Excitatory ionic currents and calcium extrusion in hippocampal neurons in epilepsy
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CHAPTER 7

GENERAL DISCUSSION

Susan O.M. Ketelaars
MAIN FINDINGS

1. In chronic epileptic rats three months after electrically-induced SE, the voltage dependence of activation of the transient sodium current in CA1 pyramidal neurons was shifted to more hyperpolarized potentials when compared with control rats. This may lower the spike threshold of the neuron. Together with a small shift of the steady-state inactivation function to more depolarized potentials, the shift of the activation function resulted in a significantly increased sodium window current in the epileptic rats. This will drive the membrane voltage closer to firing threshold and promote (high-frequency) firing. The voltage range where the window current occurred was shifted closer to resting membrane potential. Together, these alterations will contribute to increased excitability in the chronic epileptic state (Chapter 2).

2. The alterations of the sodium current properties in chronic epileptic rats were celltype-specific: voltage-activated transient sodium current characteristics in DG granule neurons were not different from those in control rats (Chapter 2).

3. In pharmacoresistant epilepsy patients with or without hippocampal sclerosis, the anti-epileptic drug LTG induced a concentration-dependent shift of the steady-state inactivation function of the transient sodium current to more hyperpolarized potentials. The effect of LTG was not different between CA1 and neocortical neurons from patients with hippocampal sclerosis. We conclude that pharmacoresistance in epileptic patients (with hippocampal sclerosis) is not associated with a reduced modulation of the sodium current steady-state inactivation by LTG in CA1 pyramidal neurons (Chapter 3).

4. Three weeks after kainate-induced epileptogenesis, voltage-activated transient sodium current properties in acutely dissociated CA1 pyramidal neurons were not different from those in control rats (Chapter 5).

5. Three weeks after kainate-induced epileptogenesis, voltage-activated HVA calcium currents in acutely dissociated CA1 pyramidal neurons inactivated significantly slower when compared with those recorded from control rats. However, the increase in calcium influx that could result, did not reach significance at this point in time (Chapter 5).

6. The sodium-calcium exchange current can be recorded from acutely dissociated rat (and human) CA1 pyramidal neurons using the whole-cell voltage-clamp technique. This current exhibits an essentially linear voltage dependence and is dependent on the sodium and the calcium gradient over the membrane (Chapter 4).
7. Three weeks after kainate-induced epileptogenesis, somatic NCX current properties in acutely dissociated rat CA1 pyramidal neurons were not different from those in control rats (Chapter 5).

8. The expression of calcium extrusion proteins in the hippocampal formation is regionally-specific altered in the course of epileptogenesis: (1) NCX3 expression was extensively and permanently downregulated in mossy fiber terminals from epileptic rats, (2) NCX1 and NCX3 expression in the inner molecular layer of the DG are permanently downregulated, (3) PMCA and NC(K)X2 expression are (transiently) upregulated in the (inner) molecular layer, and (4) NCX2 and NCKX2 expression are (transiently) upregulated in astrocytes of epileptic rats throughout the hippocampal formation. These changes most likely reflect an altered calcium homeostasis in the diseased state. Some alterations in calcium extrusion protein expression are already present 3 weeks after kainate-induced SE and are clearly involved in the early stages of epileptogenesis (Chapter 6).
Chapter 7

REGULATION OF CELLULAR EXCITABILITY

Neurons have the ability to process and transmit incoming stimuli in many different ways to make sense of the wide range of information that enters our brain. Neurons can even adapt their properties to facilitate neuronal signalling. Such plasticity can also be of disadvantage: it may for example lead to epilepsy, a chronic condition of enhanced excitability that regularly triggers seizures in which large populations of neurons fire in a highly synchronized way.

In this thesis I have investigated several aspects of cellular excitability in the soma of isolated hippocampal neurons in relation with epilepsy. In addition, I have investigated alterations in the expression levels of calcium extrusion mechanisms within the hippocampal formation.

Cellular excitability is primarily determined by intrinsic membrane conductances in the plasma membrane (Na\(^+\), K\(^+\), Ca\(^{2+}\) and Cl\(^-\) currents gated by voltage-activated ion channels). These membrane conductances do not operate independently of each other: together they determine the membrane potential, while at the same time they are directly dependent on this membrane potential. A major part of our research was focused on the two main excitatory ionic currents in hippocampal neurons: voltage-dependent sodium and calcium currents. Both currents are important for cellular excitability and neuronal stability. Figure 7.1 shows a schematic overview of how these two excitatory ionic currents are involved in the regulation of cellular excitability. Other ionic currents such as potassium currents and chloride currents also contribute to stability of neuronal networks, but were not included in our study.

