Absence epilepsy-associated spike-wave discharges are initiated in the peri-oral somatosensory cortex and maintained by the rostral reticular thalamic nucleus. To elucidate mechanisms underlying initiation and maintenance of spike-wave discharges, a number of factors potentially determining neuronal signal input, signal recognition and signal transduction in thalamocortical brain centres have been studied using the WAG/Rij rat model for human absence epilepsy. The results lead to a model which predicts that spike-wave discharge activity is initiated in the peri-oral somatosensory cortex, as a result of a reduced GluR4-rich AMPA receptor-mediated glutamatergic stimulation of cortical GABAergic interneurones together with a prolonged Ca\(^{2+}\)-activated K\(^+\) channel activity caused by a loss of PV-mediated Ca\(^{2+}\)-buffering. The model furthermore indicates that the rostral reticular thalamic nucleus can maintain spike-wave discharge activity due to an increased postsynaptic GluR4-rich AMPA receptor-mediated excitation together with an intense glutamatergic neurotransmission that is mediated by presynaptic Cav2.1 channels.
Cellular and Molecular Determinants Underlying Spike-Wave Discharge Initiation and Maintenance in the Absence Epileptic WAG/Rij Rat

Maartje Carin van de Bovenkamp-Janssen
Cellular and Molecular Determinants Underlying Spike-Wave Discharge Initiation and Maintenance in the Absence Epileptic WAG/Rij Rat

Een wetenschappelijke proeve op het gebied van de Natuurwetenschappen, Wiskunde en Informatica

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CHAPTER 1

General introduction
Absence epilepsy

Epilepsy is a collective noun for chronic neurological disorders of which the main feature is the reoccurrence of epileptic seizures (Pedley et al. 1995). An epileptic seizure can be defined as an intermittent, stereotyped disturbance of consciousness, behaviour, emotion, motor function or sensation, and results from abnormal neuronal activity in the brain (Chadwick 1994). There are various forms of epilepsy, mainly characterised by a specific type of seizure but each associated with their own particular behavioural, etiological, anatomical, physiological and psychological characteristics (Commission 1981, 1989).

One major form of epilepsy is absence epilepsy or ‘petit mal’, which is a typical childhood disease that, with age, may disappear, or transform into a different type of epilepsy (Coenen et al. 1992; Porter 1993). Human absence epilepsy is characterised by a paroxysmal decrease in consciousness of abrupt onset and offset accompanied by generalised, bilaterally, synchronous, regular, stereotyped and symmetrical 3 Hz spike-wave discharges (SWD) in the cortical electroencephalogram (EEG), preceded and immediately followed by normal EEG activity (Porter 1993; Snead III 1995; Steriade and Contreras 1995). During an absen-ce epileptic attack the patient interrupts ongoing activities, stares, appears dazed and is hardly or not responsive. There is a blank facial expression with the eyes drifting upwards, and mild rhythmical myoclonic twitches occur, such as a rapid blinking of the eyelids, slight chewing of the jaw and small movements of the head. The attack rarely lasts longer than 15-20 sec, and after its cessation ongoing behaviour is normally resumed (Niedermeyer 1993; Porter 1993). SWD are the electroclinical hallmark of absence epilepsy, and therefore it is important to know how they are initiated and maintained in the brain. Investigating cellular and molecular determinants that underlie the initiation and maintenance of SWD forms the central aim of this thesis.

The thalamocortical basis of SWD activity

The neuronal basis of the initiation and maintenance of SWD has received considerable attention in animal (rat, mouse and cat) studies, but is still far from understood. SWD activity in the EEG reflects synchronous oscillations in neuronal discharge activity in various parts of the so-called thalamocortical network (e.g. Gloor et al. 1990; Steriade and Contreras 1995; Avanzini et al. 2000; McCormick and Contreras 2001; Meeren 2002; Meeren et al. 2002). This network consists of three major parts, mutually connected by elaborate fibre pathways: the cortex, the thalamic nuclei and the reticular thalamic nucleus (RTN) (Fig. 1). SWD activity can only be initiated if the thalamocortical network is anatomically and functionally intact. This does not mean that all components have the same role, because in particular the cortex and the RTN are key players in absence epilepsy. Below, these structures will be considered into some detail.
Role of the cortex in SWD

Gloor (1968, 1969) has proposed a prominent role for the cortex in SWD initiation. In his ‘cortico-reticular’ theory he suggested that abnormal, pathological thalamocortical activity, visible as SWD, is the result of a hyperexcitable cortex that overreacts to normal, thalamocortical oscillatory input. According to Seidenbecher et al. (1998) this process would particularly take place in the somatosensory cortex. This was recently confirmed by Meeren et al. (2002), who showed that SWD originate from that part of the somatosensory cortex where the nose and upper lip are represented, the so-called peri-oral region. From this cortical region, SWD rapidly spread over the entire cortex, establishing large-scaled synchronisation of SWD that are subsequently imposed upon all thalamic areas. Apparently, this massive synchronised electrical activity suppresses normal brain activities resulting in the various motor and sensory changes so characteristic for absence epilepsy.

Role of the RTN in SWD

About half a century ago, Penfield and Jasper (1947, 1954) suggested in their ‘centrencephalic’ theory that SWD have a thalamic origin, and subsequently spread bilaterally over
the cortex that, in turn, would act as a passive follower. More recently, especially the RTN became implied as a key structure in thalamocortical activities such as SWD (e.g. Steriade and Deschênes 1984; Steriade et al. 1985, 1987; Mulle et al. 1986; Steriade and Llinás 1988; Avanzini et al. 1992). Individual RTN neurones can be considered as ‘endogenous oscillators’, due to their intrinsic membrane currents (e.g. Avanzini et al. 1989; Bal and McCormick 1993; Contreras et al. 1993) and the connections between these ‘oscillator cells’ might provide the synchronisation of oscillatory activity of individual neurones, leading to SWD (Spreafico et al. 1991). In several rat models for absence epilepsy, the RTN appears to be essential for SWD activity (Buzsáki et al. 1990; Avanzini et al. 1992, 1993, 2000; Snead III 1995; Tsakiridou et al. 1995; Avoli et al. 2001) and recently, Meeren (2002) has indicated that especially the rostral pole of the RTN (rRTN) is crucial for SWD maintenance.

HYPOTHESIS UNDERLYING THE THESIS RESEARCH

The precise mechanisms underlying SWD initiation and maintenance by neurones in the cortex and the RTN, respectively, are unclear. Therefore, we have studied a number of factors essential for neuronal signal input, signal recognition and signal transduction that potentially are involved in these mechanisms, in both brain areas, with emphasis on the rRTN. The following aspects have been studied for the rRTN: (1) signal input: the general ultrastructure and synaptic organisation, (2) signal recognition: the expression of glutamatergic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and (3) signal transduction: (a) the expression of high voltage-operated (HVA) Ca\(^{2+}\) channels, (b) the expression of the Ca\(^{2+}\)-buffering protein parvalbumin (PV), and (c) the occurrence of intracellular, periodic changes in the intracellular Ca\(^{2+}\) concentration (the so-called Ca\(^{2+}\) oscillations). Signal recognition by NMDA and AMPA receptors and PV as a signal transduction protein were also investigated in the somatosensory cortex.

The investigations were performed using the WAG/Rij rat, a well-established animal model to study the neuronal mechanism of human absence epilepsy (Coenen et al. 1992; van Luijtelaar and Coenen 1997). In the next paragraphs, first this animal model will be considered and then an outline will be given of the research performed on each of the factors possibly involved in the initiation and maintenance of SWD in this rat strain.

THE WAG/RIJ RAT MODEL

There are various experimental animal models for human absence epilepsy. This type of epilepsy has a strong genetic predisposition, and many of the models share the major disadvantage that they do not possess this predisposition and, hence, SWD have to be experimentally induced. On the contrary, the WAG/Rij rat is a genetic model for absence epilep-
and is a valid tool for studying the pathogenesis of this disorder (for review see van Luijtelaar and Coenen 1997). In this animal, absence epilepsy has an age-dependent onset. The first signs of SWD activity appear in rats three months of age, and at the age of six months they show periods of massive SWD activity with up to 400 seizures per day (Coenen and van Luijtelaar 1987; Inoue et al. 1990; de Bruin et al. 2000). SWD are accompanied by typical absence epileptic behavioural phenomena such as head tilting, eye twitching, breath acceleration, muscle shocks and frequent movements of the vibrissae. Analysis of the cortical EEG revealed that SWD consist of trains of 8-10 Hz, large, sharp spikes and slow, small waves (Fig. 2). They may last from one to some tens of seconds (van Luijtelaar and Coenen 1986). SWD suddenly emerge from a normal background EEG and end abruptly. Electrophysiological studies showed that the SWD are bilaterally symmetrical and generalised over the entire cortex, but the hippocampal area is not involved (van Luijtelaar and Coenen 1986; Inoue et al. 1993; Kandel et al. 1996). In fact, Meeren (2002) and Meeren et al. (2002) indicated that the SWD originate from the peri-oral region of the somatosensory cortex and that the rRTN controls SWD maintenance.

In studying the possibility of a relation between absence epilepsy and 1) the ultrastructure and synaptic organisation (‘synaptology’) of the rRTN and 2) PV expression, we used 6 months old WAG/Rij rats and age-matched ACI control rats. ACI rats are considered as non-epileptic controls, since they show no or hardly any SWD activity at all ages (Inoue et al. 1990; de Bruin et al. 2000). In our investigations on HVA Ca\(^{2+}\) channels and the expression of glutamate receptors, we studied 3 months old, non-epileptic and 6 months old, absence epileptic WAG/Rij rats, and age-matched ACI rats. This experimental design provides insight in the neuronal properties correlated with the development of SWD (e.g. Inoue et al. 1990; van Rijn et al. 1991; Coenen et al. 1992; Lason et al. 1992, 1994; van Luijtelaar and Coenen 1997). Ca\(^{2+}\) oscillations were studied in young, postnatal day 8-20, WAG/Rij rats prone to develop SWD, and in age-matched ACI rats, to examine if the aberrant occurrence of spontaneous Ca\(^{2+}\) oscillations during postnatal brain development of WAG/Rij rats is related to the emergence at later age of SWD and absence epilepsy.

Figure 2:
Cortical electroencephalographic recording of spike-wave discharge (SWD) activity in the absence epileptic WAG/Rij rat.
Outline of the thesis

The Chapters 2 and 3 concern signal input to the rRTN, investigated at the ultrastructural level. Although the ultrastructure of the rat RTN has been studied before (e.g. Ohara and Lieberman 1985; Ohara 1988; Liu and Jones 1999), those studies do not concern the WAG/Rij rat nor do they focus on the rostral part of the RTN. In Chapter 2, the presence is demonstrated of three neurone populations within the RTN, characterised by their biosynthetically functional state, together with different types of glial cells. Moreover, various types of structurally and neurochemically different synapses have been distinguished. The organisation of synaptic inputs to and within the rRTN has been studied in detail in Chapter 3. Quantitative and semi-quantitative ultrastructural determinations of the respective synapse types and subtypes indicate that SWD maintenance probably does not depend on the synaptic organisation of the rRTN.

In Chapters 4, 5 and 6 it is described how quantitative immunocytochemistry reveals that a number of crucial proteins are differentially expressed in relation to absence epilepsy. Chapter 4 deals with signal recognition, describing excitatory, glutamatergic neurotransmission mediated by NMDA and AMPA receptors. The role of these glutamate receptors in absence epilepsy has been indicated before (e.g. Peeters et al. 1990, 1994; Pu-main et al. 1992; Koerner et al. 1996). Our data suggest that an increased AMPA receptor expression in the rRTN plays a role in SWD maintenance, while a decreased expression of NMDA and AMPA receptors in the peri-oral somatosensory cortex suggests a role in SWD initiation. Chapter 5 focuses on HVA Ca$^{2+}$ channels involved in signal transduction. Besides the generally accepted role of low voltage-activated Ca$^{2+}$ channels in SWD (e.g. Tsakiridou et al. 1995; McCormick and Bal 1997; Gomora et al. 2001; Kim et al. 2001) also HVA Ca$^{2+}$ channels are assumed to play a role. Disturbances in HVA Ca$^{2+}$ channels caused by gene mutations (Burgess and Noebels 1999) and changes in translation processes (Lakaya et al. 2002), protein compositions (McEnery et al. 1998), channel dynamics (Lorenzon et al. 1998; Wakamori et al. 1998) and pharmacological regulation (van Luijtelaar et al. 1995, 2000), correlate with absence epilepsy-associated SWD activity. We describe the expression of HVA Ca$^{2+}$ channel $\alpha_1$ subunits in the rRTN and demonstrate that the development of SWD in WAG/Rij rats is concomitant with an increased expression of Ca$\gamma_{2.1}$ channels in this nucleus. In Chapter 6 the distribution and expression of the signal transduction protein PV have been investigated in the RTN and in the cortex. Ca$^{2+}$-buffering proteins like PV play a key role in the regulation of the intracellular Ca$^{2+}$ concentration (Kawaguchi et al. 1987) and this concentration is crucial for neuronal electrical and secretory activity. Moreover, these proteins protect the neurone from excitotoxicity by Ca$^{2+}$ overload, which may occur as a result of opening of HVA Ca$^{2+}$ channels upon depolarisation or upon activation of glutamate receptors (e.g. Heizmann et al. 1990). Therefore, a change in the PV concentration could reflect a disturbed neuronal activity and explain the occurrence of SWD. The study presented suggests that PV plays a role in SWD generation indeed, as
in the somatosensory cortex the number of PV-containing neurones in absence epileptic WAG/Rij rats is considerably lower than in ACI control rats.

The last aspect of the transduction process of extracellular signals that might be involved in SWD, is presented in Chapter 7, and concerns spontaneously occurring Ca\(^{2+}\) oscillations in the rRTN of young (p8-20) WAG/Rij rats. The hypothesis has been investigated that differences in intracellular Ca\(^{2+}\) signalling by rRTN neurones in postnatal WAG/Rij rats underlie the predestination of these young rats to develop, at a later age, SWD activity, since these oscillations are associated with synaptogenesis (Mizutani et al. 1996; Tanaka et al. 1996; Garaschuk et al. 1998; Stosiek et al. 2003). It appears that such young animals have a remarkably lower number of spontaneously oscillating rRTN neurones than age-matched ACI rats.

Finally, in Chapter 8 the results obtained in the thesis research are placed into a broader perspective, and the possible mechanisms of initiation and maintenance of absence epilepsy-associated SWD are discussed.
CHAPTER 2

Ultrastructure of the reticular thalamic nucleus of the WAG/Rij rat

In press as:
ABSTRACT

The aim of this study was to describe the ultrastructure of the reticular thalamic nucleus (RTN) in rats of the WAG/Rij strain, which is an established model for human absence epilepsy. The RTN appears to have complex architectonics. Most RTN neurones are medium- to large-sized and have either a dark or light appearance depending on their functional state. Dark neurones are in a biosynthetically active state, contain electron-dense vesicles and may produce neuropeptides. Light neurones show less biosynthetic activity. Moreover, small-sized neurones with short axons are present. Glial cells are especially represented by cytoplasmatic astrocytes. The close topographical connection between astrocytes and RTN neurones suggests a functional relationship. Furthermore, oligodendrocytes and microglial cells occur. Pinocytosis and endocytosis phenomena in the blood-brain barrier correspond with the barrier’s permeability properties. In terms of topography and symmetry, various types of synapses were found, but no gap junctions were seen. On the other hand, some light neurones that occur apposed to each other, reveal membrane junctions that may be involved in intercellular communication.

INTRODUCTION

The reticular thalamic nucleus (RTN) is involved in the pathogenic mechanisms of generalised absence epilepsy. Previously, studies have been carried out on its cytoarchitecture, neuronal organisation and electron microscopic characteristics in mammals, including rat (e.g. Ohara and Lieberman 1985; Hallanger and Wainer 1988; Sprefisco et al. 1991; Lübke 1993) but not in the WAG/Rij rat strain, a well-established genetic model to study the mechanisms underlying absence epilepsy (e.g. van Luijtelaar and Coenen 1997; Meeren 2002; Meeren et al. 2002). In order to provide a basis for future investigations on structure-function relationships in the RTN with respect to absence epilepsy, we here present a general ultrastructural description of the RTN in this rat strain.

METHODS

Studies were performed with 14 rats of the WAG/Rij strain, both males and females, at the mature age of 6 months, reared in the vivarium of the Department of Human and Animal Morphology and Physiology at Bashkir State University in Ufa. The animals had free access to food and water, and 14 hrs of light daily. After nembutal anaesthesia (60 mg/kg) and decapitation, the RTN was quickly dissected under an MBS-10 dissection microscope (LOMO, St. Petersburg, Russia).
For electron microscopy, samples from the dorsal and ventral part of the RTN at the level of Bregma –1.60 mm and Interaural 7.40 mm (Paxinos and Watson 1997) were fixed by immersion in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), for 2 hrs at 20 °C, and postfixed in 2% OsO₄, for 1 hr at 4 °C. Ultrathin Epon 812 cross-sections were cut with an LKB-III ultramicrotome, contrasted with lead citrate (Reynolds 1963) and examined in a JEM 200 EX transmission electron microscope (Jeol, Tokyo, Japan).

**Results and discussion**

Below a survey is given of the ultrastructural features of the RTN of the WAG/Rij rat, which basically applies to all RTN parts. However, the dorsal part contains more myelinated axon fibers, which likely are afferents and efferents connecting the RTN with the cortex and the thalamus (Jones 1975), whereas the ventral part shows more axon terminals containing electron-dense vesicles, presumably storing bioamines and/or neuropeptides (Destexhe et al. 1994; Garlov 2002).

Most RTN neuronal somata have a spindle-like or round shape and are medium- to large-sized (diameter: ca. 15-30 µm). Some somata occur single, others are located in small groups of two or three neurones, with the somata separated from each other by a space of only a few nm (Fig. 1a). The ultrastructure of reticular neurones enables to assess their functional state, as two types of medium- to large-sized neurones can be distinguished: dark and light ones. Dark neurones have nuclei with abundant electron-dense, granular material consisting of interchromatin, perichromatin granules and perichromatin fibers (Fig. 1a,b), indicating a high level of mRNA biosynthesis. This is reflected by the appearance of the cytoplasm: the rough endoplasmic reticulum (RER) is extensive, many ribosomes and polysomes give the cytoplasm a dark appearance, and the Golgi complex is hypertrophied (Fig. 1c). The somata, dendrites and axons contain electron-dense vesicles (mean diameter ca. 70-200 nm) (Fig. 1c), which possibly store bioamines and/or neuropeptides (Destexhe et al. 1994; Garlov 2002). Some RTN neurones express preproenkephalin-mRNA (Hermanson et al. 1995), which is of special interest since enkephalins may be involved in absence epilepsy (Lason et al. 1990, 1992, 1994).

Light neurones share many morphological features with dark ones, but are characterised by a lack of clear morphological signs of transcriptional and translational activity, showing no extensive RER and a rather inconspicuous Golgi apparatus (Fig. 1d). Groups of light neurones reveal intercellular contacts like tight and septate-like junctions between their somata (Fig. 1d, inset). Perhaps such contacts represent intra-RTN communication sites, which are believed to act as ‘desynchronisers’, dampening synchronous, oscillatory activities within the thalamocortical system (Huntsman et al. 1999).

Besides dark and light neurones, we also found small, ovoid-shaped RTN neurones (diameter ca. 10 µm), which possess distinctive features. Their proximal dendrites are poor in cell organelles and the short axon is strongly curved and sometimes seems to be twis-
Figuer 1:
Reticular thalamic nucleus (RTN) of the WAG/Rij rat. [a] Closely apposed dark (D) neurones, with in [b] nucleus with different chromatin configurations (C), extensive endoplasmic reticulum (R) and numerous ribosomes and polysomes (P), and in [c] well-developed Golgi apparatus (G) and secretory granules (S). [d] Two closely apposed light (L) reticular neurones, connected by septate-like junction (arrow), which is shown in detail in inset. M: myelinated fibre. Bars: 2 µm (a), 0.5 µm (b, c, d) and 0.2 µm (d: inset).
ted around the soma. The axons may form axo-axonic and axo-dendritic synapses (cf. Leontovich 1978) that contain presynaptic, small (diameter ca. 50 nm) electron-lucent vesicles, presumably containing GABA (Houser et al. 1980; DeBiasi et al. 1986). These cells probably represent the small, spindle-like neurones described by Spreafico et al. (1991) that mainly occur in the caudal part of the RTN in a region that receives afferents from sensory cortical areas (Jones 1975; Battaglia et al. 1989). Interestingly, these cortical areas include the peri-oral somatosensory cortex, which is the starting point for absence epileptic SWD in the WAG/Rij rat (Meeren et al. 2002).

Considering intra-RTN communication, the question whether gap junctions are present in the RTN is of interest. Gap junctions form electrical synapses between adjacent neurones (Bennett 1977), and the main structural compound of RTN gap junctions is connexin-36 (Cx36) (Condorelli et al. 2000; Rash et al. 2000). In recent years these Cx36 junctions have received close attention since it was discovered that they are critical for the generation of widespread, synchronous inhibitory activity (Deans et al. 2001; Hormuzdi et al. 2001). Gap junctions may also be important during seizures as are taking place during epileptogenesis (Dudek et al. 1998; Landisman et al. 2002). A remarkable observation was made by Carole et al. (2002) who, based on electrophysiological recordings of thalamocortical slices, proposed that gap junctions between RTN neurones determine the synchronisation of delta rhythms, whereas chemical synapses would evoke the opposite effect, i.e., desynchronise high-frequency activities (sleep spindles). However, gap junctions are very small and notoriously difficult to be detected by routine transmission electron microscopic analysis. Possibly, that is why we were unable to observe them. However, at places where light neurones occur closely apposed to each other, septate-like junctions were seen, which, according to Nemecek et al. (1978) may be involved in ionic, intercellular interactions.

