Gulledge and Stuart, 2003), and thereby would increase neuronal excitability instead of decreasing it. Whether depolarizing GABA responses really play a role in pharmacoresistance to GABAmimetic drugs remains to be seen. These considerations do, however, illustrate the need to consider changes in drug targets within the more general setting of a chronically epileptic brain.

Molecular mechanisms underlying altered target sensitivity

So far, most of the mechanisms implicated in altered AED targets are changes in the transcription of ion channel subunits. Seizures appear to cause a highly coordinated change in transcription of certain groups of ion channel subunits, both in rat models of epilepsy (Brooks-Kayal *et al.*, 1998) and in human epilepsy patients (Brooks-Kayal *et al.*, 1999; Bender *et al.*, 2003). This seizure-induced transcriptional plasticity appears to be differentially regulated in different neuron types (cf. Bender *et al.*, 2003; Shah *et al.*, 2004). These transcriptional changes most probably affect both the density of ion channels in the neuronal membrane, as well as the subunit stochiometry of multisubunit channel complexes (Brooks-Kayal *et al.*, 1998). In addition to transcriptional mechanisms, seizure activity may also evoke multiple post-translational modifications of ion channel proteins, such as altered protein transport and targeting, phosphorylation or glycosylation (Bernard *et al.*, 2004). Indeed, increased phosphorylation of I_{Na} by protein kinase C has been shown to affect responsiveness to the AED topiramate in one study (Curia *et al.*, 2004). It is quite possible that other post-transcriptional modifications of ion channel proteins induced by seizures may profoundly affect their drug sensitivity. How seizures may modify the pharmacosensitivity of AED targets is summarized schematically in Fig. 3.

Relationship of molecular changes in AED sensitivity to pharmacoresistance observed *in vivo*

How is the loss of AED sensitivity on the level of an ion channel such as I_{NaT} related to pharmacoresistance observed in human epilepsy patients or intact animals? In epilepsy patients, properties of I_{NaT} seemed to differ when patients are separated into two groups, one resistant to carbamazepine and a smaller one responsive to carbamazepine. In the former, the use-dependent block of I_{NaT} proved to be abolished, similar to the findings in the pilocarpine model of epilepsy. In contrast, carbamazepine responsive patients showed potent use-dependent effects of carbamazepine on I_{NaT}. Thus, the sensitivity to carbamazepine on a cellular level appeared to correlate with the clinical responsiveness to the same drug. These results should be interpreted with caution on two levels. Firstly, the number of patients for which both clinical and *in vitro* data could be obtained is still limited, particularly when considering the group of pharmacoresponsive epilepsy patients (Remy *et al.*, 2003*a*). Secondly, patients who are resistant to carbamazepine very frequently are resistant also to other AEDs (Kwan and Brodie, 2000). However, available data indicate that altered sensitivity of Na^+ channels may not be able to account for altered efficacy of other AEDs such as valproic acid or lamotrigine (Remy *et al.*, 2003*b*). This finding may indicate that resistance to AEDs in epilepsy patients is a complex phenomenon that possibly relies on multiple mechanisms. On a genetic level, association studies are beginning to yield candidate gene polymorphisms that may be associated with AED sensitivity (Tate *et al.*, 2005).