The sodium current is primarily responsible for the upstroke of the action potential (Hodgkin and Huxley, 1952), while the calcium current also contributes, by depolarization, to changes in excitability such as synaptic efficacy, firing threshold and firing frequency (Hille, 1992). Altered sodium or calcium current characteristics may have important consequences for neuronal excitability and are important in CNS diseases associated with altered excitability, such as chronic epilepsy. In addition to the direct effects of the calcium current on cellular excitability by depolarization, calcium currents result in an increase in the intracellular free calcium concentration ([Ca\(^{2+}\)]). The [Ca\(^{2+}\)] is an important factor in the regulation of excitability. It influences excitability directly, by activating calcium-dependent potassium channels, by mediating calcium-dependent inactivation of voltage-activated calcium channels or by inducing neurotransmitter release, and indirectly, by acting as a second messenger in intracellular processes such as protein phosphorylation, gene transcription and plasticity.

Increases in [Ca\(^{2+}\)], represent an important intracellular signal. The calcium gradient over the plasma membrane is very large ([Ca\(^{2+}\)] \(\sim\) 100 nM) \(<<<\) [Ca\(^{2+}\)] \(\sim\) 2 mM). As a result, even a small calcium influx will result in a large signal. It is essential that this signal be regulated both temporally and spatially. Calcium homeostasis is accomplished
by interplay of calcium influx mechanisms such as voltage-activated and receptor-operated calcium channels, calcium buffering by calcium-binding proteins (and calcium sequestration in intracellular organelles) and calcium extrusion systems. Together, these factors determine the calcium balance in a neuron (Blaustein and Lederer, 1999).

Calcium buffering by calcium-binding proteins can shape the calcium signal but, to maintain a steady-state calcium concentration, in the end all calcium that has entered the cell during activity has to be extruded. Therefore, calcium extrusion over the plasma membrane is of crucial importance. Calcium ions are extruded from the cytoplasm via the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) or the sodium-calcium exchange (NCX). During epileptic seizures, [Ca\(^{2+}\)]\(_i\) may rise to high levels as a result of continuous activation of voltage-activated calcium channels and persistent glutamate activation of NMDA-receptor operated channels. [Na\(^+\)] levels will also rise, but the fractional change due to a similar current is many orders of magnitude smaller and the sodium gradient is rapidly restored by the sodium-potassium pump. Due to its high affinity for intracellular Ca\(^{2+}\), the PMCA will already saturate at moderate calcium concentrations. In contrast, the NCX has a low-affinity for intracellular Ca\(^{2+}\) and will therefore become the dominant regulator for calcium extrusion during and after seizures. Because of its large transport capacity, it is able to quickly extrude many
calcium ions from the cytoplasm. In epileptic patients and in animal models for TLE, persistent alterations in calcium currents have been reported to occur in hippocampal neurons. If other circumstances stay the same, such changes should result in persistent changes in calcium influx. As stated above, to maintain calcium balance on the long-term, changes in calcium influx in turn have to be accompanied by changes in calcium extrusion.

Which parameters are the key determinants in the regulation of cellular excitability and how are they related to each other? The answer to this comprehensive question is extremely complex. Still, understanding the (hierarchy of the) mechanisms that determine excitability in single neurons is an important step in understanding how information is processed in the brain. In addition, such new insights may lead to improved (pharmacological) treatment of conditions that are associated with altered excitability, such as epilepsy. When combined with insights from literature, the findings in this thesis allow some speculation on this interesting topic. Regulation of excitability in the brain can be pictured as a hierarchical pyramid with distinct, strongly interacting levels ranging from neuronal network properties to specific ionic current characteristics (Figure 7.2).

The impact of alterations of excitability on brain functioning depends on the level at which they occur. Changing a single parameter of an ionic current (e.g. amplitude, kinetics or voltage dependence) in a specific cell type will have profound effects on the excitability of the whole network (bottom-up). Changing network properties (e.g. connectivity) will affect network excitability, but does not necessarily result in altered cellular properties of the neurons integrated in this network (top-down). In a rather exceptional case, alterations in network properties may even cancel altered cellular properties, or vice versa, resulting in unaltered excitability.

![Figure 7.2. Levels of regulation of excitability in the brain](image)

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From a clinical point of view, it may seem most beneficial to change network properties, since this has the highest probability of successfully suppressing seizure generation. On the other hand, as changing network properties affects the whole system, the effect of this approach may be too powerful, resulting in many adverse effects. Changing excitability at a lower level of the regulation pyramid will probably result in more subtle effects. For example, by changing one single ionic current parameter of the voltage-activated sodium current, high-frequency firing may be effectively suppressed, while normal action potential firing remains undisturbed. Many anti-epileptic drugs (AEDs), such as carbamazepine, phenytoin, valproate and lamotrigine, are known to be effective due to such a selective mechanism: they delay the recovery from inactivation of the voltage-activated sodium current, while normal action potential generation is unaffected.