Glial cells are represented by astrocytes (Fig. 2a), oligodendrocytes (Fig. 2b) and microglial cells (Fig. 2c). Cytoplasmatic astrocytes are most numerous, especially near (GABAergic) reticular neurones, which underlines their participation in the process of GABA-shunting (Ashmarin and Stukachev 1996). The functional interaction between astrocytes and neurones also appears from phenomena suggesting endo- and exocytosis. Microglial cells closely resemble pericytes around blood vessels, suggesting that these cell types are involved in transport processes in the RTN.

We identified special features of the blood-brain barrier in the RTN. In some areas the capillary basement membrane is located very close to somata of light and dark neurones, and endothelial cells seem to take up material by endocytosis (Fig. 2d), suggesting that these reticular neurones actively interact with the circulatory system. Also, in such areas axon terminals containing small, electron-lucent vesicles are present, which corroborates the well-established fact that axon terminals are involved in the regulation of the regional blood flow (Vinogradova 2000). Interestingly, the blood-brain permeability seems to determine seizure susceptibility (Ates et al. 1999).

Synapses in the RTN are known for their structural diversity. In terms of their ultrastructure, they may be perforated (Fig. 3a) or unperforated, and symmetrical or asymmetrical (Fig. 3c). All these forms were encountered throughout the RTN of the WAG/Rij rat.
Figure 2:

Non-neuronal cell types in the RTN. [a] Astrocyte. [b] Oligodendrocyte. [c] Microglial cell (M) apposed to dark neurone (D). [d] Endothelial cell (E) showing endocytotic activity (arrows) near light neurone (L). Bars: 0.5 µm (a, c, d) and 1 µm (b).
Figure 3:

Moreover, we saw multiple synaptic configurations in which either several synaptic terminals contacted one postsynaptic element, the so-called convergent synapses, or one terminal contacted more than one postsynaptic element, so-called divergent synaptic configurations (Fig. 3c,d). We also observed synapses invaginated into neuronal somata and being surrounded by several processes of oligodendrocytes.

According to the appearance of their vesicles, axon terminals in the RTN can be divided into two groups: one group, representing the majority of terminals, has small (diameter ca. 50-80 nm), electron-lucent vesicles (Fig. 3a,c), the other contains larger vesicles, with an electron-dense core (diameter 70-200 nm) (Fig. 3b). The lucent vesicles most likely contain amino acid transmitters like glutamate, GABA or acetylcholine (Ohara and Lieberman 1985; Hallanger and Wainer 1988; Spreafico et al. 1991; Kharazia et al. 1996; Liu and Jones 1999), whereas the electron-dense vesicles may contain bioamines and/or neuropeptides. Bioamines are supposed to reach the RTN, mostly its ventral part, from the brainstem, and are perhaps released in both a synaptic and diffuse, extrasynaptic way (Asanuma 1992; Destexhe et al. 1994), influencing RTN neuronal activity. Electron-dense vesicles may contain (in addition) various neuropeptides, such as enkephalin (Hermanson et al. 1995), vasoactive intestinal peptide (VIP) and thyrotropin-releasing hormone (TRH) (Burgunder et al. 1999).

As to synaptic innervation, neuronal somata reveal both symmetrical and asymmetrical axo-somatic synapses. A xo-axonic synapses may either be symmetrical or asymmetrical, always contain presynaptic, densely packed electron-lucent vesicles (Fig. 3c), and are of the D-type (small; frequent) or L-type (large; less frequent) as described by Ohara and Lieberman (1985). Axo-axonal coupling is believed to be a mechanism for ultrafast interneuronal communication (Schmitz et al. 2001). Most asymmetrical and symmetrical synapses in the RTN are axo-dendritic with the postsynaptic component being either a proximal or distal part of a dendrite (Fig. 3d), of the main dendrite stem or of a dendritic spine. In addition, in rare cases symmetrical dendro-dendritic contacts were seen that may be dendrodendritic synapses, although they lack presynaptic vesicles. These structures are sometimes accompanied by desmosome-like contacts. Such contacts have been implicated in the establishment of neurone clusters (Kositzyn 1976). This obvious variety and complexity of synapses in the RTN of WAG/Rij rats may be of special relevance in RTN-related absence epileptogenesis.
CHAPTER 3

Synaptology of the rostral reticular thalamic nucleus of absence epileptic WAG/Rij rats

In press as:
Abstract

The adult WAG/Rij rat is a well-established animal model for human absence epilepsy characterised by the presence of spike-wave discharges (SWD). The pacemaking activity of the rostral reticular thalamic nucleus (rRTN) has been demonstrated to be essential for SWD maintenance. We investigated if SWD maintenance can be related to the synaptic organisation of the rRTN, by studying the ultrastructure of the rRTN of absence epileptic WAG/Rij rats in comparison with that of non-epileptic, age-matched ACI control rats. In WAG/Rij rats, D-, L- and F-type terminals constitute the synaptic organisation of the rRTN. D-type synapses, especially axo-dendritic ones, occur frequently. L- and F-type terminals are common but less frequent than D-type terminals. Semi-quantitative observations indicate that all terminal types are present on different parts of the postsynaptic neurone, but in different numbers: they are frequent on dendrites, common on somata and axons, and occur occasionally on dendritic spines. In addition, occasionally an F-type terminal was observed on the axon hillock. The three terminal types are also involved in multiple synaptic configurations, convergent as well as divergent, with dendrites, somata, axon hillocks and axons as postsynaptic structures. Convergent synaptic configurations outnumber divergent ones.

The synaptic organisation of the rRTN of the non-epileptic ACI rat appears to be very similar to that of the epileptic WAG/Rij rat. This indicates that SWD maintenance in the WAG/Rij rat does not depend on a different synaptic organisation of the rRTN.

Introduction

Absence epilepsy is a non-convulsive type of epilepsy, characterized by a decrease in consciousness of abrupt onset and offset and minor behavioural changes including an interruption of ongoing activities, a blank facial expression with the eyes drifting upward, and a rhythmical beating of the eyelids and twitches of the mouth (Porter 1993). Absence seizures are accompanied by highly synchronised brain oscillations, the so-called spike-wave discharges (SWD), generated and maintained in the thalamocortical system and spreading over the cortex (e.g. Gloor 1968; Gloor et al. 1990; Snead III 1995; Avanzini et al. 2000). The mechanisms underlying this disorder have been studied in pharmacological and genetic animal models such as the feline penicillin model and GAERS and WAG/Rij rats (Avoli 1995; van Luijtelaar and Coenen 1997; Danober et al. 1998). For WAG/Rij rats it has been established that SWD activity starts in the peri-oral region of the somatosensory cortex (Meeren et al. 2002) and is maintained by pacemaking activity in the rostral part of the reticular thalamic nucleus (rRTN) (Meeren 2002).

In spite of its crucial importance to absence epilepsy, the mechanism by which the rRTN maintains SWD activity is unknown. In view of its characteristic neuroanatomical
organisation and fibre connectivities, the rRTN can be considered as a distinct compartment within the reticular thalamic nucleus (RTN). The vast majority of the neurones in the rRTN use g-aminobutyric acid (GABA) as neurotransmitter (Houser et al. 1980; DeBiasi et al. 1986), are medium-sized (mean diameter about 20 µm) and have a rather round soma with an extensive dendritic field (Spreafico et al. 1991). Glutamatergic thalamocortical and corticothalamic collateral fibres originating from limbic- and motor-associated areas, innervate the rRTN (Jones 1975; de Curtis et al. 1989; Cornwall et al. 1990; Lozsádi 1994; Liu et al. 2001). In addition, GABAergic, cholinergic, noradrenergic, serotonergic and peptidergic inputs enter the rRTN from the basal forebrain, cerebellum, substantia nigra pars reticulata, locus coeruleus and several other brainstem areas (Hallanger and Wainer 1988; Jourdain et al. 1989; Asanuma and Porter 1990; Cornwall et al. 1990; Paré et al. 1990; Asanuma 1992; Hermanson et al. 1995; Cavdar et al. 2002). Furthermore, the rRTN has been assumed to receive GABAergic input from the caudal part of the ipsilateral RTN and from the contralateral RTN (Chen et al. 1992; Pinault et al. 1997). Moreover, GABAergic intra-rRTN contacts has been proposed to exist between neighbouring rRTN cells through short axon collaterals and dendro-dendritic synapses, but this claim is disputed, as is the presence of gap junctions between rRTN neurones since such junctions have only been described in the caudal part of the RTN (Williamson et al. 1994; Cox et al. 1996; Ulrich and Huguenard 1996; Pinault et al. 1997; Liu and Jones 1999; Landisman et al. 2002). The output of the rRTN consists of GABAergic afferents to the limbic- and motor-associated thalamic nuclei (Cornwall et al. 1990; Gonzalo-Ruiz and Lieberman 1995; Oda et al. 1996).

It is tempting to suppose that the particular role of the rRTN in maintenance of SWD is related to the organisation of its neuronal components, especially its synaptic connections. However, ultrastructural studies of RTN synaptology do not specifically consider the rRTN but concern the RTN in general or focus on its caudal part (e.g. Ohara and Lieberman 1985; Ohara 1988; Liu and Jones 1999) and do not pay attention to a possible relation between rRTN synaptology, the occurrence of SWD and absence epilepsy.

To increase our understanding about how the rRTN maintains SWD, we have studied the synaptology of the rRTN in the WAG/Rij rat, a well-established genetic model for absence epilepsy (Coenen et al. 1992; van Luijtelaar and Coenen 1997). At an age of 6 months, WAG/Rij rats display hundreds of absence epilepsy-related SWD a day, which are maintained by the rRTN (Meeren 2002). Our study concerns a semi-quantitative, ultrastructural characterisation of rRTN synapse types and their configurations. Furthermore, by comparing 6 months old absence epileptic WAG/Rij rats with age-matched, non-epileptic ACI control rats (Inoue et al. 1990; van Rijn et al. 1990; Lason et al. 1992, 1994; de Bruin et al. 2000), the hypothesis has been tested that the maintenance of absence epilepsy-associated SWD is related to a particular synaptic organisation of the rRTN in the WAG/Rij rat.
Methods

Animals

Three epileptic WAG/Rij and 3 ACI rats, males, aged 6 months and reared under standard conditions in our Department of Biological Psychology, were used. All experiments were carried out under the guidelines of the Dutch law concerning animal welfare. Animals were deeply anaesthetised by intraperitoneal administration of 40 mg sodium pentobarbital (Sanofi Sante, Maassluis, The Netherlands) before preparation of their brains for electron microscopy. Unless stated otherwise, all reagents were from Merck (Darmstadt, Germany).

Electron microscopy

Rats were intracardially perfused with 5% glutaraldehyde (Agar Scientific, Essex, UK) and 1% Na$_2$S$_2$O$_5$ in 0.1 M sodium phosphate buffer (PB; 72 mM Na$_2$HPO$_4$, 27.5 mM NaH$_2$PO$_4$; pH 7.4), for 15 min at 20 °C. After decapitation, brains were immediately removed and postfixed in the same fixative, for 20 hrs at 4 °C. Subsequently, 200 µm sections were cut on a LEICA VT 1000S vibratome (Leica Instruments, Nussloch, Germany) and fixed for another 20 hrs at 4 °C. Then, the medial part of the rRTN (Bregma -1.35 mm and Interaural 7.65 mm according to Paxinos and Watson 1997; Fig. 1) was punched out with a siliconised Pasteur’s pipette with an inner tip diameter of 2 mm. Punches were washed in 0.1 M PB for 10 min, postfixed in 1% OsO$_4$ (Agar Scientific) in 0.1 M PB, for 2 hrs at 4 °C, dehydrated in 30 min steps of a graded ethanol series, and embedded in Spurr’s resin. Ultrathin sections of the central area of the rRTN were cut on a Reichert ultramicrotome (Leica Instruments), collected on Formvar-coated nickel grids, and stained with uranyl acetate and lead citrate.

Estimations of the relative frequencies of different synapse types were made by classifying synapses in the most central region of the rRTN, using a square of 10,000 mm$^2$ (Fig. 1), by direct microscopic examination of one pale-gold, ultrathin section per animal (3 WAG/Rij and 3 ACI rats) in a JEM-1010 electron microscope (Jeol, Akishima, Japan). In this way at least 50 synapses were observed per animal. With the same approach, F-type terminals (see Results) were studied in a larger sampling area (40,000 mm$^2$) centrally in the rRTN (Fig. 1), permitting the classification of at least 30 F-type terminals per animal.

Results

General morphology of the rRTN of the WAG/Rij rat

At the ultrastructural level, the rRTN of the WAG/Rij rat appears as a strongly inhomogeneous structure, consisting of neuronal somata, dendrites, dendritic spines, axon hillocks,
Synaptology of the rostral reticular thalamic nucleus of absence epileptic WAG/Rij rats

Axonal processes, several types of glial cells such as astrocytes, oligodendrocytes and microglial cells, and blood vessels, interrupted by bundles of large-sized, myelinated axons (Fig. 2a). Neuronal perikarya are rather large (ca. 20-30 µm), all look similar, and reveal a rather underdeveloped rough endoplasmic reticulum (RER) and Golgi apparatus, and some scattered electron-lucent vesicles with a mean diameter of about 50 nm. Some neurones also show electron-dense vesicles with a mean diameter of about 100 nm (Fig. 2b). The rRTN
is rich in synaptic contacts. In studying the synaptology of the rRTN, we distinguished three main types of axon terminal that form synapses, on the basis of the nomenclature used by Ohara and Lieberman (1985), Ohara (1988), Liu (1997) and Liu and Jones (1999), viz. D-, L- and F-type terminals.

**Synaptology of the rRTN of the WAG/Rij rat**

To obtain an impression of the amount of each of the three terminal types present in the rRTN of WAG/Rij rats, per type (D, L, F) the number of terminals in the sampling area was counted. Furthermore, for each terminal encountered, the postsynaptic element was determined (soma, dendrite, dendritic spine, axon hillock or axon) and the number of synapses belonging to each element was counted. In view of the inhomogeneity of the rRTN and, in spite of the large sampling area studied, of the low numbers of synapses present in some of the distinguished categories, no attempt was made to carry out detailed statistical analyses. Instead, counts for the three main terminal types, which occur in a reasonable high frequency, were averaged over the three animals per experimental group, and expressed quantitatively as percentages of all synapses observed (Table 1). Furthermore, for the synapse subtypes (classified according to their postsynaptic element), which occur in rather low frequencies, a semi-quantitative approach was followed that would still allow to detect possible main differences in synaptic organisation of the rRTN of the two strains (WAG/Rij versus ACI rats). For this purpose, counts of a particular terminal type per postsynaptic element were averaged over the three animals per experimental group, expressed as percentages of all synapses belonging to that particular category, and then classified as follows: ‘frequent’: >50%, ‘common’: 10-50%, and ‘occasional’: 0-10%. In an additional experiment, this classification was also carried out for F-type terminals using a larger rRTN sampling area (Fig. 1). F-type terminals are assumed by some authors to play an important role in intra-RTN coupling (e.g. Ohara et al., 1983; Williamson et al., 1994; Liu and Jones, 1999) underlying synchronous RTN firing activity such as SWD.

D-type axon terminals (Fig. 2c-e) are small (average diameter 0.6 µm) and reveal a cluster of numerous, closely packed, round or slightly oval, electron-lucent vesicles with a diameter of ca. 50 nm. Sometimes a mitochondrion (mean diameter 0.3-0.4 µm) is present. D-type terminals form asymmetrical synapses with a relatively short active site (0.2-0.5 µm). They are the most frequent synapse type in the rRTN of the WAG/Rij rat, constituting more than half (54%; Table 1) of the number of all rRTN synapses. They occur frequently on rRTN dendrites (Fig. 2c) and are common on rRTN somata (Fig. 2d), which reveal a light cytoplasm with a rather underdeveloped RER and Golgi apparatus. Furthermore, D-type terminals are common on axons, which are characterised by microtubules and elongated mitochondria (Fig. 2e).

L-type axon terminals are much larger than D-type ones (diameters ranging from 1-3 µm), contain a fair number of mitochondria (up to 5 were observed) and form long, asymmetrical active sites (0.5-1.0 µm) that sometimes are split into two or more segments (Fig. 3a). Near each segment, a cluster of rather loosely packed, electron-lucent synaptic vesicles
Synaptology of the rostral reticular thalamic nucleus of absence epileptic WAG/Rij rats

Figure 2:
The rRTN with [a] a survey of neuronal somata (S), large myelinated axons (m) and areas (A) with dendrites and unmyelinated axons [b] somata with Golgi apparatus (G) and large electron-dense vesicles (arrows), and [c-f] D-type axon terminals forming synapses on dendrite [c,f] soma [d] and axon [e]. mt: microtubules. [a-e]: WAG/Rij rat, [f]: ACI rat. Bars: 5 µm (a), 0.5 µm (b,c,d,e), 0.2 µm (f).
(mean diameter *ca.* 50 nm) occurs. L-type terminals are common (23%; Table 1) but clearly less numerous than D-type ones. They frequently make axo-dendritic contacts (Fig. 2a) and commonly form axo-somatic (Fig. 3c) and axo-axonic synapses (Fig. 3d).

F-type axon terminals, 1-2 µm in diameter, contain a variable number of mitochondria (0-3 were observed) and loosely distributed, electron-lucent, pleomorphic vesicles with a mean diameter of 50-70 nm. They can be easily distinguished from D- and L-type terminals because they form symmetrical active sites that are medium-sized (ca 0.8 µm). F-type synapses are common (23%; Table 1) and occur in similar numbers as L-type terminals. They frequently are axo-dendritic (Fig. 4a) and are common on somata (Fig. 4b) and axons (Fig. 4c). Occasionally, an F-type terminal was seen to make a synaptic contact with the axon hillock (Fig. 4d). This classification is based on the observation of no more than about 10 F-type terminals per animal. To increase accuracy, in the additional experiment a

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**Figure 3:**
L-type axon terminals in rRTN, forming synapses on dendrites [a,b] soma [c, with detail in inset] and axon [d]. Note the split active site in a, b and c, and convergent synaptic configuration in c and d. a: axon, mt: microtubules. [a,c,d]: WAG/Rij rat, [b]: ACI rat. Bars: 0.5 µm (inset: 0.25 µm).
larger sampling area was studied, thereby permitting the classification of at least 30 F-type terminals per animal. However, the outcome showed frequencies of postsynaptic F-type terminal elements that were very similar to those of the classification described above for the regular sampling area (see also Table 2).

Occasionally, axon terminals make contact with dendritic spines, which appear as thin (mean diameter 0.2-0.3 µm), stalk-like structures, often with a slightly broadened end (‘bouton’) and partly or completely surrounding the spine. On the basis of the presence of the small asymmetrical active site, a dense cluster of presynaptic vesicles and the rare presence of mitochondria, the majority of these synapses were classified as D-type terminals (Fig. 5a). The others belong to either the L- (Fig. 5b) or the F-type.

No dendro-dendritic synapses or gap junctions were found between any neuronal element in the rRTN.

**Figure 4:**
F-type axon terminals in rRTN of WAG/Rij rat, forming synapses on dendrite [a] soma [b, with detail in inset] axon [c] and axon hillock [d, with detail in inset]. Convergent synaptic configuration in [a] and [d]. a: axon, h: axon hillock. Bars: 0.5 µm.
**Table 1:**

Relative frequencies of the three terminal types (D, L, F) present in the rRTN of WAG/Rij and ACI rats, in percentages of all single synapses observed.

<table>
<thead>
<tr>
<th>Terminal type</th>
<th>WAG/Rij rats</th>
<th>ACI rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-type</td>
<td>54 %</td>
<td>57 %</td>
</tr>
<tr>
<td>L-type</td>
<td>23 %</td>
<td>22 %</td>
</tr>
<tr>
<td>F-type</td>
<td>23 %</td>
<td>21 %</td>
</tr>
</tbody>
</table>

**Table 2:**

Relative frequencies of different postsynaptic elements of F-type terminals present in the rRTN of WAG/Rij and ACI rats, in percentages of all F-type terminals examined (at least 30 per animal).

<table>
<thead>
<tr>
<th>Postsynaptic element</th>
<th>WAG/Rij rats</th>
<th>ACI rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendrite</td>
<td>67 %</td>
<td>69 %</td>
</tr>
<tr>
<td>Soma</td>
<td>12 %</td>
<td>12 %</td>
</tr>
<tr>
<td>Dendritic spine</td>
<td>8 %</td>
<td>6 %</td>
</tr>
<tr>
<td>Axon</td>
<td>12 %</td>
<td>12 %</td>
</tr>
<tr>
<td>Axon hillock</td>
<td>1 %</td>
<td>1 %</td>
</tr>
</tbody>
</table>

**Table 3:**

Relative frequencies of convergent and divergent multiple synaptic configurations, in percentages of all synaptic contacts (multiple + single) observed.

