Antiepileptic drugs targeting sodium channels: subunit and neuron-type specific interactions
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Chapter 5

General Discussion
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The main theme of this thesis is to study the antiepileptic action of Na\(^+\) channel-targeting antiepileptic drugs (AEDs). About 20-40% of epilepsy patients are pharmacoresistant and continue to have seizures (Brodie & Kwan, 2002; Loscher & Schmidt, 2002; Schmidt & Loscher, 2005; Picot et al., 2008). Alterations in the expression of mRNAs or proteins of \(\alpha\)-subunits have been observed in both epileptic human brain (Lombardo et al., 1996; Whitaker et al., 2001a) and animal models of epilepsy (Bartolomei et al., 1997; Gastaldi et al., 1997; Aronica et al., 2001). One possible factor contributing to pharmacoresistance is that different Na\(^+\) channel \(\alpha\)-subunits interact with AEDs with different pharmacological efficacy. The changed expression patterns of Na\(^+\) channel \(\alpha\)-subunits may thus lead to a diminished pharmacological response during the development of epilepsy. Furthermore, the current knowledge of this class of AEDs is that they have a higher affinity for inactivated Na\(^+\) channels, stabilize Na\(^+\) channels in this non-conducting state and prevent epileptic discharges by selectively inhibiting high frequency spike firing, with much less effects on normal action potential firing (Ragsdale & Avoli, 1998; Rogawski & Loscher, 2004). However, this understanding only focuses on the AED – target (i.e. inactivated Na\(^+\) channel) interaction at the molecular level without considering pharmacological responses of networks which are composed of different neuron types. Therefore we aimed to answer two main questions: (1) are there any differences between the biophysical and pharmacological properties of different Na\(^+\) channel \(\alpha\)-subunits? (2) Are there any differences in the sensitivity of spike firing to AEDs between different neuron types? To answer the first question, we compared the interactions between three Na\(^+\) channel-targeting AEDs (CBZ, DPH and LTG) and the four main brain Na\(^+\) channel \(\alpha\)-subunits (Na\(_V\)1.1, Na\(_V\)1.2, Na\(_V\)1.3 and Na\(_V\)1.6) stably
expressed in HEK293 cells in Chapter 2. To answer the second question, we investigated effects of LTG on spike firing in excitatory pyramidal neurons and inhibitory GABAergic interneurons in the hippocampal CA1 area in Chapter 4. As inhibitory GABAergic interneurons are extremely diverse with respect to their morphology, electrophysiological features and molecular composition, we chose a relatively homogeneous subpopulation of inhibitory GABAergic interneurons (i.e. 5HT3AR-expressing interneurons) to compare with pyramidal neurons. Furthermore, to gain more insight into Na\(^+\) channel-related alterations during the development of epilepsy, we investigated the regional and (sub)cellular protein expression of different Na\(^+\) channel α-subunits (Na\(_V\)1.1, Na\(_V\)1.2 and Na\(_V\)1.6) in the hippocampus at 1 day, 3 weeks and 2 months after kainic acid-induced status epileptus (SE) in a rat model for temporal lobe epilepsy in Chapter 3.