The correlation of target pharmacosensitivity and sensitivity to AEDs *in vivo* in experimental models of epilepsy is quite unclear. The pilocarpine model of epilepsy has been frequently used to study changes in pharmacosensitivity of drug targets. Leite and Cavalheiro (1995) have provided some evidence that high doses of common AEDs such as carbamazepine, PHT and valproate reduce the spontaneous seizure frequency in these animals. This is in apparent contradiction to the finding that Na^+ channels in the same model are resistant to carbamazepine. It is possible that this may be due to the high doses of AEDs used, or alternatively to differences in the rat strains and/or pilocarpine protocols used. Furthermore, it is likely that some groups responsive and resistant to AED may exist in chronic epilepsy models (Löscher *et al.*, 1998; Nissinen and Pitkänen, 2000). Ideally, these questions could, therefore, be resolved by first examining pharmacosensitivity in intact animals, and subsequently comparing these *in vivo* results to the cellular effects of AEDs in the same individuals. So far, this important approach has only been implemented in few experiments (Jeub *et al.*, 2002). For these experiments, kindled rats were used that could be separated into two groups based on their responsiveness to PHT (Löscher *et al.*, 1998). When rats responsive to PHT were compared to a group of rats that were not, no difference in PHT sensitivity of I_{NaT} emerged. It should be noted, however, that PHT effects on the recovery from inactivation and use-dependent block, where the most dramatic effects were seen in the pilocarpine model of epilepsy, were not examined in this study (Jeub *et al.*, 2002). Nevertheless, animal models in which groups with differential pharmacosensitivity can be defined represent a promising avenue to study mechanisms of pharmacoresistance (Nissinen and Pitkänen, 2000). It remains to be seen how far such animal models mirror mechanisms of pharmacoresistance in human epilepsy patients.

Modification in multidrug transporters as basis for pharmacoresistance

Overview of multidrug transporter molecules expressed in the brain

The second main emerging concept to explain pharmacoresistance contends that increased expression or function of multidrug transporter proteins decreases the effective concentration of AEDs at their targets (*see* Fig. 1B). Intense

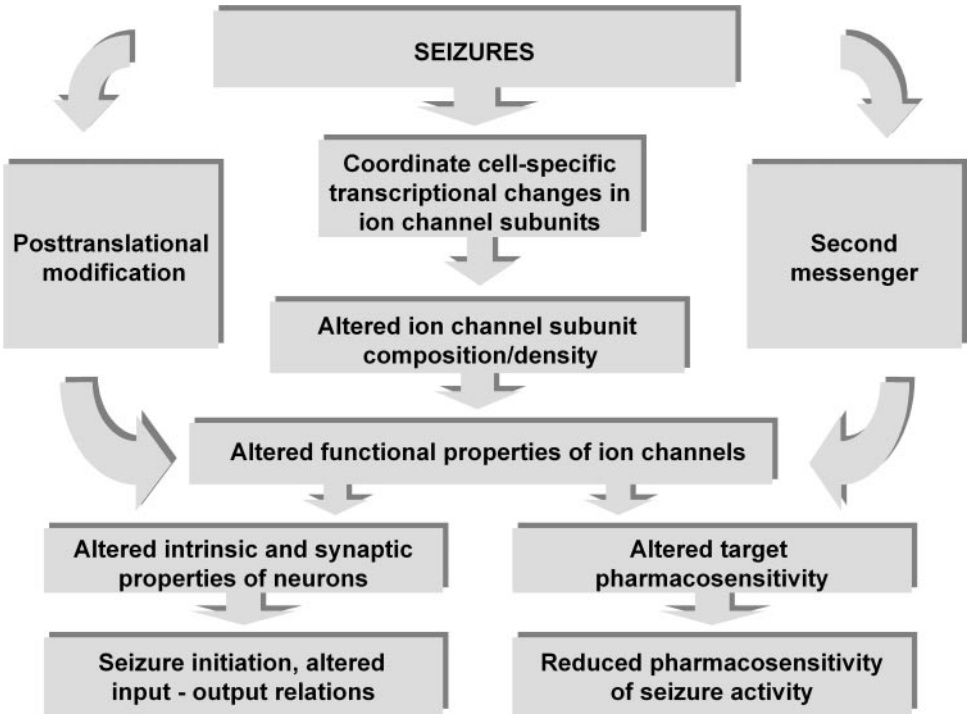

Fig. 3 Epileptic seizures trigger an activity-dependent sequence of events resulting in alterations in neuronal firing and pharmacosensitivity. Known changes triggered by seizures include coordinate changes in ion channel transcription, altered post-translational processing of ion channel proteins, or altered modification of channels by second-messenger systems. Many of these changes result in defined pharmacological and functional changes in ion channels. These changes may underlie altered responses to AEDs as well as altered excitability. It should be noted that this model depicts acquired activity-dependent changes caused by seizures. Reduced responses to AEDs may also be a pre-existing condition caused by genetic polymorphisms that affect specific AED targets (see discussion in text).