<table>
<thead>
<tr>
<th>Multiple configuration type</th>
<th>WAG/Rij rats</th>
<th>ACI rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convergent</td>
<td>16.8 %</td>
<td>14.6 %</td>
</tr>
<tr>
<td>Divergent</td>
<td>4.2 %</td>
<td>2.4 %</td>
</tr>
</tbody>
</table>
Multiple inputs
The rRTN somata, dendrites and axons of the WAG/Rij rat receive not only single but sometimes also multiple synaptic inputs, in which all terminal types (D, L and F) may participate. The postsynaptic elements are most often dendrites, but also the soma, axon hillock and axon were observed in such multiple synaptic configurations. When all synaptic contacts (single as well as multiple configurations) are considered, convergent synaptic configurations are common (16.8% of all synaptic structures; Table 3). They show several terminals (up to 5) contacting one postsynaptic element (Fig. 3c,d, 4a,d). Divergent multiple inputs are less numerous (4.2% of all contacts; Table 3). Generally, one terminal contacts up to 3 postsynaptic elements (Fig. 5c).

The rRTN of the ACI rat
The ultrastructural organisation and synaptology of the rRTN of the non-epileptic ACI rat closely resembles that of the absence epileptic WAG/Rij rat. A similar heterogeneous distribution of neuronal elements as observed in the WAG/Rij rat was seen, and D-, L- and F-type terminals are found on somata, dendrites, dendritic spines and axons (Fig. 2f, 3b).

The close resemblance in synaptic organisation between the WAG/Rij and ACI rats clearly appears from the very similar, relative frequencies of the 3 different terminal types (D-, L- and F-type for ACI vs. WAG/Rij: 54% vs. 57%, 23% vs. 22%, and 23% vs. 21%, respectively; Table 1). Also, the additional, detailed classification of postsynaptic elements of F-type terminals does not show any obvious difference between the two strains (Table 2). Moreover, the semi-quantitative observations indicate that also in the ACI rat all three terminal types are frequent in the axo-dendritic form, are common on somata and axons, and occur only occasionally on dendritic spines and, as far as F-type terminals are concerned, on the axon hillock. One slight difference is the presence of one D-type and one L-type terminal innervating the axon hillock. However, this phenomenon, which was not observed in any of the WAG/Rij rats, was observed in one ACI rat only. Therefore it does not seem to indicate a meaningful difference between the two strains.

As to multiple inputs, ACI rats again do not obviously differ from WAG/Rij rats. All three terminal types were observed to be involved in multiple synaptic configurations with dendrites, somata, axon hillock and axons (Fig. 5d). Moreover, convergent synaptic configurations occur in a similar frequency as in WAG/Rij rats (14.6 % of all synaptic contacts; Table 3) and outnumber to the same degree divergent ones (2.4 % of all contacts; Table 3).

Discussion
The present study has been carried out in the context of absence epilepsy, by comparing 6 months old, absence epileptic WAG/Rij rats with age-matched, non-epileptic ACI rats. The WAG/Rij rat is a well-characterised animal model for absence epilepsy (Coenen et al.)
The rRTN with spine synapses of D-type [a] and L-type [b] divergent synaptic configuration on axon and dendrite [c] and convergent synaptic configurations on axon [d]. a: axon, s: spine. [a-c]: WAG/Rij rat, [d]: ACI rat. Bars: 0.2 µm.

The general ultrastructure of the RTN of adult WAG/Rij rats was recently described by Akhmadeev et al. (2003; Chapter 2). However, it has been demonstrated that in the WAG/Rij rat especially the rostral part of the RTN is important for absence epilepsy, as it is responsible for the maintenance of SWD (Meeren 2002). The present data provide the first detailed description of the ultrastructural organisation of the rRTN of the WAG/Rij rat, as well as that of the ACI control strain.

Morphology of the rRTN of the WAG/Rij rat

In the WAG/Rij rat, the general ultrastructure of the rRTN is similar to that described for the RTN as a whole (e.g. Ohara and Lieberman 1985; Akhmadeev et al. 2003), revealing
areas rich in neuronal elements interrupted by tracts of myelinated, large-sized axons. In the RTN different types of perikarya have been distinguished on the basis of their size and ultrastructure, such as large ‘dark’ and ‘light’ neurones, and smaller, spindle-like neurones (Spreafico et al. 1991; Akhmadeev et al. 2003). In the present study of the rostral part of the RTN we only saw medium- to large-sized neurones, which reveal a rather underdeveloped RER and Golgi apparatus and respond to the description of light neurones (Akhmadeev et al. 2003). In view of their ultrastructural characteristics and the well-known, strong dominance of GABAergic neurons in the RTN (Houser et al. 1980; DeBiasi et al. 1986), we assume that these neurones produce GABA and are responsible for the connection of the rRTN with the limbic- and motor-associated thalamic nuclei and probably neighbouring rRTN neurones (Cornwall et al. 1990; Gonzalo-Ruiz and Lieberman 1995; Cox et al. 1996; Oda et al. 1996; Pinault et al. 1997). We show that some neurones not only contain electron-lucent but also larger, electron-dense vesicles, which suggests the presence of neuropeptides in these rRTN neurones. Neurones of the rRTN are known to contain thyrotropin-releasing hormone (TRH) (Burgunder et al. 1999) and enkephalin (Hermanson et al. 1995), the latter peptide being of special interest since (met)enkephalins may be involved in absence epilepsy (Lason et al. 1992, 1994).

The RTN, including the rRTN, is considered to be an interface between thalamus and cortex, modulating the activity of the total thalamocortical system (Shosaka 1986). The rRTN is traversed by numerous corticothalamic and thalamocortical axons and receives cortical and thalamic inputs via axon collaterals (Jones 1975; Cornwall et al. 1990; Lozsádi 1994). Consequently, it can be assumed that the large-sized, myelinated axon tracts seen in our study represent such axons. Many, if not all, of the unmyelinated axon processes may be small collaterals originating from these fibres (Schiebel and Schiebel 1966, 1972). Moreover, rRTN neurones receive inputs from the caudal ipsilateral RTN, the contralateral RTN, the basal forebrain, the cerebellum, the locus coeruleus, the substantia nigra pars reticulata and presumably from neighbouring rRTN cells (Jourdain et al. 1989; Asanuma and Porter 1990; Cornwall et al. 1990; Asanuma 1992; Chen et al. 1992; Pinault et al. 1997; Cavdar et al. 2002). Probably, these inputs are represented in the rRTN by some of the small, unmyelinated axons.

Origin of synaptic inputs

D-type axon terminals in the RTN are also named ‘corticothalamic’ terminals, because they degenerate after cortical ablation (Ohara and Lieberman 1981, 1985; Liu and Jones 1999). Our data show that D-type terminals form the most abundant synapse type in the rRTN of the WAG/Rij rat. Since especially the limbic- and motor-associated cortical areas project to the rRTN (Cornwall et al. 1990; Lozsádi 1994), these areas may form the main innervation of the rRTN by D-type terminals. The cortical innervation of the rRTN is glutamatergic and exerts a profound influence on the limbic- and motor-associated thalamus (Ohara and Lieberman 1981, 1985; de Curtis et al. 1989; Cornwall et al. 1990; Gonzalo-Ruiz and Lieberman 1995; Kharazia et al. 1996; Oda et al. 1996; Salt and Eaton 1996). Corticothalamic stimulation can set RTN activity (Steriade et al. 1972; de Curtis et al. 1989; Bal
and McCormick 1993; Contreras and Steriade 1996; Golshani et al. 2001) and in that way probably influence SWD maintenance by the rRTN.

L-type axon terminals in the RTN are glutamatergic and are called ‘thalamocortical’ terminals because they are affected by thalamic lesioning (Ohara and Lieberman 1985). The glutamatergic, thalamic input to the rRTN concerns the limbic- and motor-associated thalamic nuclei (Cornwall et al. 1990; Gonzalo-Ruiz and Lieberman 1995; Oda et al. 1996). Therefore, these nuclei are likely to be the source of the L-type terminals in the rRTN. The terminals may contribute to the reciprocal connectivities between the rRTN and these thalamic nuclei, providing a strong synaptic coupling which is necessary for re-exciting rRTN neurons and, in this way, the maintenance of oscillatory activity within the thalamocortical system (Warren et al. 1994; Bal et al. 1995). Since L-type terminals are present in a clearly lower frequency than D-type terminals, the rRTN may receive less extensive input from the thalamus than from the cortex. In this respect, the rRTN seems to be representative for the RTN as a whole, as D-type terminals dominate L-type terminals in the caudal RTN of the Wistar rat (Ohara and Lieberman 1985; Liu and Jones 1999).

In addition to glutamatergic terminals, noradrenergic and cholinergic terminals originating in the locus coeruleus and forebrain, respectively, innervate the rRTN. These terminals form asymmetrical synapses resembling D- and L-type terminals (Hallanger and Wainer 1988; Asanuma 1992). Both noradrenaline and acetylcholine may modulate thalamocortical activity via the RTN (McCormick 1989) and are involved in the control of SWD activity (Marescaux et al. 1992). Therefore, non-glutamatergic D-type and L-type terminals in the rRTN may be adrenergic and/or cholinergic and influence SWD in the rRTN.

F-type terminals form the third type of synapse in the rRTN and occur in similar numbers as L-type terminals. They are GABAergic because they contain the GABA-synthesising enzyme glutamic acid decarboxylase (Ohara et al. 1983) and therefore are presumably part of the GABAergic innervation of the rRTN by structures like the basal forebrain, the substantia nigra pars reticulata, the caudal part of the ipsilateral RTN, the contralateral RTN and, possibly, neighbouring rRTN cells (Jourdain et al. 1989; Asanuma and Porter 1990; Paré et al. 1990; Chen et al. 1992; Pinault et al. 1997). Our results indicate that the rRTN contains more F-type terminals than the caudal RTN, where F-type terminals are relatively scarce (Ohara and Lieberman 1985; Liu and Jones 1999). It may be that the relatively strong F-type terminal input to the rRTN is due to inputs from the ipsilateral caudal part of the RTN and from the contralateral RTN, as the caudal RTN is not known to be innervated by the ipsilateral rRTN or the contralateral RTN (Chen et al. 1992, Pinault et al. 1997).

Postsynaptic elements

Our finding that both D- and L-type terminals predominantly abut rRTN dendrites, is in accordance with Ohara and Lieberman (1985) and Liu and Jones (1999), who described the innervation of the caudal RTN. Also with respect to the occurrence of D- and L-type axo-somatic and axo-spinous synapses and of F-type terminals on somata, dendrites and dendritic spines, the rRTN appears to be similar to the caudal RTN (Ohara and Lieberman 1985; Liu and Jones 1999).
In the caudal RTN of the Wistar rat, the axon hillock has been described as postsynaptic element of D-, L- and F-type synapses (Pinault et al. 1997). Our study on the WAG/Rij rat confirms this finding for F-type terminals on the axon hillock of rRTN neurones. However, we did not find D- or L-type terminals on the axon hillock of rRTN neurones in this rat strain, whereas D-, L- and F-type terminals on axons are common. Apparently, D- and L-type synapses are homogeneously distributed throughout the RTN. The axon as postsynaptic structure has not been described previously as a postsynaptic element in the rRTN. In the caudal RTN small, intrinsic axonal ramifications have been observed that contact boutons in an asymmetrical way (Pinault et al. 1997). The axo-axonic synapses we show in the rRTN, possibly directly affect corticothalamic or thalamocortical axon collaterals, providing thereby a powerful tool to control the GABAergic output from the rRTN to the thalamus.

Several morphological structures such as axon collaterals, dendro-dendritic synapses and gap junctions have been proposed to provide inhibitory, intra-RTN connectivity (Cox et al. 1996; Ulrich and Huguenard 1996; Pinault et al. 1997; Liu and Jones 1999; Landisman et al. 2002). Intra-RTN inhibition controls the excitability and activity pattern of the thalamocortical system, although its role in thalamic oscillations such as absence epilepsy-associated SWD is controversial (Sanchez-Vives et al. 1997; Huntsman et al. 1999; Sohal et al. 2000; Aker et al. 2002; Shu and McCormick 2002; Slaght et al. 2002). We observed F-type synapses in the rRTN, which presumably are GABAergic (Ohara et al. 1983) and may originate from neighbouring rRTN neurones. We did not find the dendro-dendritic synapses described by Pinault et al. (1997) for the caudal part of the RTN, nor did we see gap junctions in the rRTN. Such junctions have been demonstrated in the caudal RTN by ultra-immunocytochemistry for connexin-36, the key-protein of gap junctions (Landisman et al. 2002). However, as gap junctions are difficult to detect by routine electron microscopy, we do not want to rule out their presence in the rRTN.

Multiple synapses

In the WAG/Rij rat rRTN, postsynaptic structures may act as target for several (different) presynaptic axon terminals. Also, one terminal can make a synaptic contact with more than one postsynaptic element. Such multiple synaptic connectivity has not been described before for the rRTN, nor for the RTN in general, but it is not an uncommon phenomenon in the brain of both vertebrates and invertebrates (e.g. Roubos and Moorer-van Delft 1981; Sesack and Pickel 1992; Cobb et al. 1997; Svingos et al. 2000). These multiple synaptic configurations may be involved in the synchronisation of the activities of different neuronal structures and, perhaps, in that way contribute to the maintenance of SWD activity in the rRTN of the WAG/Rij rat.

Synaptology of the rRTN in relation to absence epilepsy

As to the ultrastructural organisation and synaptic organisation of the rRTN, ACI and WAG/Rij rats appear to be very similar, in both qualitative and quantitative respects. We
show that all terminal types and subtypes have very similar frequencies in both strains. This holds not only for the occurrence of single synaptic terminals of the L-, D- and F-type, but also for convergent and divergent synaptic configurations, which do not obviously differ in composition or frequency among the two rat strains. In one ACI rat we detected one D- and one L-type terminal contacting the axon hillock. However, in view of the absence of such synapses in all other animals (including the two other ACI rats), this rare observation does not give enough ground to relate this phenomenon to absence epilepsy. Therefore, the occurrence of SWD in WAG/Rij rats does not seem to be related to obvious qualitative or quantitative differences in their rRTN synaptic organisation compared to ACI control rats.

Conclusions

The elaborate synaptic organisation of the rRTN in WAG/Rij rats is in line with the role of this brain structure as a pacemaker maintaining oscillatory activity, such as SWD, in the extensive corticothalamic network. However, it also appears to be not obviously different from the synaptic organisation of the rRTN in non-epileptic ACI rats. This indicates that the role of the rRTN in WAG/Rij rats in the maintenance of absence epilepsy-associated SWD does not depend on this synaptic organisation per se. Either the absence of SWD in ACI rats is due to the fact that no SWD are generated in the somatosensory cortex of these animals, or other properties of the rRTN are involved in SWD maintenance, such as the functioning of their neuronal elements at the molecular level, which are not visible in our present study. In this respect it is noteworthy that we recently showed that, compared to the ACI rat, the rRTN of the WAG/Rij rat expresses a higher number of high-voltage-operated Ca^2+ channels of the 2.1 type (van de Bovenkamp-Janssen et al. 2003, Chapter 5).
CHAPTER 4

NMDA-NR₁ and AMPA-GluR₄ receptor subunit expression in the absence epileptic WAG/Rij rat

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Abstract

From an age of 3 months onwards, the WAG/Rij rat, a genetic model for human absence epilepsy, develops spike-wave discharges (SWD) that are characteristic for absence epilepsy. The peri-oral somatosensory cortex is the site of SWD initiation, whereas the rostral reticular thalamic nucleus (rRTN) maintains SWD activity. We hypothesised that changed expressions of the NMDA-NR1 receptor subunit and the AMPA-GluR4 receptor subunit in the peri-oral somatosensory cortex and in the rRTN are involved in, respectively, the initiation and maintenance of SWD activity. To test this hypothesis, we compared 3 months old, non-epileptic and 6 months old, absence epileptic WAG/Rij rats with age-matched, non-epileptic ACI control rats. NMDA-NR1 and AMPA-GluR4 receptor subunits were visualised by immunocytochemistry, demonstrating the presence of NMDA-NR1- and AMPA-GluR4-containing receptors, respectively. Quantification of the presence of subunit immunostaining revealed no differences in the amount of NMDA-NR1 or AMPA-GluR4 subunits in the hippocampus, an area which is not involved in absence epilepsy-associated SWD. However, in the peri-oral somatosensory cortex, WAG/Rij rats of both ages showed less NMDA-NR1 and AMPA-GluR4 subunit staining than ACI rats. Furthermore, in the rRTN of WAG/Rij rats, AMPA-GluR4 subunit staining more strongly increases from 3 to 6 months, than in the rRTN of ageing ACI rats. It is proposed that in the peri-oral somatosensory cortex of the WAG/Rij rat, SWD start as a result of reduced NMDA- and AMPA-GluR4-mediated glutamatergic stimulation, whereas in the rRTN of this rat strain SWD maintenance may be promoted by increased excitation as a result of increased GluR4-rich AMPA receptor presence.

Introduction

Oscillations within the thalamocortical system, especially the somatosensory cortex and the reticular thalamic nucleus (RTN), are considered to underlie the initiation and maintenance of absence epileptic spike-wave discharge (SWD) activity (e.g. Gloor 1968; Gloor et al, 1990; Snead III 1995; Avanzini et al., 2000; Kostopoulos 2000; Meeren 2002; Meeren et al. 2002). The subcellular mechanisms underlying the initiation and maintenance of SWD are not understood but there is some evidence that the glutamate receptors N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolopyridine acid (AMPA) are involved (e.g. Peeters et al. 1990, 1994; Pumain et al. 1992; Koerner et al. 1996; Caddick et al. 1999; Destexhe 1999; D’Arcangelo et al. 2002). The NMDA receptor subunit 1 (NMDA-NR1) is the constant component of the NMDA receptor, and forms a functional, heteromeric receptor with the NMDA-NR2A-D and/or the NMDA-NR3 subunit. AMPA receptors are composed of various combinations of four subunit proteins, AMPA-GluR1-4. Both
receptors form ion channels that mediate excitatory postsynaptic currents in central neurons (Michaelis 1998; Ozawa et al. 1998; Dingledine et al. 1999). These receptors are abundantly present in the thalamocortical system, including the somatosensory cortex and the RTN (e.g. Petralia and Wenthold 1992; Petralia et al. 1994; Spreafico et al. 1994; Liu 1997; Mineff and Weinberg 2000).

We have studied the possible roles of NMDA and GluR4-rich AMPA receptors in SWD activity in the WAG/Rij rat. This rat strain is a well-established animal model for human absence epilepsy (van Luijtelaar and Coenen 1997). From an age of 3 months onwards, WAG/Rij rats develop SWD activity and at 6 months they display hundreds of absence epileptic seizures a day (Coenen and van Luijtelaar 1987; Inoue et al. 1990; de Bruin et al. 2000). For WAG/Rij rats it has been established that SWD activity starts in the peri-oral region of the somatosensory cortex, the part of the parietal cortex area 1 (Par1) (Zilles 1985) where the nose and upper lip are represented (Meeren et al. 2002). After initiation, SWD activity is maintained by the pacemaking activity of the rostral part of the RTN (rRTN)(Meeren 2002), while the hippocampus is not involved (Inoue et al. 1993; Kandel et al. 1996). We hypothesise that SWD activity is related to a changed expression of NMDA and GluR4-rich AMPA receptors in the absence epilepsy focus, viz. the peri-oral somatosensory cortex, and in the absence epilepsy pacemaker, viz. the rRTN.

To test this hypothesis we studied the presence of the NMDA-NR1 and AMPA-GluR4 subunits in these thalamocortical areas, and in the CA3 region of the hippocampus as a control region. Immunoreactivities against the NMDA-NR1 and AMPA-GluR4 receptor subunits were quantified in non-epileptic (3 months old) and absence epileptic (6 months old) WAG/Rij rats, using ACI rats as non-epileptic, age-matched controls (Inoue et al. 1990; van Rijn et al. 1991; Lason et al. 1992, 1994; de Bruin et al. 2000). On the basis of the results we propose that a reduction in cortical NMDA and GluR4-rich AMPA receptor-mediated glutamatergic excitation in the peri-oral somatosensory cortex leads to SWD initiation and that an increased glutamatergic excitation via the GluR4-rich AMPA receptor in the rRTN underlies SWD maintenance.

**Methods**

**Animals**

Experiments were carried out with minimally 10 WAG/Rij and 10 ACI rats. Per rat strain, at least 5 animals with ages of 3 and 6 months were used. They had been bred and reared under standard conditions in our Department of Biological Psychology, University of Nijmegen. All experiments were carried out under the guidelines of the Dutch law concerning animal welfare. Unless stated otherwise, chemicals were from Merck (Darmstadt, Germany).
Tissue preparation

Animals were deeply anaesthetised by intraperitoneal administration of 40 mg sodium pentobarbital (Sanofi Sante, Maassluis, The Netherlands) and intracardially perfused with 4% paraformaldehyde and 0.05% picric acid in sodium phosphate buffer (PB; 72 mM Na₂HPO₄, 27.5 mM NaH₂PO₄; pH 7.4), for 10 min. Subsequently, they were decapitated and their brains immediately dissected and postfixed in the same fixative, for 20 hrs at 4 °C. Then, brains were cryoprotected by immersion in 30% sucrose (Mallinckrodt, Deventer, The Netherlands), for 48 hr. Serial (40 μm) coronal sections were cut on a Microm HM 440 E sliding freeze-microtome (Microm, Walldorf, Germany) and stored in 0.1 M PBS (PB-buffered saline: 84 mM Na₂HPO₄, 22 mM NaH₂PO₄, 137 mM NaCl, 2.7 mM KCl; pH 7.4).