1. Properties of Na\(^+\) channel α-subunits

1.1. Molecular mechanisms

As confirmed in the study described in Chapter 2 of this thesis, the biophysical properties of the four α-subunits are quite similar. The nine functionally expressed α-subunits known so far are more than 50% identical in amino acid sequence in the transmembrane and extracellular domains and the four α-subunits tested in Chapter 2 are less than 10% different (Catterall et al., 2005). Further analyses of their phylogenetic relationship revealed that Na\(_V\)1.1, Na\(_V\)1.2, Na\(_V\)1.3 and Na\(_V\)1.7 are the most closely related group and their genes are located on human chromosome 2q23-24. Na\(_V\)1.5, Na\(_V\)1.8 and Na\(_V\)1.9 are also a closely related group with the genetic location on human chromosome 3p21-24. Na\(_V\)1.4 and Na\(_V\)1.6 are set apart from the other two closely related groups and are located on human
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chromosomes 17q23-25 and 12q13, respectively (Catterall et al., 2005). The comparisons of amino acid sequence and phylogenetic relationship lead to the conclusion that all members of the voltage-gated Na\(^+\) channel family are members of a single family of proteins and that they have arisen from gene duplications and chromosomal rearrangements relatively recently in evolution (Catterall et al., 2005). On the other hand, the different biophysical properties of the four \(\alpha\)-subunits observed in Chapter 2 (e.g. Na\(_V\)1.1 and Na\(_V\)1.6 display a faster recovery from inactivation than Na\(_V\)1.2 and Na\(_V\)1.3) may result from amino acid sequence differences. Na\(_V\)1.1, Na\(_V\)1.2 and Na\(_V\)1.3 are less than 5% different in amino acid sequence, and Na\(_V\)1.6 is about 8% different from the other three brain isoforms. These differences in amino acid sequence between the four \(\alpha\)-subunits contribute to their different biophysical properties. Another factor affecting the different biophysical properties may arise from three-dimensional structure differences. Although recent studies revealed a three-dimensional structural basis of voltage-dependent gating in model Na\(^+\) channels from bacteria (DeCaen et al., 2009; DeCaen et al., 2011; Payandeh et al., 2011; Yarov-Yarovoy et al., 2012), no crystal structure of a mammalian Na\(^+\) channel is currently available. One of the obstacles to constructing the crystal structure of a mammalian Na\(^+\) channel is its stability under solubilization with detergents and difficulty in synthesizing high levels of functional Na\(^+\) channels (England & de Groot, 2009).

The subtle different pharmacological responses of the four \(\alpha\)-subunits observed in Chapter 2 (e.g. LTG shows a faster binding rate to Na\(_V\)1.1 than to the other three isoforms) suggest that there may exist differences in AED interactions with the four \(\alpha\)-subunits. Amino acid residues in the S6 segments of the III and IV domains form the AED binding site (Ragsdale et
al., 1994; Ragsdale et al., 1996; Yarov-Yarovoy et al., 2001; Liu et al., 2003). However, these amino acid residues are conserved across all nine functionally expressed α-subunits (Ragsdale et al., 1994; Ragsdale et al., 1996). The sequence identity suggests that the subtle different pharmacological properties of the four α-subunits most likely do not arise from the amino acid sequence in the AED binding site. Future constructing the crystal structure of different mammalian Na⁺ channels would provide more insights into the three-dimensional structural basis of the AED binding site of the distinct Na⁺ channels. The differences in the conformational structure may cause distinct degrees of binding site exposure, and thus lead to different pharmacological properties.

1.2. Functional implications

1.2.1. Functional implications of the different properties of Na⁺ channel α-subunits

Subtle differences in the biophysical properties of the four α-subunits were described in Chapter 2. The amplitude of the window current of Naᵥ1.1 was larger than those of the other three subunits. This could contribute to increasing the excitability of the (sub)cellular compartments where Naᵥ1.1 is densely expressed. Recent studies showed that Naᵥ1.1 is primarily expressed in GABAergic interneurons and mainly in parvalbumin- and Kᵥ3.1b-positive neuron types (Yu et al., 2006; Ogiwara et al., 2007; Lorincz & Nusser, 2010; Martin et al., 2010). The larger window current of Naᵥ1.1 might contribute to the fast-spiking behavior of these interneurons. Furthermore, the recovery from inactivation for Naᵥ1.1 and Naᵥ1.6 were relatively fast. The faster recovery from inactivation for Naᵥ1.1 may also contribute to the fast-spiking feature of Naᵥ1.1-expressing interneurons. Naᵥ1.6 is present at high densities in AISs and nodes of Ranvier (Boiko et
The faster recovery from inactivation of this subunit may facilitate action potential initiation and propagation along axons. Compared to Na\textsubscript{V}1.1 and Na\textsubscript{V}1.6, Na\textsubscript{V}1.2 and Na\textsubscript{V}1.3 displayed a slower recovery from inactivation, which could result in a reduced excitability at the (sub-cellular) sites where these subunits are present. For example, the presence of Na\textsubscript{V}1.3 in neonatal neurons may help to provide new-born mammals with a neuroprotective mechanism against hypoxic conditions (Park & Ahmed, 1991; Cummins \textit{et al.}, 1994).