interest has been focused on understanding the molecular basis of multidrug transport in the brain in recent years, primarily because of their potential importance in mediating resistance to anticancer drugs. This effort has led to the discovery of several genes encoding transmembrane proteins that function as drug efflux pumps. These genes are highly conserved and the vast majority belongs to the superfamily of adenosine triphosphate-binding cassette (ABC) proteins. A large number of human genes belonging to this superfamily have been identified, which have been systematically classified into seven subfamilies [ABCA, ABCB, ABCC, ABCD, ABCE ABCF and ABCG (Dean *et al.*, 2001)]. Most of these genes encode ATP-driven pumps that are able to transport a wide range of substrates.

Studies addressing the role of multidrug transporters in the development of pharmacoresistant epilepsy have hitherto been focused mainly on a subset of these transporters. *MDR1* (belonging to the ABCB subfamily, systematic nomenclature ABCB1) encodes P-glycoprotein (PGP, Silverman *et al.*, 1991; Ueda *et al.*, 1993), which transports a wide range of lipophilic substances across cell membranes. A further family of genes [multidrug-resistance associated proteins or MRPs, systematic nomenclature ABCC subfamily (Borst *et al.*, 2000)] transports a range of substances partially overlapping with those transported by PGP. Most of the proteins encoded by these genes (i.e. MRP1 to 6 and PGP) are expressed in endothelial cells of the blood–brain or blood–CSF barrier (Schinkel *et al.*, 1996; Huai-Yun *et al.*, 1998; Rao *et al.*, 1999; Zhang *et al.*, 1999). In addition, MRP1 and one of the two rodent isoforms of PGP are present in astrocytes (Pardridge *et al.*, 1997; Regina *et al.*, 1998; Golden and Pardridge, 1999; Decleves *et al.*, 2000). The functional role of this expression is currently a matter of debate (Golden and Pardridge, 2000).

Altered expression of multidrug transporters in human and experimental epilepsy, and consequences for intraparenchymal AED concentrations

Several lines of evidence indicate a role of multidrug transporters in the development of resistance to AEDs, which are set out in more detail as follows. Firstly, drug transporters transport some AEDs in isolated cell systems (Batrakova *et al.*, 1999; Marchi *et al.*, 2004). Secondly, drug transporters appear to regulate intraparenchymal drug concentrations *in vivo* in many cases. Mice or rats lacking certain drug transporters display increased accumulation of AEDs (Schinkel *et al.*, 1996, 1997; Rizzi *et al.*, 2002; Potschka *et al.*, 2003*b*, but see Sills *et al.*, 2002; *see* Table 3). It should be noted that interpretation of data from such animal models is complicated by two issues: firstly, there may be a compensatory regulation of other drug transporter molecules and, secondly, the wide

Table 3 Drug efflux transporters and their anticonvulsant drug substrates (adapted from Loescher and Potschka, 2005)

Drug efflux transporter	Substrate
PGP (MDR1, ABCB1)	Phenytoin (Tishler *et al.*, 1995; Schinkel *et al.*, 1996; Potschka and Löscher, 2001; Rizzi *et al.*, 2002)
	Carbamazepine (Potschka *et al.*, 2001)
	Phenobarbital (Potschka *et al.*, 2002)
	Lamotrigine (Potschka *et al.*, 2002)
	Felbamate (Potschka *et al.*, 2002)
	Topiramate (Sills *et al.*, 2002)
MRP2 (ABCC2)	Phenytoin (Potschka *et al.*, 2003*b*)

expression of drug transporters in other tissues may result in complex pharmacokinetic effects of deleting drug transporter genes. These issues do not apply when drug transporters are inhibited pharmacologically *in vivo*. Indeed, pharmacological inhibition of drug transporters alters brain distribution of some AEDs (Potschka and Löscher, 2001; Potschka *et al.*, 2001, 2003*b*; Löscher and Potschka, 2002, *see* Table 3). It should be stated, however, that the drug transporter inhibitors employed thus far are not very specific, and that studies using more specific novel inhibitors are currently being undertaken.