Immunocytochemistry

Sections were processed for free-floating immunocytochemistry, to visualise NMDA-NR1 or AMPA-GluR4 subunits, using the avidin-biotin complex (ABC) method (Hsu et al. 1981). Mouse anti-NMDA-NR1 and rabbit anti-AMPA-GluR4 sera were purchased from Chemicon (Temecula, CA, USA). Sections were incubated in 0.1 M PBS containing 0.3% H₂O₂ (Lamers and Pleuger, ’s Hertogenbosch, The Netherlands), for 30 min. Non-specific binding sites were blocked by 1% BSA (ICN Biomedicals, Aurora, Ohio, USA) and tissue permeability was increased with 0.5% Triton-X 100 (Sigma, St. Louis, MO, USA) in 0.1 M PBS (PBS-BT) for 30 min. Then, sections were incubated either in mouse anti-NMDA-NR1 (specifically recognising all known NMDA-NR1 splice variants) (1:500 in PBS-BT) or rabbit anti-AMPA-GluR4 (1:1500 in PBS-BT) serum, for 60 hr at 4 °C, followed by incubation with either goat anti-mouse biotin-conjugated IgG (NMDA-NR1) or goat anti-rabbit biotin-conjugated IgG (AMPA-GluR4) (both diluted 1:1500 in 0.1 M PBS-BT), for 90 min. Both secondary antisera were from Vector Laboratories (Burlingame, CA, USA). For signal amplification, sections were incubated for 90 min in ABC (Vector Laboratories) (diluted 1:800 in 0.1 M PBS-BT) and visualised by 0.025% w/v 3,3’-diaminobenzidine (DAB; Sigma) in 50 mM Tris buffer with 0.25% w/v nickel ammonium sulphate (BHD Laboratory Supplies, Poole, UK), for 10 min. Sections were mounted on poly-L-lysine-coated glass slides, dehydrated and coverslipped with Entellan.

Control sections were treated as described above, but with omission of the respective primary antisera.

Quantifications and statistics

Per animal, quantifications were made in a 40 μm thick section at the level of Bregma −1.40 mm and Interaural 7.60 mm (Paxinos and Watson 1997) in 1) the medial part of the layers I, II+III, IV, V and VI of the peri-oral somatosensory cortex and 2) the most medial section of the rRTN. The cortical layers II and III were studied as one area, as they
could not be completely distinguished from each other. Quantifications were made in both the dorsal and ventral part of the rRTN (Fig. 1a,b). In addition, immunoreactivity was quantified in a 40 µm thick section of the molecular layer of the CA3 region of the hippocampus (Bregma −2.56 mm and Interaural 6.44 mm; Paxinos and Watson, 1997).

NMDA-NR1- and AMPA-GluR4-immunoreactive spots in the peri-oral somatosensory cortex layers, the dorsal and ventral rRTN, and the CA3 region of the hippocampus were counted by direct light microscopic (Nikon, Tokyo, Japan) examination using a x100 oil immersion objective and a square graticule at a final magnification of x1000, covering 2500 mm² (Fig. 1a,b), and expressed as numerical densities per 1000 µm². In case of the AMPA-GluR4 immunostaining of the rRTN, spots associated with neuronal somata could easily be distinguished from spots outside rRTN somata. Therefore, in addition, the AMPA-GluR4 ‘extrasomal’ spot density (spots outside soma/1000 µm²) and the ‘somal’ spot density (spots per labelled soma) were determined.

Data obtained in the peri-oral somatosensory cortex and rRTN were graphically presented as means ± standard error of the mean (SEM), and tested for significance between experimental groups by a three-way ANOVA with ‘rat strain’, ‘age’ and ‘brain region’ (i.e., cortical layer I, II+III, IV, V, VI and dorsal + ventral part of the rRTN) as independent variables. Hippocampal data were tested by a two-way ANOVA with ‘rat strain’ and ‘age’ as independent variables. The ANOVA was followed by Duncan’s multiple range test (a=5%), using Statistica (StatSoft, Tulsa, OK, USA) after appropriate transformation of the original data on the basis of tests for normality (Shapiro-Wilk test) and for homogeneity of variance (Bartlett’s Chi-square test) (Shapiro et al. 1968; Winer et al. 1991).

Results

In general, clear immunoreactivities were observed against both NMDA-NR1 and AMPA-GluR4 subunits, in all four rat groups tested. Staining was present throughout the brain areas studied, including substantial immunoreactivity in the cerebral cortex (Fig. 1a), the rRTN (Fig. 1b), the hippocampus (Fig. 1c) and the other thalamic nuclei (Fig. 1d). At high magnification, NMDA-NR1 and AMPA-GluR4 immunoreactivities appear as distinct spots (Fig. 1e,f). Omission of the respective primary antisera abolished all immunoreactivity, which holds for all of the four experimental groups.

Attention was focused on the peri-oral somatosensory cortex and on the rRTN. In addition, the CA3 region of the hippocampus was studied. In the peri-oral somatosensory cortex and the rRTN, NMDA-NR1 spots occur rather evenly distributed, making it impossible to distinguish neuronal elements (soma, axon, dendrites) from each other (Fig. 1e). The same holds for AMPA-GluR4 spots in the peri-oral somatosensory cortex. In contrast, staining of the rRTN with the AMPA-GluR4 antiserum revealed distinct, immunoreactive spots that appeared to be present in high numbers over lightly stained neuronal somata, making it possible to distinguish these somal spots from spots located outside the neuronal somata, the so-called extrasomal spots (Fig. 1f). In the molecular hippocampal area, both
Figure 1:

Immunoreactive (ir) spots indicating NMDA-NR1 and AMPA-GluR4 subunits in brains of 3 and 6 months old WAG/Rij rats (W3, W6) and ACI (A3, A6) rats. [a] NMDA-NR1 ir in the peri-oral somatosensory cortex, located within the parietal cortex area 1, Par1, according to Zilles (1985), of W3 demonstrating the sample areas in cortical layer I, II+III, IV, V and VI (squares). [b] AMPA-GluR4 ir in the rRTN of A3 showing the dorsal and ventral sample area (squares). [c] NMDA-NR1 ir in the CA3 region of the hippocampus of W3. [d] AMPA-GluR4 ir in thalamus and adjacent caudal RTN of A6. [e] NMDA-NR1 ir in layer V of the peri-oral somatosensory cortex of W6, demonstrating the presence of dark ir spots (arrows). [f] AMPA-GluR4 ir in the medial section of the rRTN of W3. Note the ir spots associated with rRTN somata (‘somal’ spots; arrowheads) and the presence of ir spots outside somata (‘extrasomal’ spots; arrows). Cc: corpus callosum, Ci: capsula interna, cRTN: caudal part of the RTN, G: globus pallidus, Po: posterior thalamic nuclear group, Th: thalamus, VPL: ventral posterolateral thalamic nucleus. Bars: 250 µm (a,c,d), 100, µm (b), 10 µm (e,f).
antisera revealed similar immunoreactive spots that were rather evenly distributed over all neuronal elements.

In the following paragraphs the quantitative data will be presented for each of the three brain areas studied.

The peri-oral somatosensory cortex

NMDA-NR1 immunoreactivity

Independently from age, rats belonging to the absence epileptic WAG/Rij strain show less NMDA-NR1-immunoreactive spots than ACI rats (P<0.01) (reduction over all cortical layers by –14.7% ± 2.9%). Only in cortical layer I the two rat strains do not clearly differ, neither in 3 nor in 6 months old animals (Fig. 2a). Moreover, the distribution of NMDA-NR1 spots differs among the various layers (P<0.001) in both rat strains (Fig. 2a). The numerical density of NMDA-NR1 spots is high in cortical layers II+III and low in the layers I and VI (Fig. 2a). This differential laminar distribution shows an age-dependency (P<0.01), which is particularly obvious in layer I where in both 6 months old ACI and 6 months old WAG/Rij rats the NMDA-NR1 spot density is remarkably lower than in 3 months old rats (ACI rats: –38.2% ± 7.2%; WAG/Rij rats: –49.2% ± 9.9%; Fig. 2c).

AMPA-GluR4 immunoreactivity

Independently from age, a lower AMPA-GluR4 spot density occurs in WAG/Rij rats compared to ACI rats (P<0.01) (reduction over all cortical layers by –8.7% ± 3.2%). Only in the cortical layers I and II+III no difference can be seen between the two rat strains (Fig. 2b). The AMPA-GluR4-immunoreactive spots are not equally distributed over the cortical layers (P<0.001) as the spot density decreases with cortical depth (Fig. 2b). This phenomenon is independent from rat strain or age (Fig. 2d).

The rRTN

NMDA-NR immunoreactivity

No differences were found in the density of NMDA-NR1-immunoreactive spots between 3 months old ACI and age-matched WAG/Rij rats (Fig. 3a). The same holds for 6 months old rats (Fig. 3a). In addition, no age-effect was observed. These conclusions hold for both the dorsal and ventral part of the rRTN.

AMPA-GluR4 immunoreactivity

In both the dorsal and ventral part of the rRTN in both rat strains, an age-dependent increase (P<0.001) appears in the numerical AMPA-GluR4 spot density (ACI rats: +5.8% ± 2.1%; WAG/Rij rats: +15.2% ± 2%; Fig. 3b). This increase was not seen for the density of rRTN somal spots (Fig. 3c). However, compared to young rats, 6 months old ACI and WAG/Rij rats revealed a higher density (P<0.001) of extrasomal spots (ACI rats: +16.4% ± 11.4%; WAG/Rij rats: +49.8% ± 9.4%; Fig. 3d). This age-dependent increase in extra-
Figure 2:
Numerical NMDA-NR1 subunit [a,c] and AMPA-GluR4 subunit [b,d] spot densities in cortical layers I, II+III, IV, V and VI of the peri-oral somatosensory cortex. [a,b] rats belonging to the ACI ( ■) and WAG/Rij ( ●) rat strain (both 3 and 6 months old rats are represented together). [c,d]: 3 and 6 months old ACI (A3, A6) and WAG/Rij (W3, W6) rats. Note the large, age-dependent decrease in NMDA-NR1 spot density in layer I [c]. Vertical bars represent SEM.

Figure 3:
Quantitative data of NMDA-NR1 subunit [a] and AMPA-GluR4 subunit [b,c,d] spot densities in the rRTN (data from dorsal and ventral sample areas are averaged) of 3 and 6 months old ACI (A3, A6) and WAG/Rij (W3, W6) rats. [a] Numerical NMDA-NR1 spot density. [b] Numerical AMPA-GluR4 spot density. Note the age-dependent increase in both strains. [c] Numerical AMPA-GluR4 spot density associated with rRTN somata. [d] Numerical AMPA-GluR4 extrasomal spot density. Note the age-dependent increase in ACI and especially in WAG/Rij rats (significant strain x age interaction; P<0.01). *: P<0.01; **: P<0.001. Vertical bars represent SEM. Numbers of animals per experimental group are indicated in the bar graphs.
somal spots shows a ‘strain x age’ interaction, as 6 months old WAG/Rij rats have a much higher (P<0.01) spot density than 3 months old WAG/Rij rats (+49.8% ± 9.4%; P<0.001), 3 months old ACI rats (+45.3% ± 8%; P<0.001) and 6 months old ACI rats (+24.8% ± 6.8%; P<0.01; Fig. 3d).

The CA3 region of the hippocampus

NMDA-NR1 and AMPA-GluR4 immunoreactivity

In the CA3 area of the hippocampus, for both antisera similar spot densities were found in all four experimental groups (Table 1).

<table>
<thead>
<tr>
<th>Rat group</th>
<th>NMDA-NR1</th>
<th>AMPA-GluR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>202.7 ± 3.0</td>
<td>208.2 ± 2.3</td>
</tr>
<tr>
<td>A6</td>
<td>202.7 ± 5.3</td>
<td>207.9 ± 1.8</td>
</tr>
<tr>
<td>W3</td>
<td>203.5 ± 7.8</td>
<td>209.9 ± 4.8</td>
</tr>
<tr>
<td>W6</td>
<td>203.4 ± 4.0</td>
<td>208.2 ± 1.9</td>
</tr>
</tbody>
</table>

Table 1:
Numerical NMDA-NR1 and AMPA-GluR4 spot densities (per 1000 µm²) in the CA3 region of the hippocampus of 3 and 6 months old ACI (A3, A6) and WAG/Rij (W3, W6) rats. N=5

Discussion

WAG/Rij rats provide a suitable and well-established model to investigate the mechanisms underlying absence epilepsy (e.g. Inoue et al. 1990; van Rijn et al. 1991; Coenen et al. 1992; Lason et al. 1992, 1994; van Luijtelaar and Coenen 1997). We have used WAG/Rij and ACI rats with ages of 3 and 6 months, to study possible differences in NMDA and GluR4-rich AMPA receptor presence that may be associated with the initiation and maintenance of SWD in WAG/Rij rats in, respectively, the peri-oral somatosensory cortex and the rRTN. WAG/Rij rats develop SWD activity from an age of 3 months onwards, and at 6 months of age they display hundreds of SWD per day. SWD activity starts in the peri-oral somatosensory cortex (Meeren et al. 2002) and is maintained by the pacemaking activity of the rRTN (Meeren 2002). ACI rats have been used as non-epileptic controls, because they show hardly or no SWD activity (Inoue et al. 1990; de Bruin et al. 2000).

Identification of NMDA and GluR4-rich AMPA receptors

Using immunocytochemistry, we have identified NMDA-NR1 and AMPA-GluR4 subunits of glutamate receptors in WAG/Rij and ACI rats of both 3 and 6 months old. As these sub-
units are characteristic for NMDA and GluR4-rich AMPA receptors, respectively (Michaelis 1998; Ozawa et al. 1998; Dingledine et al. 1999), our results indicate the presence of these receptors. At high magnification, NMDA-NR1 and AMPA-GluR4 immunoreactivities are clearly visible as distinct spots that probably represent receptors located closely together, since both NMDA and AMPA receptors have been described to be concentrated in so-called (postsynaptic) ‘hot-spots’ (Jones and Baughman 1991). The presence of NMDA-NR1 and AMPA-GluR4 spots in the cortex, hippocampus and rRTN is in agreement with immunoelectron microscopy indicating that these subunits are enriched in postsynaptic densities of asymmetrical synapses. In addition, our observation that rRTN somata slightly stain with the AMPA-GluR4 serum is consistent with the cytoplasmic synthesis, processing and transport of these proteins (e.g. Petralia and Wenthold 1992; Petralia et al. 1994; Spreafico et al. 1994; Liu 1997).

The peri-oral somatosensory cortex

NMDA and GluR4-rich AMPA receptors display significant, strain-independent, variations in their quantitative distribution across the layers of the peri-oral somatosensory cortex. A similar layer-specific distribution of NMDA receptors was demonstrated in the somatosensory cortex of 3 months old Sprague-Dawley and Wistar rats, using autoradiography (Insel et al. 1990; Jaarsma et al. 1991). As to AMPA receptor binding sites in the somatosensory cortex, these authors did not find a layer-specific distribution, but data on the presence of, specifically, the GluR4-rich AMPA receptor in the (peri-oral) somatosensory cortex are lacking, so that our study is the first to describe that this AMPA receptor subtype is not equally distributed in this area of the cortex.

A special observation is the age-dependent decrease in NMDA receptor staining in cortical layer I. The significance of this decrease is not known, but as such a large age-dependent change in NMDA-NR1 staining was not found in the primary motocortex (M.C. van de Bovenkamp-Janssen, unpubl. res.), the phenomenon seems to be specific for the peri-oral somatosensory cortex. Layer I of the somatosensory cortex is the site where diffuse somatosensory thalamocortical and reciprocal corticocortical projections have excitatory synaptic contacts with the apical dendrites of pyramidal neurones in layer II+III and V (Koralek et al. 1990; Vogt 1991; Cauller and Conners 1994; Cauller et al. 1998). Therefore, NMDA receptors in layer I exert a significant influence on the lateral cortical propagation by modulating the activities of these pyramidal neurones. The expression of the cortical NMDA receptor is known to be related to ageing (e.g. Magnusson 1995; Johnson et al. 1996; Migani et al. 2000; Ossowska et al. 2001; Magnusson et al. 2002), but layer-specific age-dependent changes in NMDA receptor expression in the cortex have not been reported elsewhere.

SWD initiation

SWD activity is initiated in the peri-oral somatosensory cortex of WAG/Rij rats (Meeren et al. 2002). We demonstrate lower NMDA and GluR4-rich AMPA receptor densities in rats
belonging to the WAG/Rij rat strain compared to ACI control rats, a phenomenon especially apparent in the cortical layers IV, V and VI. These deep layers contain the major input-output connectivity with the thalamus and RTN (e.g. Jones 1985; Bode-Greuel et al. 1987; Jensen and Killackey 1987; Bourassa et al. 1995; Steriade et al. 1997). The CA3 region of the hippocampus, an area not involved in SWD activity (Inoue et al. 1993; Kandel et al. 1996), does not show differences in NMDA-NR1 and AMPA-GluR4 receptor subunit immuno-reactivities between the WAG/Rij and the ACI rat strain. This suggests that in the WAG/Rij strain, the low receptor densities in the somatosensory cortex are related to the initiation of SWD. As 3 months old WAG/Rij rats do not yet show substantial SWD activity, a low amount of NMDA and GluR4-rich AMPA receptors might precede the development of SWD activity and perhaps even be the cause of it.

The question arises how lower NMDA and GluR4-rich AMPA receptor densities can stimulate the initiation of SWD. Previously, it was shown that various absence epileptic animal species have hyperexcitable cortical neurones (e.g. Gloor and Fariello 1988; Pumain et al. 1992; Luhmann et al. 1995; Avanzini et al. 1996; Destexhe 1999), which might be due to an increased efficacy of NMDA and AMPA receptor-mediated glutamatergic transmission (Pumain et al. 1992; Avanzini et al. 1996; Koener et al. 1996; Destexhe 1999). Such an increased efficacy would not seem to fit with our observation of reduced receptor presence. It would be also explained by another mechanism, viz. that SWD arise from cortical disinhibition, which can also lead to (postsynaptic) cortical neuronal hyperexcitability (Pumain et al. 1992; Luhmann et al. 1995; Avanzini et al. 1996). Luhmann and Prince (1990) have suggested that even a small decrease in the efficacy of the intracortical GABAergic inhibitory system might play an important role in cortical susceptibility to epileptogenesis. Excitatory synapses on cortical GABAergic interneurones primarily involve AMPA receptors (Thomson and Deuchars 1994). Indeed, in the somatosensory cortex, the AMPA-GluR4 subunit is largely restricted to non-pyramidal GABAergic cells which might represent such interneurones (Ong et al. 1996; Kondo et al. 1997; Munoz et al. 1999). A lower number of AMPA receptors would lead to a decrease in the strength of the inhibitory system and hence to hyperexcitability as seen during absence epilepsy. Therefore, we propose that a low GluR4-rich AMPA receptor expression in WAG/Rij rats reduces cortical inhibition, thus creating the possibility to initiate SWD activity. If such an explanation also holds for the reduced NMDA receptor presence remains to be investigated.

The rRTN

No age or strain differences were observed in NMDA receptor presence in the rRTN. However, we see an age-dependent increase in the numerical density of spots immunoreactive to the AMPA-GluR4 subunit (spots per 1000 µm²), in the rRTN of both WAG/Rij and ACI rats. This strain-independent increase indicates that GluR4-rich AMPA receptors in the rRTN neuronal processes, but not on rRTN somata, increase in number. Indeed, AMPA-GluR4 immunoreactivity is exceptionally high in the rRTN of 3 months old Sprague-
Dawley and Wistar rats (Martin et al. 1993; Spreficco et al. 1994; Mineff and Weinberg 2000) and not in young, postnatal (p1-p20) animals (Spreficco et al. 1994). Our data suggest that this increase continues during ageing after 3 months.

**SWD maintenance**

In WAG/Rij rats, SWD activity is maintained by the pacemaking activity of the rRTN (Meeren 2002). The present immunocytochemical data show that SWD activity in 6 months old, absence epileptic WAG/Rij rats is concomitant with an increase in extrasomal, GluR4-rich AMPA receptor density in the rRTN when compared to presymptomatic (3 months) WAG/Rij rats and age-matched ACI rats.

The rRTN receives a powerful cortical drive provided by a strong glutamatergic corticothalamic input (Ohara and Lieberman 1985; Ohara 1988; Cornwall et al. 1990; Lozsádi 1994; Bourassa et al. 1995; Liu 1997; Liu and Jones 1999). In turn, the GABAergic rRTN neurones (Houser et al. 1980; DeBiasi et al. 1986) inhibit the thalamus (Jones 1985; Steriade et al. 1997). The basis for the generation of low-frequency, synchronous thalamocortical oscillations such as sleep spindles and presumably SWD, lies in an increase in this RTN-generated inhibition of thalamic neurones, which overrides the direct corticothalamic excitatory input to these neurones (von Krosigk et al. 1993, 1999; Warren et al. 1994; Contreras and Steriade 1996; Contreras et al. 1996; Destexhe et al. 1998a). It has been demonstrated that corticothalamic stimulation leads to a stronger excitation of RTN cells than of thalamic neurones, which may be explained by a higher number of postsynaptic GluR4-rich AMPA receptors in the RTN than in the thalamus (Golshani et al. 2000, 2001; Mineff and Weinberg 2000; Liu et al. 2001). We therefore propose that the increased GluR4-rich AMPA receptor density shown by us in the rRTN of 6 months old WAG/Rij rats provides an intensification of the corticothalamic drive to the rRTN, thereby promoting the maintenance of SWD activity in the thalamocortical system in absence epileptic WAG/Rij rats.