In \textit{Chapter 2}, LTG was found to bind to Na\textsubscript{V}1.1 faster than to the other three isoforms. As mentioned above, Na\textsubscript{V}1.1 is primarily expressed in interneurons and Na\textsubscript{V}1.1 knockout mice displayed an epileptic phenotype (Yu \textit{et al.}, 2006; Ogiwara \textit{et al.}, 2007; Lorincz & Nusser, 2010). Compared with wild-type mice, Na\textsubscript{V}1.1 knockout heterozygous mice displayed reduced Na\textsuperscript{+} currents in interneurons, whereas no significant differences in Na\textsuperscript{+} currents in pyramidal neurons were found between wild-type and knockout mice (Yu \textit{et al.}, 2006). Thus reduced Na\textsuperscript{+} current densities in interneurons cause the decreased excitability of interneurons and the epileptic phenotype. The faster binding rate of LTG to Na\textsubscript{V}1.1 we observed suggests that LTG could preferentially bind to Na\textsuperscript{+} channels in these Na\textsubscript{V}1.1-expressing inhibitory interneurons. CBZ and DPH did not show different binding rates to the four α-subunits, which suggests that these two drugs would bind to Na\textsuperscript{+} channels in both inhibitory interneurons and excitatory neurons and could reduce the excitability of inhibitory interneurons as well as that of excitatory neurons. Epilepsy is a disorder in which seizures arise from abnormal excitability in the brain. How networks composed of diverse components (including excitatory neurons and inhibitory interneurons)
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respond to AEDs is thus of importance for clinical AED application (see the discussion in the section “Factors contributing to AED efficacy”).

1.2.2 Na\(^+\) channel β-subunits

Voltage-gated Na\(^+\) channels in the brain contain a primary α-subunit associated with one non-covalently (β1 or β3) and one covalently (β2 or β4) linked β-subunit (Yu & Catterall, 2003; Catterall et al., 2005). Na\(^+\) channel β-subunits have been shown to modulate the gating behavior of their associated α-subunits in HEK293 cells. The β1 subunit shifts the voltage dependence of activation, fast and slow inactivation of Na\(_V\)1.2 in a depolarizing direction (Qu et al., 2001; Xu et al., 2007). Co-expression of the β1 subunit facilitates recovery from inactivation of the skeletal muscle Na\(^+\) channel α-subunit (Tammaro et al., 2002). The β2 and β3 subunits have been found to shift the voltage dependence of activation and inactivation of Na\(_V\)1.2 and Na\(_V\)1.3 in a depolarizing direction (Qu et al., 2001; Cusdin et al., 2010; Merrick et al., 2010), and β3 accelerates recovery from inactivation of the Na\(^+\) current carried by Na\(_V\)1.2 (Merrick et al., 2010). Furthermore, the β3 subunit is unique in causing increased persistent Na\(^+\) currents (Qu et al., 2001).

Although β-subunits likely affect the biophysical and pharmacological properties of Na\(^+\) channels via modulating the gating behavior of α-subunits, the transmembrane α-subunit of the brain Na\(^+\) channels is sufficient for expression of functional Na\(^+\) channels (Goldin et al., 1986; Isom, 2002). The AED binding site resides in the S6 segments in the III and IV domains of a Na\(^+\) channel α-subunit (Ragsdale et al., 1994; Ragsdale et al., 1996). We investigated interactions between three AEDs and the four α-subunits. A heterologous system (HEK293 cells) allowed us to perform the experiments in a constant and controllable environment. Therefore, the results
objectively reflected the biophysical and pharmacological properties of the primary $\alpha$-subunits.