Interestingly, the results in the literature with regard to one of the most frequently employed AEDs, carbamazepine, are not uniform. This drug is not transported in PGP-containing cell systems, and its brain concentration remains unaltered in mice lacking PGP (Owen *et al.*, 2001), or animals lacking MRP2 (Potschka *et al.*, 2003*a*). On the other hand, pharmacological (Potschka *et al.*, 2001) or genetic (Rizzi *et al.*, 2002) inhibition of PGP does appear to be associated with a change in intraparenchymal carbamazepine concentration under some conditions. The reasons for this discrepancy are currently under scrutiny. That not all AEDs may be transported equally by drug transporters has also been suggested in the case of some benzodiazepines, which appear not to be transported by MDR1 (Schinkel *et al.*, 1996). As a further possibility, carbamazepine has been shown to itself inhibit the activity of human P-glycoprotein, albeit at high concentrations which may not be clinically relevant (Weiss *et al.*, 2003).

A large body of evidence suggests that different drug transporter molecules are indeed upregulated in human epilepsy, as well as in experimental models of epilepsy. For instance, increased MDR1 expression on the mRNA and/or protein level occurs in patients with different forms of epilepsy (Tishler *et al.*, 1995; Lazarowski *et al.*, 1999; Sisodiya *et al.*, 1999, 2002; Aronica *et al.*, 2003; Volk and Löscher, 2005) and after chemically-induced status epilepticus or audiogenic seizures (Zhang *et al.*, 1999; Kwan *et al.*, 2002; Rizzi *et al.*, 2002). Similar findings have been obtained for MRP1 [(Sisodiya *et al.*, 2001, 2002), MRP2 (Dombrowski *et al.*, 2001)] and major vault protein (Van Vliet *et al.*, 2004). These studies have also shown that expression of drug transporter genes in epileptic foci is observed in cell types that do not usually express them. For instance, PGP and MRP1 appear to be strongly upregulated on the protein level in astrocytes, especially surrounding blood vessels (Sisodiya *et al.*, 2002). Likewise, there may be upregulation of drug transporter expression in dysplastic neurons in focal cortical dysplasia (Sisodiya *et al.*, 2001), as well as in hippocampal neurons in temporal lobe epilepsy (Marchi *et al.*, 2004; Volk *et al.*, 2004). It is yet unclear how this ectopic expression might contribute to altered pharmacosensitivity. It is entirely possible, however, that expression of multidrug transporters in neuronal membranes inhibits access of AEDs to intracellular sites of action.

Relationship of molecular changes in AED sensitivity to pharmacoresistance observed *in vivo*

If multidrug transporters play a significant role in pharmacoresistance, then upregulation of transporters on the molecular or functional level should correlate with the clinically observed responsiveness to AEDs. Indeed, increased drug transporter expression appeared to be correlated with less efficient seizure control in one study (Tishler *et al.*, 1995). This correlation is also found in an animal model of resistance to AEDs. Rats resistant to phenobarbital showed a dramatic overexpression of PGP in limbic brain regions compared to rats responsive to phenobarbital (Volk and Löscher, 2005). Similar findings have been obtained in an elegant study using MRP2-deficient rats. In this study, MRP2-deficient kindled rats have higher PHT brain levels than wild-type rats, and are more susceptible to PHT treatment (Potschka *et al.*, 2003*b*). These findings are interesting because they constitute the first controlled experiment in which deficiency in a specific drug transporter is associated with differential susceptibility to AED treatment.

Even though, collectively, these findings appear to support a role for multidrug transporters in pharmacoresistant epilepsy, there are a number of conceptual questions that remain enigmatic. Firstly, epileptic seizures are known to result in a disruption of the BBB, which would be expected to result in better access of AEDs to brain parenchyma despite the upregulation of multidrug transporters. Secondly, patients are in many cases treated with AEDs until CNS side effects develop. This seems to indicate that relevant CNS concentrations of AEDs are reached despite transporter upregulation, yet, these patients are resistant to treatment. This apparent discrepancy could potentially arise via local upregulation of drug transporters that only affects AED concentrations at the epileptic focus.