**Conclusion**

On the basis of the data obtained in the present study, we propose that in the peri-oral somatosensory cortex of the WAG/Rij rat, SWD start as a result of a reduction of NMDA- and GluR4-rich AMPA-mediated glutamatergic stimulation, whereas in the rRTN of this rat species SWD maintenance may be promoted by a stronger excitation by glutamate via increased GluR4-rich AMPA receptor presence.
CHAPTER 5

Differential expression of high voltage-activated Ca\(^{2+}\) channel types in the rostral reticular thalamic nucleus of the absence epileptic WAG/Rij rat

In press as:
Abstract

In the WAG/Rij rat, a model for human absence epilepsy, spike-wave discharges (SWD) and absence epileptic behaviour develop from an age of 3 months. The rostral part of the reticular thalamic nucleus (rRTN) is involved in the maintenance of SWD. Ca\(^{2+}\) channels play a central role in the initiation and maintenance of burst firing activity of thalamic cells. We hypothesise that a changed expression of \(\alpha_1\) subunits of one or more high voltage-activated Ca\(^{2+}\) channel types in the rRTN underlies the development of SWD. To test this hypothesis we compared 3 and 6 months old WAG/Rij rats with non-epileptic, age-matched control rats. By immunocytochemistry, the expressions of \(\alpha_{1.3}\), \(\alpha_{1.2.1}\), \(\alpha_{1.2.2}\) and \(\alpha_{1.2.3}\) subunits were shown in both strains, demonstrating the presence of Ca\(_{V1.3}\), Ca\(_{V2.1}\), Ca\(_{V2.2}\) and Ca\(_{V2.3}\) channels, respectively. Quantification of channel expression indicates that the development of SWD in WAG/Rij rats is concomitant with an increased expression of Ca\(_{V2.1}\) channels in the rRTN. These channels are mainly presynaptic, as revealed by double-immunofluorescence involving the presynapse marker syntaxin. The mechanism by which this increase could be related to the occurrence of SWD has been discussed.

Introduction

Absence epilepsy is a non-convulsive type of epilepsy, characterised by a decrease in consciousness of abrupt onset and offset accompanied by generalised, bilateral, synchronous, regular, stereotyped and symmetrical spike-wave discharges (SWD). Behavioural changes concern, in human, a sudden interruption of ongoing activities, a blank facial expression with the eyes drifting upward, rhythmical beating of the eyelids and twitches of the mouth (Porter 1993). In rats, absence seizures are associated with a reduced level of consciousness and responsiveness with orofacial myoclonic twitches, most conspicuously of the vibrissae (van Luijtenaar and Coenen 1986; Drinkenburg et al. 2003). The SWD appear to reflect oscillations generated in the thalamocortical network (e.g. Gloor 1968; Gloor et al. 1990; Snead III 1995; Avanzini et al. 2000). This network contains the cerebral cortex, the thalamic relay nuclei, the intralaminar thalamic nuclei and the reticular thalamic nucleus (RTN). All these structures have been implied to play a role in the occurrence of sustained, synchronised, generalised absence epileptic SWD activity (e.g. Vergnes and Marescaux 1992; Inoue et al. 1993; Avanzini et al. 2000; Kostopoulos 2000; Seidenbecher and Pape 2001; Meeren 2002; Meeren et al. 2002). In this study we focus on the RTN, as it has been clearly demonstrated that thalamocortical oscillations are maintained by the pacemaking activity of the RTN (Steriade et al. 1985, 1987; Avanzini et al. 1992). More specifically, recently Meeren (2002) provided evidence that especially the rostral pole of the RTN (rRTN) is essential
for SWD maintenance. How the rRTN controls SWD activity is unknown, but the involvement of low voltage-activated (LVA) T-type Ca\(^{2+}\) channels in SWD initiation has been demonstrated (e.g. Snead III 1995; Tsakiridou et al. 1995; McCormick and Bal 1997; Avanzini et al. 2000; Gomora et al. 2001; Kim et al. 2001). Furthermore, also high voltage-activated (HVA) Ca\(^{2+}\) channels seem to play a role (e.g. McEnery et al. 1998; Burgess and Noebels 1999; van Luijtelaar et al. 1995, 2000; Fletcher and Frankel 1999; Lakaya et al. 2002).

HVA Ca\(^{2+}\) channels comprise the \(\alpha_{v1.3}\), \(\alpha_{v2.1}\), \(\alpha_{v2.2}\) and \(\alpha_{v2.3}\)-types (nomenclature by Ertel et al. 2000), representing L-, P/Q-, N- and R-type currents, respectively. They consist of \(\alpha_1\), \(\alpha_2\), \(\delta\), \(\beta\) and \(\gamma\)-subunits, but are individually characterised by channel-specific isoforms of the \(\alpha_1\) subunit (\(\alpha_{1.1,3}\), \(\alpha_{1.2,1}\), \(\alpha_{1.2,2}\) and \(\alpha_{1.2,3}\); Catterall 1998, 2000). There is an increasing body of evidence for the role of HVA Ca\(^{2+}\) channels in absence epilepsy. In the tottering mouse, a mutation in the \(\alpha_{1.2.1}\) gene has been demonstrated (Fletcher et al. 1996; Burgess and Noebels 1999; Fletcher and Frankel 1999). In addition, reshuffling of the \(\beta\) subunit of \(\alpha_{v2.1}\) and/or \(\alpha_{v2.2}\) channels may cause the pathological phenotype of the lethargic mouse, including SWD activity (McEnery et al. 1998; Burgess et al. 1999; Burgess and Noebels 1999). Interestingly, pharmacological inhibition of P/Q-type currents inhibits SWD, while inhibition of L-type currents promotes absence epilepsy in the WAG/Rij rat (van Luijtelaar et al. 1995, 2000). Moreover, a decreased expression in \(\alpha_{1.2.3}\) mRNA was demonstrated in the brainstem and cerebellum of the epileptic GAERS rat (Lakaya et al. 2002).

We hypothesise that absence epilepsy is related to a changed expression of \(\alpha_1\) subunits of HVA Ca\(^{2+}\) channels in the rRTN. To test this hypothesis we used a well-characterised animal model for human absence epilepsy, the WAG/Rij rat, which displays SWD without further pathophysiologic symptoms (Coenen et al. 1992; van Luijtelaar and Coenen 1997). SWD activity develops between 3 and 6 months of age, with full symptoms in 6 months old WAG/Rij rats. The expression of \(\alpha_{1.1,3}\), \(\alpha_{1.2,1}\), \(\alpha_{1.2,2}\) and \(\alpha_{1.2,3}\) subunits was studied in the rRTN which is responsible for the maintenance of SWD (Meeren 2002). Immunoreactivity was quantified in non-epileptic (3 months old) and absence epileptic (6 months old) WAG/Rij rats, using ACI rats as non-epileptic, age-matched controls (Inoue et al. 1990; van Rijn et al. 1991; Lason et al. 1992, 1994; de Bruin et al. 2000). We show that SWD in WAG/Rij rats are associated with an increased expression of \(\alpha_{v2.1}\) channel-related \(\alpha_{1.2.1}\) subunits.

**Methods**

**Animals**

Experiments were carried out with four groups of minimally 5 male rats, i.e., WAG/Rij and ACI rats with ages of 3 and 6 months. They had been bred and reared under standard conditions in our Department of Biological Psychology, University of Nijmegen. All experiments were carried out under the guidelines of the Dutch law concerning animal welfare. Unless stated otherwise, chemicals were from Merck (Darmstadt, Germany).
Tissue preparation

Animals were deeply anaesthetised by intraperitoneal administration of 40 mg sodium pentobarbital (Sanofi Sante, Maassluis, The Netherlands) and intracardially perfused with 4% paraformaldehyde in sodium phosphate buffer (PB; 72 mM Na₂HPO₄, 27.5 mM NaH₂PO₄; pH 7.4) containing 0.05% picric acid. Subsequently, they were decapitated and their brains immediately dissected and postfixed in the same fixative, for 20 hr at 4 °C. For single labelling immunocytochemistry, brains were cryoprotected by immersion in 30% sucrose (Mallinckrodt, Deventer, The Netherlands), for 48 hr. Serial (40 µm) coronal sections including the rRTN were cut on a Microm HM 440 E sliding freeze-microtome (Microm, Walldorf, Germany) and stored in 0.1 M PBS (PB-buffered saline: 84 mM Na₂HPO₄, 22 mM NaH₂PO₄, 137 mM NaCl, 2.7 mM KCl; pH 7.4). For double-labelling immunofluorescence, 20 µm thick brain sections were cut on a Vibratome VT 1000S (Leica, Rijswijk, The Netherlands) and stored in 0.1 M PBS.

Immunocytochemistry

Sections were processed for single labelling, free-floating immunocytochemistry, to visualise α₁ subunits of HVA Ca²⁺ channels, using the ABC method (Hsu et al. 1981). Rabbit anti-α₁₁.1, anti-α₁₁.2 and anti-α₁₁.3 sera had been raised in our Department of Neurosciences, Physiology and Biophysics, and Physics, Case Western University School of Medicine. Rabbit anti-α₁₂.1 serum was purchased from Alomone Research (Jerusalem, Israel). Sections were incubated in 0.1 M PBS containing 0.3% H₂O₂ (Lamers and Pleuger, ’s Hertogenbosch, The Netherlands), for 30 min. Non-specific binding sites were blocked and tissue permeability was increased by 1% BSA (ICN Biomedicals, Aurora, Ohio, USA) and 0.5% Triton-X 100 (Sigma, St. Louis, MO, USA), respectively, in 0.1 M PBS (PBS-BT), for 30 min. Then, sections were incubated either in rabbit anti-α₁₂.1 (1:400 in PBS-BT), rabbit anti-α₁₂.2 (1:200 in PBS-BT), rabbit anti-α₁₂.3 (1:200 in PBS-BT) or rabbit anti-α₁₁.3 (1:200 in 0.1 M Tris-saline buffer: 50 mM Tris, 150 mM NaCl; pH 7.4, with 1% BSA and 0.5% Triton-X 100), for 60 hr at 4 °C. After incubation, sections were incubated in goat anti-rabbit biotin-conjugated IgG (Vector Laboratories, Burlingame, CA, USA) (diluted 1:1500 in 0.1 M PBS-BT), for 90 min. For signal amplification, sections were incubated for 90 min in ABC (Vector Laboratories) (diluted 1:800 in 0.1 M PBS-BT) and visualised by 0.025% w/v 3,3’-diaminobenzidine (DAB) (Sigma) in 50 mM Tris buffer with 0.25% w/v nickel ammonium sulfate (BHD Laboratory Supplies, Poole, UK), for 10 min. Sections were mounted on poly-L-lysine-coated glass slides, dehydrated and coverslipped with Entellan.

For double-labelling immunofluorescence with rabbit anti-α₁₂.1 and mouse anti-syntaxin (generous gift from Dr. Hong Ying, Department of Psychiatry, University of British Colombia, Vancouver, Canada) free-floating sections were incubated in 0.1 M PBS containing 0.5% Triton-X 100, for 30 min, to increase tissue permeability. Non-specific binding sites were blocked by 2% normal donkey serum in 0.1 M PBS, for 30 min. Subsequently, sections were incubated in a cocktail of rabbit anti-α₁₂.1 (1:500 in 0.1 M PBS) and mouse
anti-syntaxin (1:100 in 0.1 M PBS), for 16 hr at room temperature. Then sections were rinsed, incubated in 0.1 M PBS containing both Cy2-conjugated donkey anti-mouse (Jackson Immunoresearch Labs, Inc., West Grove, PA, USA; 1:100 in 0.1 M PBS) and Cy3-conjugated donkey anti-rabbit (Jackson Immunoresearch Labs; 1:100 in 0.1 M PBS), for 3 hr. They were mounted on poly-L-lysine-coated glass slides and coverslipped with Fluorsave (Calbiochem, San Diego, CA, USA). Immunofluorescent sections were examined with a BioRad MCR 1024 using Lasersharp 2000 software (Hemel Hempstead, Hertfordshire, UK).

Control sections were treated as described above, but with omission of the respective primary antisera. Moreover, controls were performed with antisera previously absorbed with 1000x excess of the native subunit protein, for 3 hr at 22 °C.

Quantifications and statistics

Per animal, quantifications were made in the centre of the 40 µm thick, medial section of the rRTN, at the level of Bregma –1.40 mm and Interaural 7.60 mm (Paxinos and Watson 1997) (Fig. 1a). Previously, we have found that the centre of the medial rRTN can be considered to be representative for the rRTN considering numerical cell density and mean numbers of immunoreactive spots per cell soma, for each antiserum. The numbers of anti-α1.3, anti-α1.2.1 and anti-α1.2.3 labelled cells were counted by direct microscopic (Nikon, Tokyo, Japan) examination, using a square graticule at a final magnification of x100, and expressed per mm². Furthermore, the numbers of anti-α1.3-, anti-α1.2.1- and anti-α1.2.3-immunoreactive spots were counted per labelled cell soma, at a final magnification of x1000, using a x100 oil immersion objective. Counts were made in a focus plane medially through the soma. All data are presented as means ± SEM.

Data were tested by a two-way ANOVA followed by Duncan’s multiple range test (α=5%), using Statistica (StatSoft, Tulsa OK, USA), after appropriate transformation of the original data on the basis of tests for normality (Shapiro-Wilk test) and for homogeneity of variance (Bartlett’s Chi-square test) (Shapiro et al. 1968; Winer et al. 1991).

Results

Generally, clear immunoreactivity against each of the four α1 subunits was found in all experimental groups throughout the brain sections studied, including adequate staining of the cerebral cortex, the hippocampus, the thalamic nuclei and the rRTN (Fig. 1a,b). At low magnification, immunoreactivity was concentrated in neuronal somata (Fig. 1c). At high magnification, immunoreactivity appeared in two ways: as smoothly, moderately stained areas, and as distinct spots (Fig. 2a-d). Omission of primary antisera abolished all immunoreactivity and no immunoreaction was observed when a serum was used that had been previously absorbed with its native subunit protein antigen. These control data hold for all experimental rat groups.
Figure 1:

Immunoreactivity (ir) spots indicating different Ca$^{2+}$ channel $\alpha_1$ subunits in brains of 3 and 6 months old ACI (A3, A6) and 6 months old WAG/Rij (W6) rats. [a] $\alpha_{1\,2.3}$ ir in the medial section of the rRTN and anterior thalamic nuclei (T) of A6, at the level of Bregma –1.40 mm and Interaural 7.60 mm (Paxinos and Watson 1997). [b] $\alpha_{1\,1.3}$ ir in hippocampus (H) of A3. [c] $\alpha_{1\,2.3}$ ir in the medial section of the rRTN of A6, demonstrating the clear presence of dark immunoreactivity in neuronal somata (arrows) and smoothly, moderately stained areas (asterisks) that were interrupted by only faintly or unstained areas with neuronal fibres (F). [d] $\alpha_{1\,2.2}$ ir in the medial section of the rRTN of W6. Notice the absence of clearly identifiable immunoreactive somata. All immunoreactivity appears in smoothly, moderately stained areas (asterisks), interrupted by faintly or unstained areas with neuronal fibres (F). C: corpus callosum, Ci: capsula interna. Bars: 1 mm (a,b), 50 $\mu$m (c,d).

Attention was focused on the rRTN. At low magnification, staining with the $\alpha_{1\,1.3}$, $\alpha_{2\,1}$ and $\alpha_{1\,2.3}$ antisera revealed in all experimental rat groups dark stained neuronal somata and smoothly, moderately stained areas throughout the rRTN that were interrup-
ted by only faintly or unstained regions, possibly as a result of an uneven distribution of \( \alpha_1 \) subunits over different neural elements (e.g. neuronal somata, axon fibers and glial cells) (Fig. 1c). Moreover, at high magnification, these antisera revealed immunoreactive spots that appeared to be predominantly and consistently present over neuronal somata, making these spot-labelled somata easily identifiable (Fig. 2a-c). Occasionally, spots were seen outside the neuronal somata. The density of spots on cell somata was high for each antiserum, with at least minimally 15 spots per soma. Therefore, individual \( \alpha_{1.3} \), \( \alpha_{1.2.1} \) and \( \alpha_{1.2.3} \) labelled cells could be readily identified at low magnification (Fig. 1c). For each of the antisera the spot size was fairly constant (mean diameter 0.5-0.7 \( \mu \)m, with about 10% of the spots having a diameter between 0.7 and 1.0 \( \mu \)m) and no spot size difference between the four experimental rat groups, nor between the different antisera, were visible.

Staining with the \( \alpha_{1.2.2} \) antiserum did not reveal clear immunoreactive neuronal somata at low magnification (Fig. 1d) and at high magnification, spots occurred rather evenly distributed throughout the rRTN (Fig. 2d), a situation which was qualitatively equal among the four experimental rat groups. Neuronal somata could not be distinguished with sufficient accuracy to permit reliable quantification of the number of spot-labelled somata and the number of spots per cell soma.

Therefore, we quantified the \( \alpha_{1.1.3} \), \( \alpha_{1.2.1} \) and \( \alpha_{1.2.3} \) immunoreactivity in the rRTN of 3 and 6 months old WAG/Rij and ACI rats. Quantification was done by determining the numerical densities of spot-labelled somata per tissue area (number of somata per mm\(^2\)) and the numbers of spots per spot-labelled soma (Fig. 3). In the following paragraphs the quantitative data will be presented for each of the three subunits studied.

The \( \alpha_{1.2.3} \) subunit

No differences were found in the density of the \( \alpha_{1.2.3} \) labelled cells between young WAG/Rij and young ACI rats (Fig. 2a, 3a). Six months old ACI rats show a small increase in the density of \( \alpha_{1.2.3} \) labelled cells (+8% ± 7%; P<0.05) compared to 3 months old ACI rats (Fig. 3a). No increase was seen between young and old WAG/Rij rats. As to \( \alpha_{1.2.3} \)-immunoreactive spots per soma, both young and old rats show very similar numbers, independent of rat strain.

The \( \alpha_{1.1.3} \) subunit

Quantifying the numerical density of \( \alpha_{1.1.3} \) labelled cells in the rRTN of young (3 months) animals shows no difference between WAG/Rij and ACI rats (Fig. 3b). Also with regard to the number of immunoreactive spots per soma, the young WAG/Rij and ACI rats are very similar. Equal numbers of both the density of cells and spots per soma were also observed in the old (6 months) WAG/Rij and ACI rats (Fig. 2b, 3b). However, compared to young rats, old ACI and WAG/Rij rats demonstrated a remarkable decrease in the density of \( \alpha_{1.1.3} \) labelled somata (WAG/Rij -44% ± 6%; P<0.001, ACI -50% ± 7%; P<0.001) (Fig. 2e,f, 3b). Also the numbers of \( \alpha_{1.1.3} \)-positive spots per soma are lower with age in both rat strains (WAG/Rij -13% ± 4%; P<0.001, ACI -14% ± 3%; P<0.001) (Fig. 3b).
Figure 2:
Rat rRTN with immunoreactive (ir) spots indicating α1 subunits of four different Ca\(^{2+}\) channels, predominantly located on neuronal somata (arrowheads) but also outside somata (arrows), e.g. in areas with neuronal fibres (F). Asterisks are in moderately stained areas. [a] α\(_{12.3}\) ir spots in 6 months old ACI rat. [b] α\(_{11.3}\) ir spots in WAG/Rij rat of 3 months. [c] α\(_{12.1}\) ir spots in 6 months old WAG/Rij rat. [d] α\(_{12.2}\) ir spots dispersed over various nervous elements in 6 months old WAG/Rij rat. [e,f] ACI rats show more α\(_{11.3}\) labelled cells at 3 months [e] than at 6 months [f]. Bars: 10 \(\mu\)m [a-d], 50 \(\mu\)m [e,f].
Figure 3:
Quantitative data of α1 subunit expression in rRTN of 3 and 6 months old ACI (A3, A6) and WAG/Rij (W3, W6) rats. [a] α12.3, [b] α11.3, [c] α12.1. The number of labelled cells is given in the left column, the number of labelled spots per soma in the right column. Vertical bars represent means ± SEM. Asterisks indicate significant differences (*P<0.05, **P<0.01, ***P<0.001). Numbers of animals per experimental group are given in the bar graphs.
The $\alpha_{1.2.1}$ subunit

The density of $\alpha_{1.2.1}$ labelled somata was not different between young ACI rats and young non-epileptic WAG/Rij rats (Fig. 3c). In old (6 months) ACI rats a similar density was found, but in old, absence epileptic WAG/Rij rats the density of $\alpha_{1.2.1}$ labelled somata is significantly higher: compared to old ACI rats, the cell density has increased by $+29\% \pm 15\%$ ($P<0.01$) and in comparison to young ACI and young WAG/Rij rats increases of $+44\% \pm 13\%$ ($P<0.001$) and $+44\% \pm 17\%$ ($P<0.001$), respectively, are present (Fig. 3c). The picture is similar for the number of $\alpha_{1.2.1}$-positive spots per labelled soma (Fig. 2c). Old, absence epileptic WAG/Rij rats differ from the other three experimental groups, the number of spots being significantly higher compared to young WAG/Rij and ACI rats (+10% ± 5%; $P<0.01$, and +8% ± 4%; $P<0.05$, respectively) and old ACI rats (+14% ± 3%; $P<0.001$) (Fig. 3c).