From the fact that many epileptic patients develop pharmacoresistance during the course of their epileptic condition, it is clear that the complex of interacting mechanisms that regulate excitability is subject to continuous alterations, which reflects the upward and downward interactions between the elements in the pyramid. Therefore it is extremely important to gain more insight into the hierarchy of the mechanisms that regulate network and cellular excitability. In this way, it may even become possible to determine the optimal level of regulation, which may result in new leads for the development of more effective AEDs. Influencing the critical parameter (or parameter set) in the regulation system should then result in an optimal effect on cellular and, finally, network excitability, while adverse effects may be minimized. An even more optimistic scenario would be the possibility of intervening epileptogenesis, thereby preventing the chronic epileptic state. For that reason, understanding how higher levels in the pyramid cope with alterations (mistakes) at a lower level is of prime importance. The network is able to counterbalance the malfunctioning of a small number of neurons, in this way guaranteeing neuronal stability at all levels. Likewise, network alterations may trigger cellular changes (activity-dependent plasticity), which in turn stabilize excitability at all levels. However, at some point this compensation will become insufficient and epileptogenesis may start.

Given the complexity of this matter, it is clear that this ultimate goal cannot be achieved in one thesis. By studying some cellular aspects of excitability, I hope to contribute (a little) to solving this challenging quest.

The research described in this thesis addressed the following questions:

- Are voltage-activated sodium and calcium current characteristics from hippocampal neurons altered in chronic epilepsy? (Chapter 2 & 5)
- Is the expression of the calcium extrusion proteins in the hippocampal formation altered in chronic epilepsy? (Chapter 6)
• Is NCX functioning in CA1 pyramidal neurons altered in chronic epileptic rats? (Chapter 4 & 5)

• Is the sensitivity of the voltage-activated sodium current to the anti-epileptic drug lamotrigine altered in hippocampal neurons from pharmacoresistant epileptic patients? (Chapter 3)

The first three topics will be discussed in more detail below.

EXPERIMENTAL FINDINGS

Voltage-activated sodium currents in epilepsy

In CA1 pyramidal neurons from chronic epileptic rats (post-SE rats, three months after an electrically-induced SE) we have found alterations in the voltage-activated transient sodium current characteristics (Chapter 2): when compared with control rats, the sodium current activated at relatively more negative membrane potentials ($\Delta V_{h,a} = 6.5$ mV). This will result in a lower threshold for action potential generation. In addition, the window current (a persistent sodium current that only exists at membrane potentials where a sodium current can be activated and the current does not completely inactivate) was significantly larger and shifted closer to the resting membrane potential in epileptic rats. The resulting sustained influx of sodium ions at relatively more hyperpolarized membrane potentials will contribute to depolarization (several mV) of the cell, resulting in a reduced threshold for action potential firing. The altered sodium current characteristics that we reported would therefore contribute to increased cellular excitability in these chronic epileptic rats.

What could be the mechanisms underlying these altered sodium current characteristics in chronic epileptic rats? As discussed in Chapter 2, changes in modulation of the sodium channels (e.g. phosphorylation) are a possibility, but they may have a profound effect on other proteins (e.g. other ion channels!) as well. Alterations in the expression level of the various sodium channel subtypes/alpha subunits are another possibility to account for the long-term effects in chronic epilepsy. As described in the introduction of this thesis, several authors already have reported alterations of the expression of sodium channel subtypes or subunits in human and experimental epilepsy (Aronica et al., 2001; Bartolomei et al., 1997; Ellerkmann et al., 2003; Gastaldi et al., 1997; Gastaldi et al., 1998; Lombardo et al., 1996; Whitaker et al., 2001).

The observed increase of the window current in electrically-induced post-SE rats together with the shift of the activation curve of the sodium current to more negative membrane potentials are very intriguing in light of the fact that these post-SE rats exhibited spontaneous, recurrent seizures. In the kindling model for epileptogenesis, no alteration of the voltage dependence of activation of the transient sodium current was found (Vreugdenhil, M. et al., 1998a). In contrast to post-SE rats, kindled rats
generally do not exhibit spontaneous epileptic seizures. Could an increase of the window current and/or a shift of the activation function be sufficient for generation of spontaneous seizures? Computer simulations using the “Neuron” simulation environment of Hines and Carnevale (Hines and Carnevale, 1997) indeed showed that an increased window current can result in spontaneous epileptic seizures (Kager et al., 2004). To be more precise, these simulations showed that any net persistent inward current component is sufficient for seizure generation. There are several redundant pathways to accomplish this: NMDA-receptor mediated currents, the persistent sodium current ($I_{Na}$) and as described before, the window current resulting from the overlap between the activation and steady-state inactivation curve of the voltage-activated transient sodium current.