2. Factors contributing to AED efficacy

As shown in the study described in Chapter 2, Na$^+$ channel-targeting AEDs bind to all four $\alpha$-subunits. Na$_V$1.1 has been shown to be primarily expressed in inhibitory interneurons (Yu et al., 2006; Ogiwara et al., 2007; Lorincz & Nusser, 2010). These findings suggest that Na$^+$ channel-targeting AEDs could inhibit the excitability of both inhibitory interneurons and excitatory neurons. However, so far it was not known whether there exists a neuron-type specific interaction with AEDs. Therefore, we investigated how different neuron types respond to Na$^+$ channel-targeting AEDs in Chapter 4. We performed current-clamp recordings in brain slices to study the effects of LTG on action potential firing in pyramidal neurons and 5-HT$_3$AR-expressing interneurons in the hippocampal CA1 area. Spike firing in pyramidal neurons was more inhibited by LTG than interneurons. This neuron type-specific response suggests a complicated mechanism underlying the treatment of epilepsy with LTG and other AEDs. Under voltage-clamp conditions, we examined AED – Na$^+$ channel interactions in terms of AED effects on steady-state inactivation of Na$^+$ channels and AED binding to and unbinding from Na$^+$ channels. These parameters were found to be not different between these two neuron types, which suggest that other factor(s), instead of a direct AED – Na$^+$ channel interaction, contributes to this neuron type-specific response to LTG. Since LTG and other Na$^+$ channel-targeting AEDs have a high affinity for inactivated Na$^+$ channels, the efficacy of LTG is also determined by the fraction of Na$^+$ channels which are inactivated during spike firing.
One possible factor contributing to the fraction of Na\(^+\) channels which are inactivated during spike firing is the timing or shape of the action potential. Two recent studies investigated Na\(^+\) entry and Na\(^+\) channel availability (non-inactivated Na\(^+\) channels) during the action potential in different neuron types (Carter & Bean, 2009; Carter & Bean, 2011). They first recorded the action potential in a neuron and then used it as the voltage command in voltage-clamp recording to record the evoked ionic currents (Carter & Bean, 2009). Na\(^+\) entry was almost completely confined to the rising phase of the action potential in hippocampal CA1 and cortical pyramidal neurons, whereas a large portion of Na\(^+\) entry was still present in the falling phase in cerebellar Purkinje cells and cortical interneurons. This finding suggested that a large fraction of Na\(^+\) channels reached complete inactivation during the falling phase in pyramidal neurons (Carter & Bean, 2009). The average half-maximal spike widths in pyramidal neurons (cortical pyramidal neurons 1.2 ms and hippocampal pyramidal neurons 0.8 ms) were wider than those in cortical interneurons (0.3 ms) and Purkinje cells (0.2 ms) (Carter & Bean, 2009). To further investigate whether the difference in Na\(^+\) entry arises from the shape of the action potential, they measured Na\(^+\) entry in each neuron type using the pre-recorded action potential waveforms from all four neuron types and found that the difference in Na\(^+\) entry largely depended on the shape of the action potential. For example, all four neuron types showed a large portion of Na\(^+\) entry during the falling phase of an applied Purkinje cell action potential, while with a cortical pyramidal neuron action potential, Na\(^+\) entry was almost confined to the rising phase in all four neuron types (Carter & Bean, 2009). Furthermore, they investigated Na\(^+\) channel availability (non-inactivated Na\(^+\) channels) during and after the action potential by applying an action
potential waveform followed by a test pulse to -8 mV at various times during the falling phase (Carter & Bean, 2009). The average minimum availability in Purkinje cells (0.15) and cortical interneurons (0.07) was higher than those in pyramidal neurons (0.003 for cortical pyramidal neurons and 0.02 for hippocampal pyramidal neurons). In Purkinje neurons and cortical interneurons, availability returned to >75% within 2.5 ms whereas in pyramidal neurons, availability was still < 75% after 5 ms (Carter & Bean, 2009). They further applied the action potential waveforms from all four neuron types followed by a test pulse to -8 mV in each neuron type. They found that the difference in Na$^+$ channel availability (non-inactivated Na$^+$ channels) depended primarily on the shape of the action potential (Carter & Bean, 2009). As shown in the study described in Chapter 4, the average spike widths in interneurons (half-maximal width 1.2 ms and decay time 1.8 ms) are narrower than those in pyramidal neurons (half-maximal width 1.8 ms and decay time 4.8 ms). Thus the narrow action potential in interneurons could contribute to more Na$^+$ channel availability (non-inactivated Na$^+$ channels) during spike firing in interneurons and therefore provide fewer substrates for LTG to bind to.