The genetic basis of pharmacoresistance

How can the wide spectrum of pharmacoresistance observed in human epilepsy patients and some animal models be explained? A number of recent studies have suggested that

sequence variants in drug transporter or ion channel genes affect either function or expression of the corresponding proteins. In the case of drug transporters, a number of functionally relevant polymorphisms have been identified (Kerb *et al.*, 2001*a*, *b*). Furthermore, a polymorphism (C3435T) has been identified in exon 25 of the gene encoding MDR1 that is associated with increased expression of the protein (CC-genotype). Based on these findings, Siddiqui *et al.* (2003) conducted a population-based association study testing the hypothesis that the C3435T polymorphism is associated with resistance to AED treatment. They found that patients with drug-resistant epilepsy were more likely to have the CC-genotype than the TT-genotype [OR 2.66, 95% CI 1.32–5.38, $P = 0.006$ (Siddiqui *et al.*, 2003)]. As suggested by the authors of this study, the C3435T polymorphism by itself is very unlikely to confer a biologically relevant effect. Since this variant is localized in an extensive block of linkage disequilibrium spanning the gene, the as yet unidentified causal variant is supposed be in linkage disequilibrium with the C-allele of the C3435T polymorphism. It should be noted that the results of Siddiqui *et al.* (2003) have not been confirmed in a subsequent study by Tan *et al.* (2004). To further address how polymorphisms can contribute to drug resistance, two major obstacles will have to be overcome. Firstly, it will be necessary to address experimentally whether polymorphisms found in association studies have biologically relevant effects. Secondly, it will be necessary to significantly increase the size of carefully matched patient cohorts to increase reproducibility of such results (Soranzo *et al.*, 2004; Cavalleri *et al.*, 2005). Finally, it will be interesting to extend current studies to include polymorphisms in other multidrug transporters. In this respect, the development of single nucleotide polymorphism tagging for classes of genes important in resistance is a very important step that may enable screening of large numbers of patients (Ahmadi *et al.*, 2005).

It is important to note that gene polymorphisms relevant for pharmacoresistance may occur both in promoter regions as well as in introns and exons. Gene polymorphisms within the coding regions of such genes would result in a difference in ion channel or transporter proteins that precedes the onset of epilepsy. Polymorphisms in promoter regions, which affect the transcription of such genes, may affect activity-dependent transcriptional regulation of these genes by seizures. This provides a potential mechanism for the acquisition of a pharmacoresistant phenotype during epileptogenesis in pharmacoresistant—as opposed to pharmacoresponsive—patients.

Interplay between target and transporter-mediated mechanisms

Collectively, the experimental results described in the above sections indicate that functionally relevant alterations in both AED targets and AED transporters exist. Clearly, these mechanisms are not mutually exclusive. It is entirely possible that decreased permeation of AEDs into brain tissue, in synergy with changes in targets for these drugs, mediate pharmacoresistance. This does not exclude that—for some AEDs—predominant mechanisms underlying pharmacoresistance to these drugs can be identified.

Carbamazepine, for instance, appears not to be a substrate of some multidrug transporters (Owen *et al.*, 2001); rather, sodium channels display a potent loss of sensitivity to carbamazepine (Remy *et al.*, 2003*a*). These and other results imply that expression of multidrug transporters at the BBB is not the main factor in the development of resistance, and imply a target mechanism for this drug. For other AEDs, this may be different. For instance, intraparenchymal PHT concentration is potently regulated by multidrug transporters (Potschka and Löscher, 2001; Rizzi *et al.*, 2002; Potschka *et al.*, 2003*b*), and epileptic animals lacking a multidrug transporter protein (MRP2) are more sensitive to PHT treatment than wild-type animals (Potschka *et al.*, 2003*b*). In contrast, target mechanisms seem to be less important for PHT compared to carbamazepine (Jeub *et al.*, 2002; Remy *et al.*, 2003*b*). Although the evidence supporting this view is far from conclusive, it is tempting to speculate that predominant resistance mechanisms may exist for specific AEDs.