To investigate whether the staining observed with the $\alpha_{1.2.1}$ antiserum is associated with presynaptic terminals on rRTN cell somata, we carried out free-floating immunofluorescence double-labelling with syntaxin as a presynaptic marker (cf. Westenbroek et al. 1995). Immunoreactivity with the $\alpha_{1.2.1}$ serum appears in a similar way as was seen with single label-DAB immunocytochemistry, and staining with syntaxin antiserum highlighted nerve terminals scattered throughout the rRTN. The merged image of the staining pattern for anti-$\alpha_{1.2.1}$ (red spots) and anti-syntaxin (green spots) demonstrated the co-existence of the two antisera, visible as yellow spots. The co-existence is clearly present on the cell soma (Fig. 4).

**Discussion**

We have used WAG/Rij and ACI rats with ages of 3 and 6 months, to study possible differences in HVA Ca$^{2+}$ channel expressions associated with the development of SWD in WAG/Rij rats. WAG/Rij rats develop SWD activity after the age of 3 months and at 6 months of age they display hundreds of SWD per day. ACI rats are considered as non-epileptic controls, since they show no, or hardly any SWD activity at the age of either 3 or 6 months (Inoue et al. 1990; de Bruin et al. 2000). Therefore, WAG/Rij rats, in combination with age-matched ACI control rats, provide a suitable and well-established model to investigate fundamental neuronal properties underlying the generation of SWD (e.g. Inoue et al. 1990; van Rijn et al. 1991; Coenen et al. 1992; Lason et al. 1992, 1994; van Luijtelaar and Coenen 1997).

**Identification of HVA Ca$^{2+}$ channels**

Using immunocytochemistry, we have identified $\alpha_{1.1.3}$, $\alpha_{1.2.1}$, $\alpha_{1.2.2}$ and $\alpha_{1.2.3}$ subunits of HVA Ca$^{2+}$ channels in WAG/Rij and ACI control rats, thereby indicating the presence of Cav1.3, Cav2.1, Cav2.2 and Cav2.3 channels, respectively. For the first time, HVA Ca$^{2+}$ channel expression has been quantitatively compared between these two rat strains in relation to absence epileptogenesis. Although the $\alpha_1$ subunit is the pore-forming subunit of HVA Ca$^{2+}$ channels and by itself sufficient to produce a functional channel, co-expres-
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Figure 4:
Coronal sections through the rRTN of 6 months old WAG/Rij rats stained with anti-\(\alpha_{12.1}\) (red) and anti-syntaxin (green). Yellow spots indicate coexistence of \(\alpha_{12.1}\) subunits with syntaxin in presynaptic terminals contacting neuronal somata. Asterisks indicate somata. Bars: 10 \(\mu\)m (a), 5 \(\mu\)m (b).

Expression with the auxiliary subunits can modulate channel function, and the assembly, trafficking and membrane localisation of the subunit (Catterall 1998, 2000). Therefore, the expression of the subunits demonstrated in this study is likely, but not necessarily, related to the activity of the respective HVA Ca\textsuperscript{2+} channels. Similarly, although it is likely that the demonstrated changes in \(\alpha_1\) immunoreactivity reflect changes in membrane-bound channel expression, we cannot rule out that part of the observed changes underlies de novo synthesised subunits that are on their cytoplasmic way to the cell membrane. Immuno-electron microscopy could provide qualitative information about the subcellular location of the different \(\alpha_1\) subunits.

At high magnification, some immunoreactivity appears as smoothly and moderately stained areas, which possibly indicate evenly distributed, single HVA Ca\textsuperscript{2+} channels. Moreover, immunoreactivity is visible as distinct spots, which probably represent channels that are located closely together or are clustered. Similar spots, immunoreactive with HVA Ca\textsuperscript{2+} channel \(\alpha_1\) subunit antisera, were found in the adult Sprague-Dawley rat brain (Westenbroek et al. 1992, 1995; Hell et al. 1993; Yokoyama et al. 1995). For Ca\textsubscript{v}1.3 channels a clustering on thalamic neuronal somata and their processes has been described (Budde et al. 1998). Therefore, the \(\alpha_{1.3}\)-immunoreactive spots observed in our study most likely are clusters of Ca\textsubscript{v}1.3 channels. In analogy, we assume that the observed \(\alpha_{1.2.1}\), \(\alpha_{1.2.2}\) and \(\alpha_{1.2.3}\)-immunoreactive spots in our material represent clusters of Ca\textsubscript{v}2.1, Ca\textsubscript{v}2.2 and Ca\textsubscript{v}2.3.
channels, respectively. Our data indicate that the average size of the channel clusters is fairly constant between different types of channel, ages and rat species studied.

**Ca\textsubscript{V}2.2 channels**

The $\alpha_{12.2}$-type $\text{Ca}^{2+}$ channel is widely expressed in the rat brain. In line with our observations, the $\text{Ca}_v^{2.2}$ channels seem to be evenly distributed along the surface of neuronal elements including dendrites, cell bodies and synaptic sites (Westenbroek et al. 1992), indicating a role in dendritic action potentials and neurotransmitter release (Catterall 2000). No reliable quantification of the number of spots per soma spots nor of the numerical cell soma density could be made. However, no obvious differences were observed in the $\alpha_{12.2}$ expression in the rRTN between the four groups suggesting that no clear correlation exists between the expression of $\alpha_{12.2}$ subunits in the rRTN and the generation of SWD in the WAG/Rij rat. On the other hand, we can not rule out that the $\text{Ca}_v^{2.2}$ channel in the rRTN is involved in absence epilepsy in the WAG/Rij rat, because we could not quantify the expression, neither did we study other subunits of this channel type. A mutation in the $\beta_4$ subunit may be related to increased epileptic seizure susceptibility of the lethargic mouse (McEnery et al. 1998).

**Ca\textsubscript{V}2.3 channels**

The physiological significance of $\text{Ca}_v^{2.3}$ channels is only partly known, but evidence exists that the channel promotes presynaptic neurotransmitter release (Wu et al. 1998; Allen 1999; Catterall 2000; Gasparini et al. 2001). Our quantification of the $\alpha_{12.3}$ subunit expression demonstrates a slight, but significant, age-dependent increase in the density of $\alpha_{12.3}$ labelled rRTN somata in the ACI rats. No such increase appears in the WAG/Rij rat strain ageing from 3 to 6 months old, the period in which this rat strain develops absence epileptic SWD. This absence of an increase in $\text{Ca}_v^{2.3}$ expression might reflect an impaired development of this channel in WAG/Rij rats, and therefore be related to the generation of SWD and, hence, of absence epilepsy.

A decreased expression of $\text{Ca}_v^{2.3}$ channel mRNA has been observed in the cerebellum and brain stem of epileptic GAERS rats but not in young, non-epileptic GAERS rats or non-epileptic control rats (Lakaye et al. 2002). The brain stem is not known to be a site of SWD generation, although there are many projections to the rRTN (Cornwall et al. 1990), which can modulate oscillatory activity (e.g. Filakovszky et al. 1999; de Bruin et al. 2001; Deransart et al. 2001). Moreover, the deep nuclei of the cerebellum show phase-locked firing with cortical SWD (Kandel and Buzsáki 1993).

**Ca\textsubscript{V}1.3 channels**

Expression of $\alpha_{1.3}$ subunits is mainly seen on neuronal somata and their proximal dendrites (Hell et al. 1993). Calcium entry through these channels is thought to influence the cyto-
solic calcium levels controlling various processes such as gene expression, regulation of cellular signalling pathways and membrane excitability (Catterall 1998, 2000; Finkbeiner and Greenberg 1998). We have found in the rRTN a strong, age-dependent decrease in the expression of the Cav1.3 channel, as appears from the numerical densities of $\alpha_{1.3}$ labelled somata and the number of $\alpha_{1.3}$-immunoreactive spots per soma. A similar age-associated decrease in Cav1.3 channel expression was previously demonstrated in the rat thalamus, between the age of 3 weeks and 6 months (Araki et al. 1997). The decrease observed in our study is similar in WAG/Rij and ACI rats, indicating that it is not strain-specific. Therefore, the data do not strengthen the hypothesis that a change in Cav1.3 channel expression in the rRTN plays a main role in the occurrence of SWD. As a consequence, earlier data demonstrating that pharmacological blocking of L-type currents promotes SWD in WAG/Rij rats (van Luijtelaar et al. 1995, 2000) may not concern the expression of the Cav1.3 channel but rather its assembly, modulation and/or biophysical properties. Moreover, L-type Ca$^{2+}$ currents formed by $\alpha_{1.2}$ and/or $\alpha_{1.4}$ subunits may account for the pharmacological results observed by van Luijtelaar et al. (1995, 2000), or it may be that the intraperitoneally injected channel blocker nimodipine used in these studies did not act on the rRTN but somewhere else in the thalamocortical system.

**Cav2.1 channels**

Cav2.1 channels are present in high densities in central synapses (Westenbroek et al. 1995) and there is extensive evidence for an important presynaptic role of these channels in neurotransmitter release (Takahashi and Momiyama 1993; Dunlap et al. 1995; Tareilus and Breer 1995; Catterall 1998, 1999, 2000). Our demonstration of co-existence of the $\alpha_{2.1}$ subunit with the presynaptic protein syntaxin shows that also in the rRTN Cav2.1 channels are located at presynaptic nerve terminals, and that they contact neuronal somata. The present immunocytochemical data show that the development of absence epilepsy in 6 months old WAG/Rij rat is concomitant with an increase in Cav2.1 channel expression in the rRTN when compared to presymptomatic (3 months old) WAG/Rij rats and age-matched ACI rats. This increase appears not only from the strong increase in the density of $\alpha_{2.1}$ labelled somata but also from the increased number of $\alpha_{2.1}$-immunoreactive spots per soma. We therefore conclude that the increase in the expression of Cav2.1 channels in the rRTN of 6 months old WAG/Rij rats is correlated with the manifestation of SWD activity in this animal. The fact that the increased Cav2.1 channel expression is correlated with the occurrence of SWD does not provide direct information about whether this increase is causative, compensatory or reactive to absence epilepsy. To get in an experimental way insight in this issue, the functioning of the Cav2.1 channel might be studied in WAG/Rij rats chronically exposed to an anti-epileptic drug that does not influence Ca$^{2+}$ channels, such as valproate (Peeters et al. 1988; Loscher 2002) or ZK 91296 (Petersen 1984; Coenen and van Luijtelaar 1988). Moreover, in the future it might become possible to study the effect of Cav2.1 channels on absence epileptogenesis by genetically engineered, tissue (rRTN)-specific deletion or overexpression of the $\alpha_{2.1}$ gene.
Role of \( \text{Ca}_v2.1 \) channels in SWD activity

In cerebellar Purkinje cells of the tottering and the leaner mouse, two strains exhibiting SWD, a reduction rather than an increase in \( \text{Ca}_v2.1 \) channel-related P/Q-type current density was found (Lorenzon et al. 1998; Wakamori et al. 1998). This reduction has been associated with a reduced channel open probability, rather than a reduced channel expression (Dove et al. 1998; Lau et al. 1998). Theoretically, a reduced open channel probability might be compensated for by an increased expression of such a channel. In the present study we only investigated the expression of \( \alpha_{12.1} \) subunits so that we cannot comment on possible changes in P/Q-type \( \text{Ca}^{2+} \) currents in the rRTN of 3 and 6 months old WAG/Rij rats. Patch clamp studies on rRTN neurones could provide a more detailed insight in the HVA \( \text{Ca}^{2+} \) dynamics in relation to the development of SWD in the WAG/Rij rat.

\( \text{Ca}_v2.1 \) channels might change the excitation of rRTN neurones

Afferent pathways to the rRTN, arising from the cortex and thalamus but also from the basal forebrain and brain stem areas (Cornwall et al. 1990) influence the balance between excitation and inhibition within the rRTN. Therefore, it seems likely that the \( \alpha_{12.1} \)-immunoreactive spots shown in our material on neuronal somata, represent (clusters of) \( \text{Ca}_v2.1 \) channels located in presynaptic nerve endings that originate from one or more of these brain areas, and abut somata and/or dendritic processes in the rRTN. An increased expression of these \( \text{Ca}_v2.1 \) channels might lead to a changed balance between excitation and inhibition of rRTN neurones. Oscillations generated within the thalamocortical loop are known to be sensitive to small changes in excitation or inhibition that lead to abnormal synchrony (Gloor et al. 1990; Huntsman et al. 1999) and, possibly, to SWD. It is therefore interesting to pursue whether \( \text{Ca}_v2.1 \) channel number and, perhaps, \( \text{Ca}_v2.1 \) channel functioning in relation to neurotransmitter release in the rRTN, are crucial in the process of SWD generation.

\( \text{Ca}_v2.1 \) channels might affect LVA \( \text{Ca}^{2+} \) currents

Another possibility is that the observed increase in \( \alpha_{12.1} \) subunit expression in the rRTN changes the activity of the \( \text{Ca}_v2.1 \) channel. Zhang et al. (2002) demonstrated that a change in \( \text{Ca}_v2.1 \) channel activity potentiates LVA T-type currents in the tottering mouse. The role of LVA \( \text{Ca}^{2+} \) channels in absence epilepsy is well established and potentiation of the T-type current enhances SWD activity (Tsakiridou et al. 1995; Zhang et al. 2002). Therefore, the found increase in \( \text{Ca}_v2.1 \) channels in the rRTN of the absence epileptic WAG/Rij rat might affect LVA \( \text{Ca}^{2+} \) currents and in that way be involved in SWD activity.
CHAPTER 6

Parvalbumin in de thalamocortical system of absence epileptic WAG/Rij rats

Slightly modified in press as:
Abstract

In the WAG/Rij rat, a model for human absence epilepsy, spike-wave discharges (SWD) and absence epileptic behaviour are fully symptomatic at an age of 6 months. The somatosensory cortex contains the site of SWD initiation, whereas the rostral part of the reticular thalamic nucleus (rRTN) maintains SWD activity by acting as a pacemaker. The hypothesis is tested that the brain disorder underlying absence epilepsy is related to a changed distribution of neuronal parvalbumin (PV) within the thalamocortical system. Quantitative immunocytochemistry of PV shows that, in comparison to non-epileptic ACI control rats, the number of PV-containing neurones in WAG/Rij rats is similar in the rRTN but considerably lower in the somatosensory cortex (parietal cortex area 1 and the forelimb area). The possible significance of this reduction in the development of SWD is discussed.

Introduction

The WAG/Rij rat is a well-established model to study the neuronal mechanism of human absence epilepsy, as it shows the characteristic absence epileptic spike-wave discharges (SWD) associated with this brain disorder. The SWD appear when WAG/Rij rats reach an age older than three months, and both the number and incidence of SWD increase with age, with full symptoms in six months old rats (Coenen et al. 1992; van Luijtelaar and Coenen 1997). SWD are generated in a neuronal network involving cortical and thalamic areas in both hemispheres (e.g. Gloor et al. 1990; Avanzini et al. 2000). The somatosensory cortex is assumed to contain the site of SWD initiation (Meeren et al. 2002) whereas the rostral part of the reticular thalamic nucleus (rRTN) probably maintains SWD activity by acting as a pacemaker (Avanzini et al. 2000; Meeren 2002).

Calcium channels play an important role in absence epilepsy, since the occurrence of synchronous neuronal action potential bursting in the RTN depends on the interaction between low- and high-threshold Ca$^{2+}$ currents that are mediated by T- and L-type voltage-operated Ca$^{2+}$ currents, respectively, and on a Ca$^{2+}$-activated K$^{+}$ conductance (McCormick and Bal 1997). In WAG/Rij rats, manipulation of T-, L- and P/Q-type Ca$^{2+}$ currents regulates the number of SWD (van Luijtelaar et al. 2000). Recent quantitative immunocytochemical studies indicated the involvement of P/Q-type channels in absence epileptogenesis in the rRTN of the WAG/Rij rat (van de Bovenkamp-Janssen et al. 2003). Upon influx of Ca$^{2+}$ through Ca$^{2+}$ channels, the ion is readily bound to intracellular Ca$^{2+}$ buffers to protect cells from a Ca$^{2+}$ overload. The Ca$^{2+}$ binding protein parvalbumin (PV) has been implicated in various forms of brain disorders, including ischemia and mesial temporal lobe epilepsy (Freund et al. 1990; Johansen et al. 1990, Bouilleret et al. 2000). PV co-exists with GABA in GABAergic neurones (Celio 1986, 1990) where it protects neurones from
neurotoxic Ca\textsuperscript{2+} overload during prolonged depolarisation (Heizmann et al. 1990). Changes in neuronal PV contents are associated with changes in neuronal activity, as PV can influence the membrane potential by buffering Ca\textsuperscript{2+} ions entering the cell upon depolarisation (Kawaguchi et al. 1987).

PV is abundant within the thalamocortical system as both the cortex and especially the RTN contain numerous GABAergic neurones (Houser et al. 1980; Celio 1990). In the light of the important role of both voltage-activated Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+}-activated K\textsuperscript{+} conductances in absence epileptic SWD activity, and in view of the involvement of PV in different brain disorders, we hypothesise that absence epilepsy in the WAG/Rij rat is related to a disturbed PV distribution within the RTN and cortex. Such a disturbance could underlie the generation and/or maintenance of SWD. This hypothesis has been tested by assessing immunocytochemically the presence of PV in the thalamocortical system, including the somatosensory cortex and rRTN, the site of SWD initiation and maintenance, respectively, comparing absence epileptic WAG/Rij rats with age-matched, non-epileptic ACI control rats (Inoue et al. 1990).

**Methods**

**Animals**

Eleven ACI and 11 WAG/Rij rats, bred and reared under standard conditions in the Department of Biological Psychology, with an age of six months, were used. All experiments were carried out under the guidelines of the Dutch law concerning animal welfare.

**Tissue preparation**

Animals were anaesthetised by intraperitoneal administration of 40 mg sodium pentobarbital (Sanofi Sante, Maassluis, The Netherlands) and intracardially perfused with 4% paraformaldehyde in PB (72 mM Na\textsubscript{2}HPO\textsubscript{4}, 27.5 mM NaH\textsubscript{2}PO\textsubscript{4}; pH 7.4), for 20 min. Brains were dissected, postfixed in the same fixative for 16 hrs, and immersed in 30% sucrose for cryoprotection (Mallinckrodt, Deventer, The Netherlands), for 48 hrs. Coronal and horizontal 40 µm sections were cut on a Microm HM 440 E sliding freeze-microtome (Microm Int., Walldorf, Germany) and stored in PBS (PB-buffered saline: 84 mM Na\textsubscript{2}HPO\textsubscript{4}, 22 mM NaH\textsubscript{2}PO\textsubscript{4}, 137 mM NaCl, 2.7 mM KCl; pH 7.4).

**Histology and immunocytochemistry**

To examine general brain morphology, sections of 4 WAG/Rij and 4 ACI rats were stained 0.1% cresyl violet (CV).

For free-floating immunocytochemistry, brain slices of 7 rats per strain were incubated in 0.1 M PBS containing 0.3% H\textsubscript{2}O\textsubscript{2} (Lamers and Pleuger, ‘s Hertogenbosch, The
Netherlands), for 30 min. Incubation for 30 min in 0.1M PBS-BT (0.1M PBS containing 1% BSA (ICN Biomedicals, Aurora, Ohio, USA) and 0.5% Triton-X 100 (Sigma, St Louis, MO, USA) blocked non-specific binding sites and increased tissue permeability, respectively. To study neuronal PV, slices of 5 WAG/Rij and 5 ACI rats were incubated in monoclonal mouse anti-PV (1:5000 in PBS-BT; Sigma; Celio et al. 1988). Brain slices of 2 rats per strain were incubated in mouse anti-M30 Cytodeath (1:2000 in PBS-BT; Boehringer Mannheim, Mannheim, Germany; Leers et al. 1999) to mark early apoptosis. Then, all sections were treated with goat anti-mouse biotin-conjugated secondary antibody (1:1500 in 0.1 M PBS-BT; Vector Laboratories, Burlingame, CA, USA) for 90 min. For signal amplification, sections were incubated for 90 min in ABC (1:800 in 0.1 M PBS-BT; Vector Laboratories; Hsu et al., 1981), visualised with 0.025% 3,3’-diaminobenzidine (DAB; Sigma) in 50 mM Tris buffer (pH 7.4) with 0.25% nickel ammonium sulphate (BHD Laboratory Supplies, Poole, England), for 10 min. Finally, sections were mounted, dehydrated, cleared in xylene and coverslipped with Entellan and examined with a Zeiss light microscope. Control sections, treated as described above, but with omission of the primary antiserum, did not reveal any immunoreactivity.

Unless stated otherwise, all reagents were from Merck (Darmstadt, Germany).

**Neuroanatomy, densitometry and statistics**

Brain regions were identified using cortical maps made from coronal and horizontal sections. This provided lateral, dorsal and medial views with reliable stereotaxic coordinates. To permit comparison of the distribution of these regions between individual rats, maps were drawn by orthogonal projection on transparent sheets, aligned and superimposed on each other. Brain regions were identified according to Zilles (1985) and Paxinos and Watson (1997).