Another possible factor contributing to the reduced sensitivity of interneurons to LTG may lie in the intrinsic properties of Na$^+$ channels (i.e. recovery from inactivation). The recovery from inactivation in interneurons was 1.5 fold faster than that in pyramidal neurons, which could cause fewer Na$^+$ channels residing in the inactivated state, and again provide fewer substrates for LTG to bind to.

Taken together, these factors could explain why we found that interneurons displayed a reduced LTG sensitivity, as compared with that of pyramidal neurons in the hippocampal CA1 area.
3. Pharmacological mechanisms of Na\(^+\) channel-modulating AEDs

The current understanding of Na\(^+\) channel-targeting AEDs is limited to the direct process of the AED – Na\(^+\) channel interaction at the molecular level. However, epilepsy is a disorder with disturbed network excitability. A systems biology-based perspective and network-level pharmacology are thus needed for the treatment of epilepsy and future AED development (Margineanu, 2012). As a first approach to investigating network-level pharmacology of Na\(^+\) channel-targeting AEDs, our results revealed a neuron type-specific sensitivity to LTG (i.e. spike firing was inhibited by LTG to a greater degree in pyramidal neurons than in interneurons). These results indicate that even though an AED only targets one set of molecules (i.e. Na\(^+\) channels), it has different effects on distinct neuron types. This preferential inhibition of spike firing in excitatory pyramidal neurons is necessary for AEDs to achieve the desired anticonvulsant action against seizure activity. We only tested a subpopulation of interneurons (i.e. 5-HT\(_{3A}\)R-expressing interneurons). The different classes of GABAergic interneurons display a diverse repertoire of action potential characteristics and firing patterns (Ascoli, Alonso-Nanclares et al. 2008). Future tests on more different neuron types are expected to provide insights into how a complete neural network, with its feedforward and feedback inhibition components, responds to AEDs. Furthermore, action potential shape and firing patterns could be pathologically altered in epilepsy. How different neuron types and networks respond to AEDs is another important aspect to be investigated in animal models of epilepsy in future research.

4. The protein expression of Na\(^+\) channel \(\alpha\)-subunits in epilepsy

Recent studies in normal tissues showed that the protein expression of different Na\(^+\) channel \(\alpha\)-subunits is cell type-specific and subcellular
compartment-specific and suggested that different Na\(^+\) channel \(\alpha\)-subunits play distinct functional roles. For example, Na\(_{V1.1}\) is primarily expressed in interneurons, thus playing a role in the excitability of interneurons (Yu et al., 2006; Ogiwara et al., 2007). Na\(_{V1.2}\) is highly expressed along axons and on terminals and regulates neurotransmitter release (Gong et al., 1999; Lorincz & Nusser, 2010). Na\(_{V1.6}\) is highly expressed in axon initial segments (AISs) and nodes of Ranvier and moderately expressed in the somata and dendrites of the CA1 pyramidal cells. Na\(_{V1.6}\) is crucial for the propagation of action potentials along axons and backpropagation of action potentials in dendrites (Lorincz & Nusser, 2010).