Future directions of research

What are the key pieces of evidence that we should consider to be prerequisites in order to state that drug transporters and/or altered targets play a role in the development of resistance to a given AED? We believe that the following sets of experimental results should be available:

(i) Evidence that the multidrug transporter regulates intraparenchymal concentrations of the drug: this should include work with specific drug transporter inhibitors, and mice lacking specific drug transporter subtypes, as well as a combination of pharmacological and genetic inhibition of transporter function.

(ii) Evidence that multidrug transporter expression and/or transporter function is upregulated in human and experimental epilepsy. Regarding drug targets, evidence should be available that drug targets are less sensitive to a given AED in chronic epilepsy. In both cases, functional and molecular changes should correlate with AED sensitivity of seizures in experimental animals or epilepsy patients.

(iii) Evidence that genetic or pharmacological manipulation of drug transporters/drug targets affects sensitivity of spontaneous seizures to AEDs *in vivo* in chronic models of epilepsy.

In addition, the following data on human epilepsy patients should be obtained.

(iv) Association of polymorphisms in drug transporter/drug target genes with clinical pharmacoresistance. This could also include association of polymorphisms with the drug

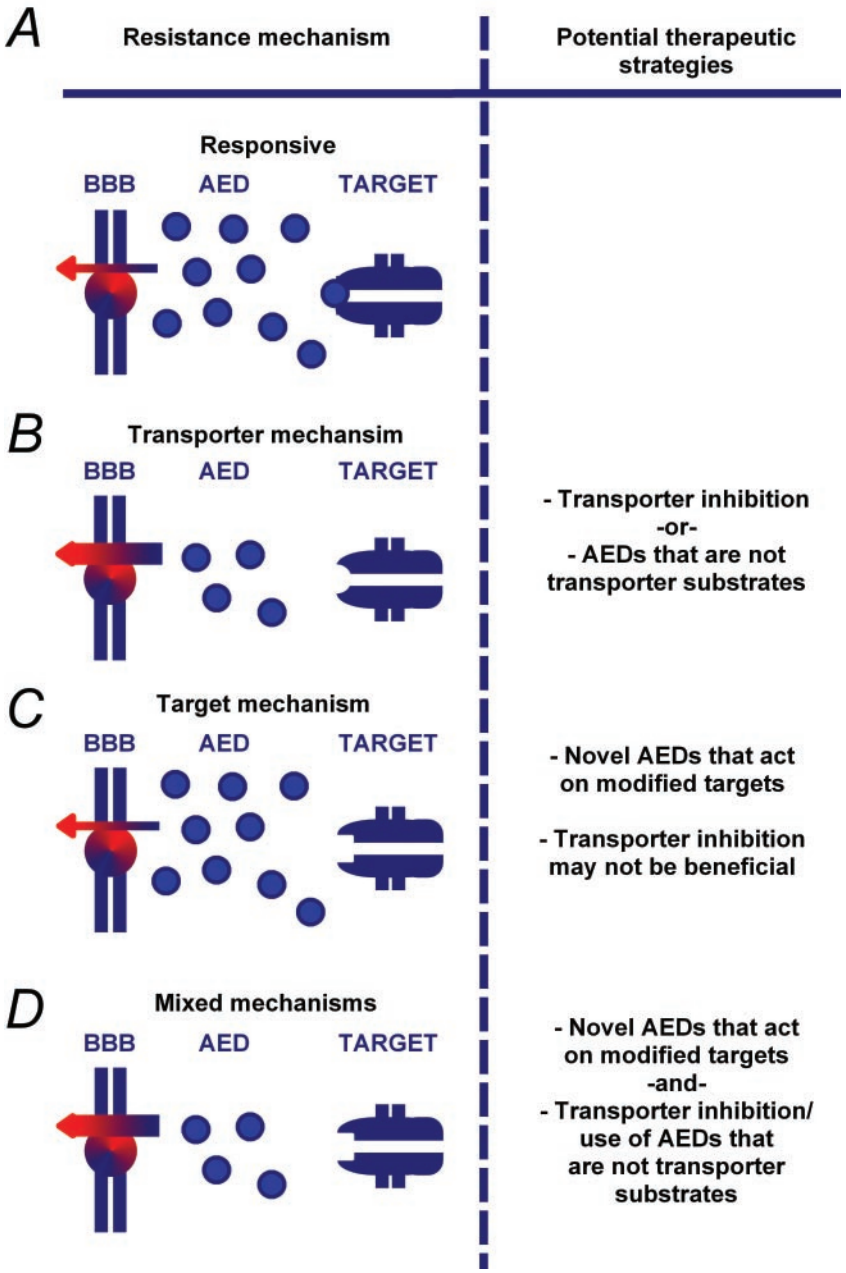

Fig. 4 Potential implications for treatment of pharmacoresistant epilepsy when taking into account different mechanisms underlying pharmacoresistance. Responsive patients (**A**): In patients responsive to a given AED, the AED gains sufficient access to a target. In addition, the AED has sufficient pharmacological effects on the target. Resistant patients, predominant transporter mechanism of resistance (**B**): In a scenario of increased drug transport without any changes in the AED target, the use of AEDs that are not transporter substrates and may additionally exhibit transporter-inhibiting properties would be advantageous in obtaining therapeutic drug levels at the site of action. Alternatively, the use of transporter inhibitors may increase the intraparenchymal drug concentration to therapeutic levels and may help to overcome the reduced efficacy of the drug. Resistant patients, predominant target mechanism of resistance (**C**): If resistance for a given AED is due to a change in its target, increasing the intraparenchymal AED concentration by comedication with transporter inhibitors would not be expected to be beneficial. In this case, the development of drugs that specifically act on the modified target would be the more appropriate approach. Resistant patients, mixed resistance mechanism (**D**): If both transporter- and target-mediated mechanisms of resistance apply, both strategies outlined in panel B and C would have to be combined.