A persistent inward current component resulting in seizure generation may be a universal mechanism for several neuronal cell types that are involved in epilepsy: both in subiculum neurons from epileptic patients (Vreugdenhil, M., Wadman, W.J., 2004) and in neurons from layer V of the entorhinal cortex from pilocarpine-induced epileptic rats, an increase of the persistent sodium current has been reported (Agrawal et al., 2003). It is interesting to note that some AEDs effectively suppress $I_{Na}$ in cortical neurons (Chao and Alzheimer, 1995; Taverna et al., 1998). This may in part be responsible for the anticonvulsive effects of these drugs, although this still has to be determined.

Is either the increased window current or the shift of the activation curve enough, or are both necessary to sufficiently increase excitability up to a level where spontaneous seizures may occur? In Chapter 2, we have already discussed that 20% of the increase of the window current in the electrically-induced post-S E rats could be attributed to the small shift of the steady-state inactivation curve to more depolarized potentials. Since the window current is the direct result of the overlap between the steady-state inactivation curve and the activation curve of the transient sodium current, this implies that 80% of the increase of the window current was directly caused by the shift of the activation curve to more hyperpolarized potentials. As the major part of the increase of the window current would not exist without the shift of the activation curve, for this situation it is impossible to evaluate the effect of each alteration alone.

However, both scenarios are at least theoretically possible. For instance, the window current may be increased, while the activation curve is not shifted. This could happen as a result of a (significantly large) shift of the steady-state inactivation function to more depolarized levels. Such a shift has been reported in kindled rats, however, it is unknown whether it was sufficiently large to significantly increase the window current in these rats (Vreugdenhil, M. et al., 1998a). When primarily caused by a shift of the inactivation function to more positive potentials, the (maximal) window current will occur at relatively more depolarized potentials. Its precise contribution to membrane depolarization critically depends on the voltage dependence of the window current. Alternatively, when the shifts of the activation and the inactivation function are equally
large and occur in the same direction, the window current amplitude remains unaltered (the minor change due to the altered driving force is negligible). Still, the voltage range where the window current occurs is shifted over the voltage axis. If the voltage range is shifted closer to the resting membrane potential, the unaltered window current amplitude will become more effective. Obviously, the above illustrates that the relevance of the window current for membrane potential is not only dependent on its amplitude but also on the voltage range where it occurs.

The firing threshold of a neuron is determined by many factors. As a first approach, simulations to determine the effect of the window current (and all other inward current components) on membrane potential (and the input resistance of the cell) or experiments to determine the firing threshold of the neurons may give a clue about the precise effect of the window current. When assuming that both situations described above result in the generation of spontaneous seizures, finding out if the seizure threshold is different between these situations would result in a better understanding of the impact on cellular stability of a shift of the activation function or an increase of the window current.

Calcium extrusion in epilepsy

Apart from alterations of voltage-activated sodium current characteristics in hippocampal neurons, the calcium balance in these neurons is also altered in chronic epilepsy. Many alterations of voltage-activated calcium current characteristics and calcium buffering have been reported in CA1 pyramidal neurons and DG granule cells from epileptic patients and in animal models of TLE (see Introduction and Chapter 5, Baimbridge et al., 1985; Beck et al., 1998; Borgdorff, 2002; Faas et al., 1996; Gorter et al., 2002; Kohr et al., 1991; Kohr and Mody, 1991; Magloczky et al., 1997; Mody et al., 1990; Nagerl et al., 2000; Su et al., 2002; Vreugdenhil, M. and Wadman, 1992; Vreugdenhil, M. and Wadman, 1994; Yang, Q. et al., 1997). In contrast, the role of calcium extrusion mechanisms in epilepsy up till now has been largely overlooked (Borgdorff, 2002; Garcia et al., 1997; Keele et al., 2000). In this thesis, I therefore decided to study NCX functioning in hippocampal neurons in relation with epilepsy. Apart from the idea that the NCX is probably the most important calcium extrusion mechanism for the recovery from calcium loads after epileptic seizures, another intriguing factor is that this exchanger couples the transport of calcium ions over the plasma membrane to that of sodium ions, both ions that play an important role in epilepsy.