The number of PV-positive cells were counted and expressed per mm², at the level Bregma 0.7 mm and Interaural 9.7 mm, in 4 cortical regions, viz. cingulate cortex area 1 (Cg1), parietal cortex area 1 (Pari), gustatory cortex (Gu) and forelimb area (FL). Furthermore, slices were studied at the level of Bregma –1.40 mm and Interaural 7.60 mm in the rRTN, at the level of Bregma –2.12 mm and Interaural 6.88 mm in the caudal RTN (cRTN) and adjacent thalamic nuclei, and at the level Bregma –2.30 mm and Interaural 6.70 mm in the CA3 region of the hippocampus. Similar regions were qualitatively examined in the Cytodeath and CV-stained slices. Quantitative data were tested by Student’s t-test (α=5%).

**Results and discussion**

In both WAG/Rij and ACI rats, PV-immunostaining is strong throughout the brain, and neurones including their processes clearly stand out against the unstained background (Fig. 1a,b). Staining is not evenly distributed. Some brain regions, like the rRTN and the pyramidal cell layers of the hippocampus, are much more strongly stained than others,
like the molecular hippocampal layers and the thalamic nuclei (Fig. 2a,b). The fact that the thalamic nuclei do not show PV-positive neurones (Fig. 2a) seems logical as PV generally co-exists with GABA (Celio 1986, 1990) and the thalamic nuclei of the rat are devoid of GABAergic (inter)neurones (Steriade et al. 1997).

Both ACI and WAG/Rij rats show some particular phenomena. Within certain brain structures known to contain GABAergic (inter)neurones, areas are poor in PV-positive cells and some structures even reveal regions that hardly or not show PV-immunoreactive cells (e.g. Par 1; Fig. 3b). CV staining reveals that these regions are not devoid of neurones. Moreover, with Cytodeath staining no apoptotic cells are visible, neither in WAG/Rij nor ACI rats. This means that these local differences in the presence of PV-positive cells in WAG/Rij rats are not the result of cell migration or cell death but are due to the inability of some neurones to stain with the anti-PV serum. Apparently, such cells do not contain (enough) PV to be immunopositive.

In order to get insight into a possible relation between the occurrence of PV-positive cells in WAG/Rij rats and the occurrence of SWD, two types of analysis were carried out. First, regions completely devoid of PV-immunostained cells (‘unstained regions’) were mapped and compared among animals within and between the two rat strains. Secondly, the numerical density of PV-positive cells in a number of distinct brain structures, including those presumed to be involved in the generation and pacemaking of SWD, were morphometrically assessed.

**Figure 1:**

PV-immunoreactive neurones in [a] piriform cortex of ACI rat. [b] Detail neurone with processes. Bars: 50 µm (a), 10 µm (b).
Unstained regions

In the rostral as well as in the caudal pole of the RTN no unstained regions were seen and the cellular composition of the nucleus appears normal (Fig. 2a). In fact, unstained regions occur exclusively in the cerebral cortex, though the location and the size of these areas strongly differs among animals. WAG/Rij rats show a tendency to have more unstained regions than ACI rats (WAG/Rij rats: 21, ACI rats: 14) but this difference is not statistically significant and no specific cortical area is consistently unstained in each WAG/Rij rat examined.

Quantification of PV-positive cells

To refine our observations on the occurrence of PV-immunoreactive neurones, determination of their numerical density was focussed on the rRTN, cRTN, the cerebral cortex and the hippocampal CA3 area. No difference exists between WAG/Rij and ACI rats in the numerical density of PV-positive neurones in the rRTN and cRTN (Fig. 4). Similar, no difference was found on the density of such neurones in the CA3 region of the hippocampus, an area not involved in absence epilepsy (Inoue et al. 1993; Kandel et al. 1996; Fig. 4). No statistically significant differences in density between WAG/Rij and ACI rats were found in the Gu and the Cgl area, although the WAG/Rij rat tends to contain less PV-immunoreactive cells compared to the ACI rat (Fig. 4). However, clear differences appear in the ParI and in the FL, where ACI rats show about 2 times as many PV-positive cells as WAG/Rij rats (Fig. 4).
Parvalbumin in the thalamocortical system of absence epileptic WAG/Rij rats

Figure 3:
Parietal cortex area 1 (Par 1). PV-positive neurones are numerous in ACI rat [a] but scarce in WAG/Rij rat [b]. CC: corpus callosum, Cp: caudate putamen. Bars: 400 μm.

Figure 4:
Number of PV positive neurones/mm² in various brain areas of WAG/Rij (n=5) and ACI (n=5) rats. CA3: CA3 region of hippocampus, Cg1: cingulate cortex area 1, cRTN: caudal reticular thalamic nucleus, FL: forelimb area, Gu: gustatory cortex, Par1: parietal cortex area 1, rRTN: rostral reticular thalamic nucleus. Asterisks and P-values indicate statistically significant difference.
The presence of PV-positive cells in the RTN is in line with the GABAergic nature of this nucleus. The fact that no differences occur in the density of PV-positive rRTN cells between ACI and WAG/Rij rats indicates that the involvement of the rRTN in maintenance of SWD activity does not depend on a changed amount of neuronal PV. On the other hand, in two distinct brain areas, viz. Par1 and FL, markedly lower densities of PV-positive cells are present in WAG/Rij rats compared to control (ACI) rats. Par1 and FL are part of the somatosensory cortex and related to SWD. In humans, an absence epileptic attack is accompanied by an immobile posture, a joining of the hands like saying one’s prayers, and head tilting. Epileptic WAG/Rij rats are also immobile except for small skeletal muscle contractions, twitching of the whiskers, and lip movements followed by head tilting (van Luijtelaar and Coenen 1986). The FL area of the cortex is possibly related to these behavioural motor aspects accompanying the absence epileptic attack. The Par1 contains the peri-oral region of the somatosensory cortex, the focus that initiates a cascade of events ultimately leading to SWD activity in the thalamocortical system of WAG/Rij rats (Meeren et al. 2002). Lack of PV in these regions may destabilise intraneuronal Ca\(^{2+}\) homeostatic processes such as excitability, intracellular signalling and neurotransmitter release. For example, prolonged Ca\(^{2+}\)-activated K\(^+\) channel activity would hyperpolarise the GABAergic neurone. Reduced GABAergic inhibition has been demonstrated in the cortex of the WAG/Rij rats (Luhmann et al. 1995). If neurones in the cortical focus of absence epilepsy, the Par1, would be affected in such a way, they are more vulnerable for factors/conditions that induce SWD activity. As a consequence, cortical SWD firing would be imposed upon the RTN and thalamus, thereby inducing the whole thalamocortical system to generate SWD.
CHAPTER 7

Spontaneous $\text{Ca}^{2+}$ oscillations in neurones of the rostral reticular thalamic nucleus of postnatal WAG/Rij and ACI rats

With:

Lieke van Diepen, Gilles van Luijtelaar, Eric Roubos en Wim Scheenen.
Abstract

Malfunctioning of the thalamus can lead to absence epilepsy. One of the animal models to study this disorder is the WAG/Rij rat. The rostral part of the reticular thalamic nucleus (rRTN) plays a crucial role in the maintenance of spike-wave discharges (SWD), the characteristic electroencephalographic manifestation of absence epileptic brain activity. We hypothesise that rRTN neurones of the postnatal WAG/Rij rat possess aberrant spontaneous, intracellular Ca\textsuperscript{2+} dynamics, so-called Ca\textsuperscript{2+} oscillations, and tested this hypothesis by comparing Ca\textsuperscript{2+} oscillations in rRTN neurones in slices of developing WAG/Rij rats between days 8 en 20 postnatal with those in age-matched ACI control rats. Between days 10 and 14 postnatal, a period coinciding with the development of main connections within the thalamus, in WAG/Rij rats only 41\% of the rRTN neurones display low-frequency and low-amplitude Ca\textsuperscript{2+} oscillations, compared to 63\% of rRTN neurones in ACI rats. The amplitude and frequency of the oscillations were similar for both WAG/Rij and ACI neurones and varied between 0.01 and 0.15 ratio units and 0.01 and 0.1 Hz, respectively. Removing Ca\textsuperscript{2+} from the extracellular medium, or adding 3 mM Ni\textsuperscript{2+}, a blocker of voltage-operated Ca\textsuperscript{2+} channels (VOCC), did not inhibit the Ca\textsuperscript{2+} oscillations in the rats strains. On the other hand, in both strains, application of thapsigargin, an inhibitor of the Ca\textsuperscript{2+}-ATPase present on the smooth endoplasmic reticulum or application of the InsP\textsubscript{3} receptor antagonist 2-APB inhibited the oscillations. These results indicate that the mechanism for generating Ca\textsuperscript{2+} oscillations in rRTN neurones of both rat strains involves an InsP\textsubscript{3} receptor-mediated Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release component and does not depend on Ca\textsuperscript{2+} influx through VOCC. The markedly lower number of cells displaying Ca\textsuperscript{2+} oscillations in postnatal WAG/Rij rats, suggests that in these animals less rRTN neurones are involved in synapse formation than in ACI rats. The possible significance of reduced synapse formation by the rRTN for the maintenance of SWD activity in adult WAG/Rij rats has been considered.

Introduction

Absence epilepsy in humans occurs between an age of 4 years and adolescence, and is characterised by a loss of consciousness and behavioural changes like rolling eye movements and a slight tilting of the head (Porter 1993). In the cortical electroencephalogram (EEG), synchronous spike-wave discharges (SWD) occur with an average frequency of 3 Hz and duration of 5-30 seconds (e.g. Snead III 1995; Steriade and Contreras 1995). A suitable animal model to study the mechanisms underlying absence epileptogenesis is the WAG/Rij rat (Coenen et al. 1992; van Luijtenaar and Coenen 1997), which displays bursts of bilateral, synchronous SWD, from an age of 3 months onwards (Coenen and van Luijtenaar 1987).
As in human, these SWD have a thalamocortical nature, with the rostral part of the reticular thalamic nucleus (rRTN) functioning as a pacemaker that maintains SWD activity (Inoue et al. 1993; Meeren 2002; Meeren et al. 2002). SWD depend on a complex interplay between several ion channels in the neuronal plasma membrane (e.g. Soltesz et al. 1991; Bal and McCormick 1993, 1996; McCormick and Bal 1997; Destexhe et al. 1998a,b). The intracellular concentration of the Ca$^{2+}$ ion ([Ca$^{2+}$]$_i$) plays an important role in SWD activity. Activation of T-type voltage-operated Ca$^{2+}$ channels (VOCC) evokes a small, transient Ca$^{2+}$ conductance that is responsible for SWD onset. Subsequent opening of other, large-conductance, Ca$^{2+}$ channels leads to the formation of an intracellular Ca$^{2+}$ transient that, in turn, activates Ca$^{2+}$-dependent K$^+$-channels in rRTN neurones that maintain SWD (e.g. Avanzini et al. 1989; McCormick and Bal 1997).

There is reason to believe that during postnatal development, repetitive Ca$^{2+}$ transients, the so-called spontaneous Ca$^{2+}$ oscillations, play a crucial role in the establishment of functional interneuronal connections, e.g. in the cortex, hippocampus and cerebellum (Mizutani et al. 1996; Tanaka et al. 1996; Garaschuk et al. 1998; Stosiek et al. 2003). The Ca$^{2+}$ oscillations often run as waves through several cell layers (Garaschuk et al. 2000) and may promote synapse formation (Mizutani et al. 1996; Tanaka et al. 1996). It is not known if such Ca$^{2+}$ oscillations also occur in the rRTN. We hypothesise that the rRTN of WAG/Rij rats differs in the dynamics of its Ca$^{2+}$ oscillations from ACI control rats, which do not, or hardly, develop absence epilepsy-associated SWD (Inoue et al. 1990; de Bruin et al. 2000) and that this difference already occurs during an early postnatal stage, i.e., before SWD and absence epilepsy emerge. This difference might underlie the formation of different interneuronal connections of the rRTN with its input and output structures, the limbic- and motor-associated cortical and thalamic areas, and the afore-mentioned thalamic nuclei, respectively (Cornwall et al. 1990; Gonzalo-Ruiz and Lieberman 1995; Oda et al. 1996; Steriade et al. 1997), and might lead later in life to differences in the occurrence of SWD between WAG/Rij and ACI rats (Inoue et al. 1990; de Bruin et al. 2000). We tested this hypothesis by studying the presence of Ca$^{2+}$ oscillations in slices of the rRTN of postnatal WAG/Rij and ACI rats. Special attention was paid to the possible role of the extracellular Ca$^{2+}$ concentration and the participation of intracellular Ca$^{2+}$ stores in the generation of Ca$^{2+}$ oscillations in rRTN neurones.

**Methods**

**Animals and slice preparation**

Both WAG/Rij and ACI rats were bred and reared under standard conditions in our Department of Biological Psychology. All experiments were carried out under the guidelines of the
Dutch law concerning animal welfare. Eleven WAG/Rij and 8 ACI, female rats, with ages between days 8 en 20 postnatal, were used. After decapitation, their brains were quickly removed and placed in an ice-cold and oxygenated solution containing in mM: KCl 5, KH$_2$PO$_4$ 1.25, CaCl$_2$ 1, MgSO$_4$ 5, NaHCO$_3$ 16, sucrose 250 and dextrose 10. Per brain, two consecutive slices of 300 µm including the whole rostral part of the reticular thalamic nucleus were cut on a vibratome (LEICA VT 1000S, Nussloch, Germany) and put in ice-cold Ringer’s solution containing in mM: NaCl 120, KCl 3, NaH$_2$PO$_4$ 1.25, NaHCO$_3$ 25, MgCl$_2$ 1, CaCl$_2$ 2, dextrose 4, Na-pyruvate 2, myo-inositol 0.5 and ascorbic acid 0.1. Throughout the studies Ringer’s solutions were always oxygenated before use. Then, slices were recuperated at 37 °C for 1 h in Ringer’s solution and subsequently kept at room temperature until use.

**Intracellular Ca$^{2+}$ measurements**

Slices were incubated in Ringer’s solution to which 10 µM fura-2/AM, 0.025% Pluronic F127 and 0.3 % w/v FCS had been added, for 15 min. Loaded slices were washed and incubated for 15 min in Ringer’s solution before starting imaging experiments, to allow full de-esterification of the dye. After manually dissecting it from a slice, the rRTN was placed in an incubation chamber containing 1 ml Ringer’s solution. To fix the slice a grid was placed on top of it. The incubation chamber was perfused with Ringer’s solution at a rate of 0.75 ml.min$^{-1}$, placed on a Zeiss Axiovert 135 TV (Zeiss, Oberkochen, Germany) and imaged using a Zeiss x40 oil-immersion, 1.3 NA fluor objective lens attached to a CoolSnap fx monochrome digital camera (Roper Scientific, Tucson, USA). Excitation wavelengths of 340 nm and 380 nm were provided by a 150 W Xenon lamp (Ushio UXL S150 MO, Ushio, Tokio, Japan) mounted in a Till Photonics Polychrome IV monochromator (spectral range 320-680 nm, band width 8-15 nm; Martinsried, Germany), and led into the microscope through an optical fibre. Fura-2 fluorescence emission was monitored at 515 nm, using a 440 nm DCLP dichroic mirror (Chroma, Rockingham, VT, USA) in front of the camera. Image acquisition and computation of ratio images (F340/F380) was operated through Metafluor v.4.6 software (Universal Imaging Corporation, Downingtown, PA, USA). Camera acquisition time was 100 ms per wavelength, and the data acquisition interval was 1 sec. To improve the S/N ratio, a camera pixel binning of 4 x 4 pixels was performed, leading to final images of 325x257 pixels. Test substances were added through the perfusion set-up. All media were continuously gassed with 95% O$_2$/5% CO$_2$.

**Data analysis**

Time series data of all experiments were analysed off-line using a dedicated data analysis software package (Origin 6.0, Microcal Software Inc., Northampton, MA, USA). Changes in ratio levels between the two excitation wavelengths were used as a measure for changes in the [Ca$^{2+}$]$_i$. 
Results

Ca\(^{2+}\) oscillations

We investigated the dynamics of Ca\(^{2+}\) oscillations, expressed as fura-2 ratio values, in neurones in \textit{in vitro} slices of the rRTN. In total 15 slices from WAG/Rij rats and 13 from ACI rats were studied. To be sure that neuronal cell bodies were examined and not astrocytes, each experiment was ended by application of 60 mM KCl (Fig. 1). It is well-known that neurones immediately respond to such treatment by depolarisation, whereas the response in astrocytes is substantially delayed (Pasti \textit{et al.} 1997; Scheenen and Carmignoto 2002). Therefore, we consider cell bodies that immediately (within 2 sec) responded to KCl to be neurones, whereas cells that typically had a delay around 30 sec are considered to be astrocytes and were left out from the analyses.

\textbf{Figure 1:}

[Ca\(^{2+}\)]\(_i\) dynamics in neurones and astrocytes in rRTN rat slices following stimulation by 60 mM KCl. [Ca\(^{2+}\)]\(_i\) is expressed as the ratio of fura-2 fluorescence upon excitation at 340 and 380 nm, respectively (ratio F340/F380). Note the delayed response of the astrocyte.
Spontaneous increases in \([\text{Ca}^{2+}]_i\) were observed in rRTN neurones of both WAG/Rij and ACI rats with ages between 10 and 14 days postnatal (see Fig. 2, 3, for examples). For WAG/Rij rats 453 neurones were studied and 186 neurones (41%) displayed spontaneous \(\text{Ca}^{2+}\) oscillations, while 294 cells of ACI rats were examined of which 184 cells (63%) show such oscillations. The oscillations varied among cells in amplitude and frequency. The amplitude ranged between 0.01 and 0.15 ratio units, with an average of 0.05 ratio units. The frequency of the oscillations varied between 1 and 10 peaks per 100 sec, with an average of 0.01 Hz. However, neither amplitude nor frequency differed between WAG/Rij and ACI rats.

**Involvement of extracellular \(\text{Ca}^{2+}\)**

To study the possible involvement of extracellular \(\text{Ca}^{2+}\) in the generation of the spontaneous \(\text{Ca}^{2+}\) oscillations we performed two types of experiment. In the first, extracellular \(\text{Ca}^{2+}\) was removed and 1 mM EGTA was added, to remove any residual extracellular \(\text{Ca}^{2+}\) in the slice (Fig. 2a1, 2a2). In this condition \(\text{Ca}^{2+}\) oscillations persisted in the majority of neurones of both WAG/Rij and ACI rats (100 out of 107 neurones and 60 out of 65 neurones, respectively). In the second experiment we assessed the possible involvement of VOCC in the generation of the oscillations by adding 3 mM Ni\(^{2+}\) to the incubation solution. This general inhibitor of VOCC had no effect in both rats strains, as \(\text{Ca}^{2+}\) oscillations continued in 31 out of 32 neurones of WAG/Rij rats and 26 out of 27 neurones of ACI rats (Fig. 2b1, 2b2).

**Involvement of intracellular \(\text{Ca}^{2+}\) stores**

To assess the possible role of intracellular \(\text{Ca}^{2+}\) stores in the generation of the \(\text{Ca}^{2+}\) oscillations, we first inhibited the smooth endoplasmic reticulum (SERCA) \(\text{Ca}^{2+}\)-ATPase by adding 500 nM thapsigargin to the incubation solution. Thapsigargin inhibited the \(\text{Ca}^{2+}\) oscillations in 29 out of 31 neurones of WAG/Rij rats and 30 out of 32 neurones of ACI rats (Fig. 3a1, 3a2). Next, we tested the involvement of InsP\(_3\) receptor-mediated CICR in \(\text{Ca}^{2+}\) oscillations, by adding 75 \(\mu\)M 2-APB. 2-APB has been described to inhibit InsP\(_3\) receptors and store-operated \(\text{Ca}^{2+}\) influx (Peppiatt *et al.* 2003). However, since removal of extracellular \(\text{Ca}^{2+}\) did not inhibit the \(\text{Ca}^{2+}\) oscillations, we can rule out a role of store-operated \(\text{Ca}^{2+}\) influx in the generation of \(\text{Ca}^{2+}\) oscillations in rRTN neurones. We found that 2-APB reversibly inhibited \(\text{Ca}^{2+}\) oscillations in 27 out of 28 neurones of WAG/Rij rats and 55 out of 59 neurones of ACI rats (Fig. 3b1, 3b2).

**Discussion**

We have characterized and compared the intracellular \(\text{Ca}^{2+}\) dynamics in neurones of the rRTN of the WAG/Rij rat with those of the ACI rat. Of these two rat strains only WAG/Rij
Figure 2:

Ca$^{2+}$ oscillations in neurones of WAG/Rij [a1, b1] and ACI [a2, b2] rat rRTN slices during [a] 0 mM Ca$^{2+}$ and 1 mM EGTA incubation and [b] 3 mM Ni$^{2+}$ incubation. Both treatments did not affect spontaneous Ca$^{2+}$ oscillations for periods up to 30 min.
Figure 3:

Ca$^{2+}$ oscillations in neurones of WAG/Rij (a1, b1) and ACI (a2, b2) rat rRTN slices during [a] 500 nM thapsigargin incubation and [b] 75 µM 2-APB incubation. Both thapsigargin and 2-APB inhibit spontaneous Ca$^{2+}$ oscillations.
rats are prone to develop SWD, from 3 months onward (Inoue et al. 1990; de Bruin et al. 2000), and these SWD are maintained by the pacemaking activity of the rRTN (Meeren 2002). Although many studies have been performed to understand the mechanisms underlying SWD activity in adult epileptic rodents (WAG/Rij rats, van Luijtelaar and Coenen 1997; GAERS rats, Danober et al. 1998; tottering, lethargic and stargazer mice, Burgess and Noebels 1999), no information is available on the possibility that the rRTN in postnatal WAG/Rij rats possesses properties that later, during adulthood, turn out to be responsible for the occurrence of SWD. We have hypothesised that an early developmental change in the generation and/or characteristics of Ca$^{2+}$ oscillations in the rRTN represents such a property, and is responsible for the development of SWD in the adult WAG/Rij rat.