transporter function or drug target pharmacology measured *in vitro* in tissue obtained from epilepsy surgery.

(v) Demonstration that drug transporter polymorphisms have functional effects resulting in a decreased intraparenchymal AED concentration (i.e. effects on either expression or function of drug transporter molecules). Likewise, polymorphisms in drug target genes should have demonstrable effects on expression or pharmacology of these targets.

Potential future clinical implications

Once we have obtained a detailed picture of the mechanisms underlying the development of pharmacoresistance to individual AEDs, this knowledge may become increasingly important both in drug development, as well as clinically. First, detailed information on drug target changes can be used to inform the development of new drugs for the treatment of epilepsy. Currently, identification of AEDs is performed mostly in acute seizure models in normal experimental animals. These animals do not show any of the chronic changes in ion channels or receptors discussed here, and may not represent the best models to develop novel compounds useful in human epilepsy. Targeting novel drugs to 'epileptic' ion channels based on information from chronic models of epilepsy or even tissue from epileptic patients represents a promising avenue for rational drug development in the future.

Information on specific resistance mechanisms might also be used to guide potential treatment with drug transporter inhibitors in conjunction with AEDs. The simplest scenario in which such substances might be used would be as comedication with an AED that is ineffective predominantly due to transporter-mediated mechanisms (depicted schematically in Fig. 4B). On the other hand, comedication with transporter inhibitors in a patient in whom resistance to an AED is predominantly target mediated (*see* Fig. 4C) would not be expected to be beneficial. In this case, the development of drugs that specifically act on the modified target would be the more appropriate approach. Ideally, these compounds would not be substrates of drug transporter, but could also be coadministered with transporter inhibitors. For some AEDs, both resistance mechanisms may be relevant and synergistic (Fig. 4D); in this case, strategies to overcome resistance would have to combine the approaches outlined in Fig. 4B and C. A further clinical issue that may gain more and more importance in the coming years is the identification of predictors that identify clinically resistant or responsive patient populations. If clear genetic polymorphisms in either transporter or ion channel genes can be identified that reliably predict the occurrence and the probable mechanism of drug resistance (Soranzo *et al.*, 2004; Tan *et al.*, 2004; Ahmadi *et al.*, 2005), these data would obviously strongly influence initial therapy, and perhaps increase the chances of its success.

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