Several authors have suggested that the calcium influx via voltage-activated calcium currents is increased in hippocampal neurons from epileptic rats. As discussed before, the increased calcium influx in epileptic rats has to be accompanied by an increase of calcium extrusion. Increased calcium extrusion can be accomplished by enhancement of the efficacy of single calcium extrusion molecules (PMCA and NCX) or by altered expression levels of these transport proteins. Modulation of the calcium extrusion
mechanisms may suffice for the recovery from short-lasting calcium increases (e.g., seizures). Increase of calcium extrusion by modulation of the NCX and/or the PMCA will almost certainly also affect the dynamics of the calcium signals and in this way interfere with many cellular functions. To our knowledge it is not yet known how baseline [Ca^{2+}]_i is determined. The calcium extrusion mechanisms described above do not have a set point: they are proportionally driven by the intracellular concentration or the ionic gradient over the plasma membrane. When calcium influx is persistently increased, it enhances [Ca^{2+}]_i, which leads to a new and higher steady-state equilibrium for [Ca^{2+}]_i, where influx and efflux are in balance again. Enhanced expression of the calcium extrusion mechanisms (NCX and/or the PMCA) in these cells in response to a persistent increase of the calcium influx in epilepsy, via the so-called excitation-transcription coupling (Atar et al., 1995; Gomez et al., 2002), is therefore the more likely option if calcium dynamics has to be preserved as much as possible. In Chapter 6 of this thesis we have shown that altered expression of calcium extrusion indeed is the case in hippocampal neurons from epileptic rats, albeit in a very specific way.

For this thesis, I have investigated both the functioning of NCX and the expression of NCX, NCKX (a potassium-dependent sodium-calcium exchanger) and PMCA in epileptic rats. It was hard to characterize NCX functioning using electrophysiological means (Chapter 4). More specifically, practical limitations restricted the range in which [Ca^{2+}]_i could be varied to values up to 360 nM, where NCX activation (K_D = 0.6–2 μM (Juhaszova et al., 2000)) is not maximal. Most of the dendritic tree and the axon are lost during the dissociation procedure, so we cannot determine specific alterations in NCX (activity) that took place in these regions after the induction of SE. There is, however, no a priori reason to believe that NCX properties change with location in the cell. Investigating NCX functioning in the soma is also relevant, because this is an important site where entering calcium ions will activate secondary processes.

Since we were unable to determine NCX activity in the CA1 pyramidal neurons at high [Ca^{2+}]_i (micromolar range), we cannot determine all relevant parameters of NCX functioning in neurons from epileptic rats. We showed that at [Ca^{2+}]_i levels around 300 nM, NCX activity in CA1 pyramidal neurons was not different between control rats and epileptic rats, three weeks after kainate-induced SE. Using immunocytochemistry we showed that the expression of the calcium extrusion proteins was not significantly altered in the somata of the CA1 pyramidal neurons at this specific time point within the chronic epileptic phase (Chapter 6). This confirmed our preliminary electrophysiological data (Chapter 4).

To investigate whether the expression of the calcium extrusion proteins is altered in the course of epileptogenesis, we used immunocytochemistry to compare the hippocampal-parahippocampal protein expression of NCX1, NCX2, and NCX3, PMCA1–4, and NCKX2 at an early and late stage after kainate injection, with that in control rats. These NC(K)X and PMCA subtypes are expressed in a highly regionally-
specific and cell-type specific manner in the hippocampal formation (Chapter 6 and Burette et al., 2003; Lytton et al., 2002; Papa et al., 2003). Regional and cellular differences in local dynamics of calcium transport, differential modulation, colocalization of the calcium extrusion proteins with specific types of calcium channels, intracellular stores or calcium-binding proteins and differences in surface-to-volume ratios between the cell types probably may underlie this differential expression (Baba et al., 2002; Burette et al., 2003; Gomez et al., 2002; Linck et al., 1998). The functional difference between the PMCA and the NCX is determined by their different affinity for intracellular Ca\(^{2+}\) and different transport capacity. However, since the overall functional properties of the three NCX subtypes are not fundamentally different, the individual physiological role for each NCX subtype is currently unclear (Blaustein and Lederer, 1999; Linck et al., 1998).

We have found several alterations of calcium extrusion protein expression in chronic epileptic rats, 3 weeks and/or 2.5 months after kainate-induced SE: (i) NCX1 was permanently downregulated in the inner molecular layer (iml) of the dentate gyrus (DG) and entorhinal cortex layer III (ECIII), related to neuronal loss in hilus and ECIII, respectively; (ii) PMCA and NCKX2 expression was transiently upregulated in the iml, and downregulated in several areas where cell loss had occurred, (iii) NCX3 expression, which in control rats is abundant in presynaptic terminals of mossy fibers (mf), was extensively and permanently decreased in stratum lucidum and hilar region. In addition, newly formed mf sprouts that project to the DG iml did not noticeably express NCX3; (iv) NCX2 and NCKX2 were (transiently) upregulated in astrocytes of epileptic rats throughout the hippocampal formation, including ECIII. These regionalspecific changes of calcium extrusion proteins in epileptic rats may contribute to altered calcium homeostasis in the diseased state.