Several studies have examined the mRNA expression levels of Na\(^+\) channels in both epileptic human brain and animal models of epilepsy. In epileptic human brain, the relative quantities of mRNAs encoding Na\(_{V1.1}\) and Na\(_{V1.2}\) were increased threefold as compared with normal human brain (Lombardo et al., 1996). In animal models of epilepsy, increased Na\(_{V1.2}\) and Na\(_{V1.3}\) mRNA expression levels were observed in neurons of the CA1-CA3 and the dentate granule cell layer after electrically-induced status epilepticus (SE) (Aronica et al., 2001). During kainate-induced seizures, an increase in neonatal Na\(_{V1.2}\) and Na\(_{V1.3}\) mRNA levels was also observed in the hippocampus (Bartolomei et al., 1997; Gastaldi et al., 1997).

However, these mRNA studies do not provide information about the regional and (sub)cellular expression of different Na\(^+\) channel \(\alpha\)-subunits and only a few studies give a general profile of the regional and (sub)cellular protein expression of different Na\(^+\) channel \(\alpha\)-subunits during the development of epilepsy. In Chapter 3, using a kainic acid rat model for temporal lobe of epilepsy, we examined the temporal and spatial expression of three voltage-gated Na\(^+\) channel \(\alpha\)-subunits. The most important findings
are: (1) Na\textsubscript{V}1.1 was expressed in a subpopulation of interneurons and the neuropil in the CA pyramidal cell layer in controls. During epileptogenesis, the number of Na\textsubscript{V}1.1-positive interneurons was reduced, but the percentage of Na\textsubscript{V}1.1-positive interneurons to total neurons was not different compared with that in controls in the hilar area. In contrast, the Na\textsubscript{V}1.1-expressing neuropil in the CA pyramidal cell layer became reduced during epileptogenesis. (2) Na\textsubscript{V}1.2 expression in the inner molecular layer of the dentate gyrus almost disappeared at 1 day after SE, but robustly reappeared at 3 weeks and 2 months after SE in association with sprouted mossy fibers. (3) Na\textsubscript{V}1.6 in the dendrites of the CA1 pyramidal cells was lost. This Na\textsubscript{V}1.6 loss most likely arose from reduced Na\textsubscript{V}1.6 expression instead of dendritic loss because MAP2 staining showed that a part of dendrites were still present. Reduced Na\textsubscript{V}1.6 expression is expected to decrease the excitability of the CA1 pyramidal cells. These findings suggest two possibilities: (1) like in normal tissues, the protein expression of different Na\textsuperscript{+} channel α-subunits is cell type-specific and (sub)cellular compartment-specific in epilepsy. (2) Although it is difficult to determine whether changes in the regional and (sub)cellular protein expression are a cause or a consequence of epileptogenesis, it is apparent that these changes could have important consequences for hippocampal excitability.

5. Concluding remarks and future directions

In Chapter 2 and Chapter 4 we aimed to study the antiepileptic action of Na\textsuperscript{+} channel-targeting AEDs at two different levels (different α-subunits and different neuron types). These results indicate that modulation of Na\textsuperscript{+} channels by AEDs is subunit-specific and neuron type-specific. Future investigation into the pharmacological responses of more different neuron types and networks in animal models of epilepsy from both behavioral and
cellular levels are expected to provide more insights into network-level pharmacology in epilepsy treatment. Furthermore, since other AEDs like CBZ and DPH have different binding rates from LTG, drug testing with these AEDs would be of interest for future studies. In Chapter 3, the results showed that the expression patterns of distinct Na$^+$ channels are differently altered during the development of epilepsy. These alterations in protein expression levels are expected to change the excitability in the hippocampus. Further investigation into the functional roles of Na$^+$ channel-related alterations in epilepsy would help understanding epilepsy pathology and improving the treatment of epilepsy by Na$^+$ channel-targeting AEDs.