The (postsynaptic) upregulation of the PMCA in the dendrites of the DG granule cells seems to compensate for the loss of NCX1 and NCX3 expression at the inner molecular layer where synaptic reorganization is progressively increasing during epileptogenesis. While the NCX has a low affinity for intracellular Ca\(^{2+}\) and a high transport capacity, the PMCA has a much higher affinity and a low transport capacity (Blaustein and Lederer, 1999). The physiological relevance of PMCA overexpression may therefore be limited: following a burst of activity, the resulting substantial elevation of [Ca\(^{2+}\)], will quickly saturate the high-affinity PMCA, while its capacity to rapidly extrude Ca\(^{2+}\), is limited. Therefore, such compensation of reduced postsynaptic NCX expression by presynaptic PMCA upregulation will result in different calcium dynamics.

In dissociated DG granule cells from chronic epileptic rats, three months after electrically-induced SE, Gorter et al. have reported a persistent increase of the calcium influx mediated by high-voltage activated calcium channels (Gorter et al., 2002). Since calcium homeostasis can only be maintained if calcium influx is balanced by calcium
extrusion, one may expect to find an overexpression of the calcium extrusion proteins in these neurons. As described above, the expression of the calcium extrusion proteins was investigated in chronic epileptic rats, 3 weeks and 2.5 months after kainate-induced SE. To our surprise, we did not observe any alterations in the expression levels of the calcium extrusion proteins in the somata of the DG granule cells from the kainate rats (neither at 3 weeks nor at 2.5 months after SE). In addition, several authors have suggested an increased calcium influx in CA1 pyramidal neurons from kindled rats and pilocarpine-treated epileptic rats (Faas et al., 1996; Su et al., 2002; Vreugdenhil, M. and Wadman, 1992), but see (Gorter et al., 2002; Karst et al., 1999). In CA1 cells, we found a small or no decrease of the expression level of the calcium extrusion proteins of the kainate-treated rats.

If the increased calcium influx in epilepsy is not compensated by an increased expression of calcium extrusion proteins, then how does the cell recover from increased calcium loads? Given the fact that the increased calcium influx has to be balanced by an increase of calcium extrusion, the unchanged or even reduced expression of the calcium extrusion proteins in these epileptic animals remains to be understood. Several hypotheses can be proposed:

1) It just takes longer to recover from increased [Ca$^{2+}$], levels. However, calcium extrusion should be sufficiently fast to restore the basal [Ca$^{2+}$], level before the next calcium influx occurs.

2) Another explanation for the discrepancy is that the increased calcium influx via voltage-dependent calcium channels is balanced by an upregulation of subtypes or splice variants of the calcium extrusion proteins that were not recognized by our antibodies or even calcium extrusion mechanisms that are presently unknown. Similar reasoning is applicable to the total calcium influx.

3) The influx-efflux mismatch could then represent an essential part of epileptic pathology. However, a brief increase of [Ca$^{2+}$], may also decrease cellular excitability: an increased [Ca$^{2+}$], will strengthen the after-hyperpolarization by activation of calcium-dependent potassium channels, and furthermore, additional calcium influx mediated by voltage-activated calcium channels is inhibited by calcium-dependent inactivation of these channels. However, while the latter processes take place during and just after repetitive firing, the calcium ions still have to be extruded from the cell afterwards.

4) The amount of intracellular calcium buffering proteins may be upregulated in the epileptic rats. The increased buffering capacity may prevent the cell from toxic calcium overloads, thereby extending the time interval in which the cell can extrude calcium. Borgdorff et al. have found evidence in support of this hypothesis: in DG granule cells from epileptic rats, 2 days after SE, an increase of the calcium buffering capacity was found together with an increased calcium extrusion rate. Calcium dynamics were not altered in these rats (Borgdorff, 2002). However, it should be mentioned that in epileptic rats
three months after SE, they reported that the amount of calcium buffering proteins had returned to control levels. Instead, the affinity of the buffers was altered, resulting in altered calcium dynamics (Borgdorff, 2002).

(5) Perhaps the most straightforward explanation is that the transport capacity of the calcium extrusion proteins is large enough to quickly cope with an increased calcium influx. This is in particular the case for the NCX, which has a low-affinity for $[\text{Ca}^{2+}]$, and a high-transport capacity (see Introduction) that, under resting conditions, is not fully utilized.

Still, the question remains why we observed a decrease of NCX expression in the (mossy fiber terminals of the) dentate granule cells.

**Consequences at the cellular level**

Alterations of calcium extrusion protein expression most likely are the consequence of altered calcium influx and/or calcium buffering. Compensation of altered calcium influx by changes in calcium extrusion is crucial for cellular excitability or neuronal plasticity, depending on the compartment of the neuron where these changes have taken place. Due to the much higher surface-to-volume ratio in the dendrites when compared with the soma, a given calcium influx will result in a much higher $[\text{Ca}^{2+}]$, and faster calcium dynamics in the dendrites. Calcium dynamics in *dendrites* is critical for processes such as long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission in the hippocampus, processes that are considered to be a substrate of learning and memory (Bliss and Collingridge, 1993; Bliss and Lomo, 1973). The induction of LTP and LTD both strongly depend on $[\text{Ca}^{2+}]$ in the dendrites of the postsynaptic neuron. The magnitude of the postsynaptic rise in $[\text{Ca}^{2+}]$, has been found to be a key factor in the determination of the polarity (LTP or LTD) of synaptic gain change, with the induction of LTP requiring a more pronounced increase of $[\text{Ca}^{2+}]$, than the induction of LTD (Brocher et al., 1992; Hansel et al., 1996). In the *soma*, compensation of changes in calcium influx by changes in calcium extrusion is important for calcium-dependent inactivation of the voltage-activated calcium current and the activation of calcium-dependent potassium currents.

**Consequences beyond the cellular level**

The most conspicuous alterations in the expression of the calcium extrusion proteins in epileptic rats were found in the dentate gyrus (3 weeks and 2–3 months after SE). The precise effect on hippocampal network excitability, however, is not immediately clear. The low expression of the calcium extrusion systems in the newly formed sprouted mossy fiber terminals in the inner molecular layer of the epileptic animals could result in a relatively slow recovery from calcium load. This in turn may lead to enhanced neurotransmitter release from these terminals especially at high frequencies (Bouron and Reuter, 1996). By enhancing recurrent excitation of the DG granule cells,
this could contribute to chronic epilepsy. Partly, however, the effect could be counteracted at the postsynaptic site by the upregulation of the PMCA in the dendrites of the DG granule cells.

The decreased NCX3 expression in stratum lucidum, where the mossy fiber terminals form synapses with the CA3 pyramidal neurons, may result in a slower clearance of elevated $[\text{Ca}^{2+}]$, from the terminal and could well affect the properties of neurotransmitter release from the mossy fiber terminals (Bouron and Reuter, 1996). The mossy fiber-CA3 synapse is mainly glutamatergic (Crawford and Connor, 1973; Henze et al., 2000), but several groups have reported a transient emergence of GABAergic neurotransmission after seizures (Bergersen et al., 2003; Gutierrez, 2000; Gutierrez and Heinemann, 2001; Romo-Parra et al., 2003; Schwarzer and Sperk, 1995; Walker et al., 2001). The net effect of the reduced NCX3 expression in the mossy fiber terminals on excitability of the CA3 pyramidal neurons in epileptic rats therefore needs to be determined.

**Time path of changes**

It is not easy to find out whether the differences in current characteristics and exchanger expression are cause or consequence of epileptic activity. The order in which alterations appear during epileptogenesis could provide an answer to this question. There is also an important clinical relevance to the question, because treatment so far is mainly aimed at seizure suppression and hardly ever with preventing epileptogenesis.

Chronic epilepsy in patients develops gradually after the first insult and the latent phase may last for years before the first spontaneous seizure occurs. The electrically-induced post-SE model and the kainate model both reproduce important features of human TLE: hippocampal sclerosis (specific cell loss and gliosis), mossy fiber sprouting and the recurrence of spontaneous seizures. In contrast to epileptic patients, rats develop a chronic epileptic state in only 2–3 weeks after SE (Babb et al., 1991; de Lanerolle et al., 1989; Gorter et al., 2001; Hellier et al., 1998; Hellier et al., 1999; Lothman et al., 1990; Mathern et al., 1997; Sloviter, 1994; Sutula et al., 1989). It is unclear what underlies this difference in time course between human and rats.

We have investigated alterations in sodium and calcium current characteristics in epileptic rats three weeks after kainate-induced SE. While these rats already exhibited a few spontaneous seizures at this early point in time, they are not yet in the ultimate chronic epileptic state, in which seizure frequency is much higher (Gorter et al., 2001). In the three-weeks KA rats, we did not find significant differences in sodium current properties when compared with control rats: the voltage dependence of activation was not shifted and the sodium window current was small and not different from control values. In contrast, these two aspects of the voltage-activated sodium current were clearly changed in epileptic rats three months after electrically-induced SE (Ketelaars et al., 2001).
We found that high-voltage activated calcium currents inactivated significantly slower in CA1 pyramidal neurons from the three-weeks KA rats; however, the resulting increase of the calcium influx did not reach significance. The fact that kainate rats already exhibited spontaneous seizures three weeks after SE, without obvious changes in sodium or calcium currents, suggests that other mechanisms also contribute to the generation of spontaneous seizures. An increase of NMDA-receptor operated currents (Kohr et al., 1993) or an increase of low-voltage activated calcium currents in the dendrites (that are lost in dissociated cells) (Beck et al., 1998; Faas et al., 1996; Su et al., 2002), are only a few of the many possibilities that need further investigation.

Apparently, the alterations of the sodium and calcium current characteristics develop gradually during the chronic epileptic phase and have not yet taken place when the first seizures appear (but see Gorter et al., 2002). Spontaneous seizures could, however, represent an important factor in the process that follows. Once alterations in the sodium or calcium currents appear and enhance excitability, this may result in a positive-feedback mechanism: the threshold for seizure generation is lowered, more seizures occur and this in turn may stimulate further alterations of current characteristics, and so on. It would be interesting to identify what factors in the end determine the extent of the alterations in the currents.

Three weeks after KA-induced SE, changes in calcium current characteristics had already appeared, while at this same time point no alterations of the voltage-activated sodium current were detected. More research is necessary to determine the relative importance of changes in these two currents for the generation of epileptic seizures.

CONCLUDING REMARKS

We now return to the central question: which parameters are the key determinants in the regulation of cellular excitability and how are they related to each other?

Changing a single ionic current parameter may already have profound effects on cellular excitability. This holds for both voltage-activated sodium and calcium currents. It is hard to determine which of these two currents is the most important for excitability. Still, we can speculate about what property of these excitatory ionic currents is the most important in the regulation of cellular excitability. A complicating factor that we have to keep in mind is that the parameters characterizing the ionic currents are not (completely) independent of each other: an alteration of one current property may (indirectly) result in an alteration of another characteristic.

It is clear that current amplitude, or current-density to be more precise, is an important parameter in the regulation of excitability. When the amplitude of the sodium current is not of sufficiently large amplitude, no action potential will be generated. The amplitude of the calcium current directly influences $[\text{Ca}^{2+}]$, which in turn regulates many intracellular processes that are activated at different $[\text{Ca}^{2+}]$ levels. Changing excitability of the regulation system at the level of the amplitude of the currents will therefore have enormous consequences.
More subtle changes in excitability may be attained by alterations of the voltage dependence or the kinetics of the currents. We have to note that changes in voltage dependence also affect the amplitude of the current at a given membrane potential. As discussed before, a shift of the voltage dependence of the sodium current may have a large impact on cellular excitability, depending on the extent and the direction of the shift and the voltage range where it occurs. This holds for both voltage-activated sodium and calcium currents. The sodium current has a much higher maximal conductance and steeper voltage dependence than the calcium current. Therefore, a given shift of the voltage dependence of the sodium current will have a much larger effect on current amplitude than an equal shift of the calcium current would have. As a result, altered voltage dependence of the sodium current will have the largest effect on the firing threshold of a neuron.

In addition, changes in current kinetics may affect action potential generation. Changes of the time course of the recovery from inactivation are particularly relevant for the fast sodium current and will have a strong effect on firing frequency. Slower inactivation kinetics of the voltage-activated calcium current may result (if current amplitude is not significantly decreased) in an increased influx of calcium, which in turn activates a variety of intracellular calcium-dependent processes. In general, altered sodium current kinetics electrogenically contribute to excitability, while altered calcium current kinetics primarily result in a change of $[Ca^{2+}]_i$.

As indicated before, current characteristics can be altered by changed modulation of the ion channels/transporters involved (e.g. by phosphorylation) or by altered expression of the ion transport mechanisms involved. $[Ca^{2+}]_i$ is an important regulator of both modulation (calcium-dependent phosphorylation) and gene transcription (via the so-called excitation-transcription coupling (Atar et al., 1995)). Although calcium extrusion mechanisms are not the primary determinants of cellular excitability in neurons, by regulating $[Ca^{2+}]_i$ levels, they do represent an important parameter in the regulation of excitability.

**Outlook**

Increased excitability in epilepsy is the result of chronic alterations in the brain. These alterations occur in specific regions, cell types or even cell compartments. While investigations on the cellular level are absolutely necessary to understand the meaning of alterations of a single aspect of excitability, the final effect of these alterations on excitability has to be determined on the network level. This should be done at several time points during epileptogenesis to get a better insight into the progression of these alterations.

The epileptic state can be developed in many different ways. Since epileptogenesis is a gradual process in which many factors play a role, there is no clear-cut point of transition from a normal brain showing normal plasticity into a chronic epileptic brain. It is therefore extremely hard to determine the critical set of parameters involved in
epileptogenesis. Identification of alterations that are relevant for cellular excitability in the epileptic state may facilitate the determination of which topics must be studied to gain a clear understanding of epileptogenesis and may in the future even lead to strategies for intervention.