We did not find a difference between WAG/Rij and ACI rats with respect to the characteristics of Ca$^{2+}$ oscillations, neither with respect to the (absence of the) role of extracellular Ca$^{2+}$ nor to the involvement of intracellular (InsP$_3$-sensitive) Ca$^{2+}$ stores. Therefore, we conclude that the Ca$^{2+}$ oscillations per se are not related to the development (at a later age) of SWD. However, rRTN neurones displaying Ca$^{2+}$ oscillations are significantly more numerous in ACI rats that in WAG/Rij rats. In view of the fact that in several brain areas, including the hippocampus, Ca$^{2+}$ oscillations in early postnatal development are important for the proper formation of synaptic contacts (Garaschuk et al. 1998), our finding might indicate that in WAG/Rij rats with an age between 10 and 14 days, less neurones are involved in synapse formation than in aged-matched ACI rats. Disturbance of neurotransmission in the reticulothalamic pathway is generally believed to lead to SWD (e.g. Hosford et al. 1997; Gibbs et al. 1996; Destexhe 1998; Huntsman et al. 1999; Lin et al. 1999). It would seem interesting to investigate how a reduced synapse formation / synaptic strength between rRTN neurones and their input and output centres would promote SWD activity.

Meanwhile, it remains to be seen if the rRTN neurones that do not display Ca$^{2+}$ oscillations are active outside the period we performed our in vitro brain slice observations or that they belong to a distinct subpopulation of rRTN neurones. With respect to the latter possibility, it is noteworthy that, in addition to the predominant GABAergic nature of the rRTN (Houser et al. 1980; DeBiasi et al. 1986), some rRTN sectors contain various types of neuropeptides (Burgunder et al. 1999; van de Bovenkamp et al., Chapter 3), indicating that multiple subpopulations of neurones exist in the rRTN. So, it seems worthwhile to investigate if there is a relation between the neurochemistry of individual rRTN neurone subtype and their ability to generate intracellular Ca$^{2+}$ oscillations.

The question arises why the [Ca$^{2+}$]$_i$ in rRTN cells start oscillating at day 10 and stop oscillating at day 14. Theoretically, two possibilities exist: (i) they possess an internal clock, and (2) the start and end of the oscillatory period depend on a changed regulatory input to the cells. Our current knowledge does not allow to make a choice between these two mechanisms. However, some insight has been obtained in the ‘motor’ that drives the oscillations, as will be outlined below.

The spontaneous Ca$^{2+}$ oscillations in rRTN neurones have a low frequency, and a low amplitude; the latter is on average 1/10th of the amplitude evoked by high KCl stimulation. Theoretically, at least two different ‘motor’ mechanisms may be involved in the generation
of such oscillations. In the majority of excitable cells displaying Ca\(^{2+}\) oscillations, the motor for the oscillations is Ca\(^{2+}\) influx through VOCC. The second mechanism that can give rise to Ca\(^{2+}\) oscillations is through CICR, which can be initiated by Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores by either ryanodine receptors or InsP\(_3\) receptors, or by a combination of both. CICR-induced Ca\(^{2+}\) oscillations are commonly found in non-excitable cells, although the CICR mechanism is also involved in oscillations in various excitable cell types. We have investigated which of these two mechanisms is responsible for the observed rRTN Ca\(^{2+}\) oscillations.

Since our slice preparation contains excitable cells, we initially focused on the role of Ca\(^{2+}\) influx. However, our experiments with lowering extracellular Ca\(^{2+}\) and application of an inhibitor of VOCC show that Ca\(^{2+}\) influx is not the initiator of the Ca\(^{2+}\) oscillations, nor does it contribute to the maintenance of the oscillations. The fact that in the absence of extracellular Ca\(^{2+}\) the oscillations normally persist, suggests that the oscillations are generated through release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores. To test this idea, we first inhibited Ca\(^{2+}\)-ATPase present on the smooth endoplasmic reticulum. Inhibition of SERCA-ATPase by thapsigargin generally depletes the intracellular Ca\(^{2+}\) stores because of leakage of Ca\(^{2+}\) into the cytoplasm. Thapsigargin treatment of rRTN neurones inhibited Ca\(^{2+}\) oscillations, indicating the importance of intracellular Ca\(^{2+}\) stores for their generation. Further evidence for an involvement of intracellular Ca\(^{2+}\) stores in the oscillatory activity of rRTN neurones comes from the study with 2-APB, an antagonist of InsP\(_3\) receptors and an inhibitor of store-operated Ca\(^{2+}\) influx (Peppiatt et al. 2003). Since lowering the extracellular Ca\(^{2+}\) did not affect the Ca\(^{2+}\) oscillations for periods up to 30 minutes, we can exclude a major contribution of the store-operated Ca\(^{2+}\) influx pathway in rRTN neurones. Therefore, the inhibitory effect of 2-APB on the intracellular Ca\(^{2+}\) oscillations must be explained from an inhibitory effect on InsP\(_3\) receptors. Taking these considerations together, it can be concluded that the low-frequency, low-amplitude Ca\(^{2+}\) oscillations in rRTN neurones are generated by a CICR mechanism that involves Ca\(^{2+}\) release from InsP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores.

Ca\(^{2+}\) oscillations in neurones generally arise through Ca\(^{2+}\) influx, although oscillations are induced via InsP\(_3\) receptors in hippocampal pyramidal cells. Our study demonstrates that also rRTN neurones can generate and maintain Ca\(^{2+}\) oscillations through InsP\(_3\)-sensitive Ca\(^{2+}\) stores without the involvement of Ca\(^{2+}\) influx. As mentioned above, the Ca\(^{2+}\) oscillations in rRTN neurones may be important in establishing proper synaptic contacts. In this respect it is interesting to note that the expression of various genes involved in cellular activity depends on distinct frequencies and amplitudes of Ca\(^{2+}\) oscillations (e.g. Dolmetsch et al. 1998; Meldolesi 1998; Hu et al. 1999). Moreover, it has been shown that gene expression can be regulated by InsP\(_3\) receptor-mediated CICR (Li et al. 1998). Future studies are needed to see whether the rRTN in (the WAG/Rij) rat expresses specific genes between p10 and p14 postnatal, and if this expression depends on the occurrence and properties of rRTN Ca\(^{2+}\) oscillations.
CHAPTER 8

General discussion
Introduction

Absence epilepsy is a non-convulsive type of epilepsy, characterised by a decrease in consciousness of abrupt onset and offset, accompanied by generalised, bilateral, synchronous, regular, stereotyped and symmetrical spike-wave discharges (SWD), and behavioural changes such as an interruption of ongoing activities, a blank facial expression with the eyes drifting upward, a rhythmical beating of the eyelids and twitches of the mouth (Porter 1993). SWD activity is generated and maintained within the thalamocortical system (e.g. Gloor 1968; Gloor et al. 1990; Snead III 1995; Avanzini et al. 2000; Kostopoulos 2000). The WAG/Rij rat is a well-characterised animal model for human absence epilepsy (for review see van Luijtelaar and Coenen 1997). From an age of 3 months, WAG/Rij rats develop SWD activity and at the age of 6 months they display hundreds of absence epileptic seizures per day (Coenen and van Luijtelaar 1987; Inoue et al. 1990; de Bruin et al. 2000), concomitant with vibrissal twitching, accelerated breathing and head tilting (van Luijtelaar and Coenen 1986). For WAG/Rij rats it has been established that SWD activity starts in the peri-oral region of the somatosensory cortex (Meeren et al. 2002) and that it is maintained by the pacemaking activity of the rostral part of the reticular thalamic nucleus (rRTN) (Meeren 2002).

In this thesis, experiments have been described to obtain insight in the mechanisms responsible for the initiation and the maintenance of the neuronal driving force for SWD activity. In six chapters, studies on the possible roles of various factors in SWD activity are presented. These factors concern signal input, signal recognition and signal transduction, that were studied in the peri-oral somatosensory cortex and in the rRTN. In the paragraphs below they will be discussed in the following order: 1) signal inputs (synaptology of the rRTN), 2) signal recognition (glutamate receptor expression in the somatosensory cortex and rRTN) and 3) signal transduction (Ca\(^{2+}\) channels in the rRTN, PV-mediated Ca\(^{2+}\) buffering/toxicity in the somatosensory cortex and rRTN, and Ca\(^{2+}\) oscillations in the rRTN).

**Signal inputs**

**Synaptology of the rRTN**

The rRTN is a distinct compartment of the RTN with respect to its characteristic neuroanatomical organisation, fibre connectivities and role in SWD activity (e.g. Cornwall et al. 1990; Spreafico et al. 1991; Lozsádi 1994; Meeren 2002). However, in this thesis it is shown that the general ultrastructure of the rRTN is similar to that described for the RTN as a whole (Chapters 2,3). The elaborate synaptic organisation of the rRTN in WAG/Rij rats consists of a dense corticothalamic excitatory input and moderate numbers of thalamocortical excitatory and GABAergic synapses (Chapter 3). This synaptology is in line with the role of the rRTN as a pacemaker that maintains SWD in the extensive corticothalamic
network. Due to a powerful cortical drive upon the rRTN, the inhibition of the thalamus by the rRTN overcomes the excitatory input from the cortex. This then enables the rRTN to impose oscillatory activity upon the thalamus, thereby maintaining thalamocortical oscillatory activity like SWD (von Krosigk et al. 1993, 1999; Warren et al. 1994; Contreras and Steriade 1996; Contreras et al. 1996; Destexhe et al. 1998a). However, the observed synaptic organisation in the rRTN of the WAG/Rij rat does not obviously differ from that of the rRTN in non-epileptic ACI rats. For instance, neither the quality nor the numbers of the various axon terminal types, nor the frequency and configurations in which the terminals contact the various postsynaptic structures significantly differ between the two rat strains. Therefore, it is concluded that the specific role of the rRTN in SWD maintenance in the WAG/Rij rat does not depend on a different organisation or synaptic input. Either the absence of SWD in ACI rats is due to the fact that no SWD are generated in the somatosensory cortex of these animals, or other properties of the rRTN are involved in SWD maintenance, such as the functioning of their neuronal elements at the molecular level, which is investigated in more detail in Chapters 4-7.

Signal recognition

Glutamate receptor expression in the somatosensory cortex and rRTN

Striking differences were found at the glutamatergic receptor level in both the peri-oral somatosensory cortex and the rRTN (Chapter 4). In the cortex, NMDA and GluR4-rich AMPA receptor densities in WAG/Rij rats are lower than in ACI rats. Moreover, SWD activity in 6 months old, absence epileptic WAG/Rij rats is concomitant with an increase in GluR4-rich AMPA receptor density on rRTN neuronal processes but not on rRTN somata, when compared to presymptomatic WAG/Rij rats and age-matched ACI control rats. Therefore, it seems plausible that in the peri-oral somatosensory cortex of the WAG/Rij rat, SWD start as a result of a decrease in NMDA and GluR4-rich AMPA receptor-mediated, glutamatergic stimulation, whereas in the rRTN of this rat strain SWD maintenance may be promoted by increased excitation mediated by GluR4-rich AMPA receptors.

Signal transduction

Ca\(^{2+}\) channels in the rRTN

Investigating the possible involvement of HVA Ca\(^{2+}\) channels in SWD maintenance (Chapter 5) we could not find differences in \(\alpha_{1.1.3}\), \(\alpha_{1.2.2}\) and \(\alpha_{1.2.3}\) subunit expressions but we demonstrated an increased expression of Ca\(_4\)2.1 channel-related \(\alpha_{1.2.1}\) subunits in the rRTN of 6 months old, absence epileptic WAG/Rij rats compared to 3 months old, non-epileptic WAG/Rij rats and age-matched ACI control rats. The \(\alpha_{1.2.1}\) subunits are located at presynaptic rRTN nerve terminals, indicating their role in neurotransmitter release.
PV-mediated Ca\textsuperscript{2+} buffering/toxicity in the somatosensory cortex and rRTN

No difference appears between WAG/Rij and ACI rats in the numerical density of PV-positive neurones in the rRTN (Chapter 6). However, clear differences were observed in the peri-oral somatosensory cortex, where WAG/Rij rats show almost 2 times less PV-positive cells than ACI rats. This difference is the result of a changed PV content and not of disappearance of neurones, e.g. by apoptosis. PV-positive neurones constitute the majority of cortical GABAergic interneurones (Celio 1986, 1990). Our results support the possibility that absence epilepsy is related to a reduction in PV-mediated Ca\textsuperscript{2+} buffering in the GABAergic interneurones of the somatosensory cortex. As a result, intraneuronal Ca\textsuperscript{2+}-homeostatic processes such as excitability, intracellular signalling and neurotransmitter release may be destabilised and SWD initiation promoted.

Ca\textsuperscript{2+} oscillations in the rRTN

In the rRTN of postnatal WAG/Rij rats, less neurones are observed showing spontaneously occurring Ca\textsuperscript{2+} oscillations than in age-matched ACI control rats (Chapter 7). Intracellular Ca\textsuperscript{2+} oscillations during postnatal development seem to play an important role in the formation of connections between neurones (e.g. Garaschuk et al. 1998). The input of the rRTN mainly consists of glutamatergic fibers arriving from the limbic- and motor-associated cortical and thalamic areas, whereas the output of the rRTN consists of GABAergic afferents to the above-mentioned thalamic nuclei (Jones 1985; Cornwall et al. 1990; Gonzalo-Ruiz and Lieberman 1995; Oda et al. 1996; Steriade et al. 1997). Our observation that in the rRTN of WAG/Rij rats relatively few neurones are oscillating may mean that in WAG/Rij rats these connections develop less well than in normal, non-epileptic rats.

A model

Taking our data together, it appears that in the absence epileptic WAG/Rij rat glutamatergic excitation mediated by NMDA and GluR4-rich AMPA receptors is changed, as well as Ca\textsubscript{v}2.1 channel and PV expression. Moreover, the connectivities between the rRTN on the one hand and the cortex and thalamus on the other may be less strong. Below, we will discuss a model explaining how these changes might lead to SWD initiation and maintenance within the thalamocortical system. The model is based on our data and on general data of SWD initiation and maintenance from the literature.
SWD activity in the thalamocortical system

Absence epilepsy is associated with a hyperexcitable cortex (e.g., Gloor and Fariello 1988; Pumain et al. 1992; Luhmann et al. 1995; Avanzini et al. 1996; Destexhe 1999). In the WAG/Rij rat a reduced GABAergic cortical inhibition leads to hyperexcitability (Luhmann et al. 1995), suggesting that SWD initiation in the peri-oral somatosensory cortex arises from cortical disinhibition. The AMPA-GluR4 receptor subunit in the somatosensory cortex is largely restricted to non-pyramidal, GABAergic interneurones (Ong et al. 1996; Kondo et al. 1997; Munoz et al. 1999). Therefore, it is likely that a low GluR4-rich AMPA receptor expression in WAG/Rij rats (Chapter 4) reduces cortical inhibition. Moreover, PV co-exists with GABA in somatosensory cortical GABAergic interneurones (Celio 1986, 1990) where it regulates the intracellular Ca²⁺ concentration (Kawaguchi et al. 1987) and in that way influences the activity of the Ca²⁺-activated K⁺ channel. Considering that only in some rare cases PV in the somatosensory cortex co-exists with other Ca²⁺-buffering proteins, such as calbindin (Celio 1990), a high Ca²⁺ concentration due to a loss of PV-mediated Ca²⁺ buffering (Chapter 6), might induce prolonged Ca²⁺-activated K⁺ channel activity. This, in turn, would hyperpolarise the GABAergic interneurones. These two phenomena would lead to a decreased and less effective cortical inhibition and as a result cortical glutamatergic neurones would become hyperexcitable and initiate SWD activity (Luhmann et al. 1995; Meeren 2002; Fig. 1).

Short- and long-range corticocortical fibres (Szentágothai 1978) mediate a fast intracortical spread of SWD activity and synchronise this activity over all cortical areas (Meeren et al. 2002). Although epileptiform activity can propagate through all cortical layers, especially layers I and V seem to be involved in this horizontal spread (Alefeld et al. 1998; Telfeian and Conners 1998). Glutamatergic corticothalamic fibres originating in layer V of the limbic- and motor-associated areas terminate in the (limbic- and motor-associated) thalamic nuclei without sending collaterals to the rRTN, whereas fibres from cells in layer VI terminate in both thalamus and rRTN (Jones 1985; de Curtis et al. 1989; Cornwall et al. 1990; Lozsádi 1994; Bourassa et al. 1995; Steriade et al. 1997).

Corticothalamic synaptic input upon dendrites, somata and axons predominates in the rRTN (Chapter 3) and is known to be glutamatergic (Ohara and Lieberman 1981, 1985; de Curtis 1989; Kharazia et al. 1996). We show that the rRTN α₇.2.1 subunits are located at presynaptic nerve terminals (Chapter 5). Since Caᵥ2.1 channels play a role in neurotransmitter release (Takahashi and Momiyama 1993; Dunlap et al. 1995; Tareilus and Breer 1995; Catterall 1998, 1999, 2000), it can be assumed that the increase in α₇.2.1 subunit expression in the rRTN is associated with increased corticothalamic, glutamate release. GluR4-rich AMPA receptors in the RTN are mainly located in the postsynaptic densities of corticothalamic synapses (Mineff and Weinberg 2000) and excite GABAergic RTN neurones (Houser et al. 1980; DeBiasi et al. 1986). A high GluR4-rich AMPA receptor density on rRTN neuronal processes (Chapter 4) suggests a strong glutamatergic excitation of these neurones.

We propose that in the absence epileptic WAG/Rij rat, these two phenomena are
Figure 1:
Model showing the involvement of the peri-oral somatosensory cortex and the rRTN in initiation and maintenance of SWD in the absence epileptic WAG/Rij rat. The model is based on the data presented in this thesis and the general literature about SWD and absence epilepsy in rat and human. Excitatory cortical neurones are disinhibited due to a reduced GluR4-rich AMPA-receptor-mediated, glutamatergic excitation in combination with a loss of PV Ca²⁺-buffering that leads to a prolonged Ca²⁺-activated K⁺ channel activity. Hyperexcitability in the peri-oral somatosensory cortex initiates SWD activity, which subsequently spreads over all cortical areas and is imposed upon the thalamic nuclei, including the rRTN. A very intense cortical stimulation of the rRTN is provided by an increased GluR4-rich AMPA-receptor and Cav2.1 channel-mediated glutamatergic excitation. Due to this excitation, the inhibition of the thalamic nuclei by the rRTN overcomes the direct cortical excitation. The resulting hyperpolarisation leads to SWD activity in the thalamic nuclei that is imposed back upon the cortical areas and the rRTN, causing SWD maintenance. D: dendritic tree; +: excitatory input; -: inhibitory input.
responsible for a strong cortical drive upon GABAergic rRTN neurones. This enables the rRTN to act as a pacemaker that maintains SWD activity by providing a strong disynaptic inhibition of the thalamus (Jones 1985; Steriade et al. 1997) that overcomes the monosynaptic thalamocortical excitation (Fig. 1). As a result, the thalamic neurones are hyperpolarised, making them prone to produce SWD (e.g. Contreras et al. 1996; Destexhe et al. 1998a; von Krosigk et al. 1999). Due to glutamatergic thalamocortical fibres and axon collaterals, that innervate the cortical layers I, IV, V and VI (Jones 1985; Bode-Greuel et al. 1987; Jensen and Killackey 1987; Steriade et al. 1997) and re-excite the RTN neurones, respectively, the complete thalamocortical network will become involved in absence epilepsy-associated SWD oscillatory activity.

**General significance of the results**

Besides the WAG/Rij rat, there are a number of other genetic animal models spontaneously displaying absence epilepsy-associated SWD activity, viz. the genetic absence epileptic rats from Strasbourg (GAERS rats, Danober et al. 1998) and several mice strains, such as the tottering (tg/tg), the lethargic (lh/lh), the stargazer (stg/stg) and the leaner (tgla/tgla) mouse (e.g. Noebels 1984; Fletcher et al. 1996; Burgess et al. 1997; Letts et al. 1998; Burgess and Noebels 1999, 2000). Since there is a strong genetic predisposition for absence epilepsy in humans (Lennox 1960), these genetic animal models are a more valid tool to investigate the mechanism underlying absence epilepsy than animal models where SWD activity has to be induced artificially.

Our electron microscope studies on the general ultrastructure and synaptology of the rRTN are the first carried out in relation to rat absence epilepsy. As to spontaneous rRTN Ca\(^{2+}\) oscillations, we suggest that in postnatal WAG/Rij rats these oscillations play a role in synapse formation and hence results in SWD activity in the adult animal. Similar studies investigating the possible role of Ca\(^{2+}\) oscillations in the development of the RTN and its relation to SWD in other animal models have not been reported.

In the GAERS rat and in some mouse models, the involvement in SWD activity of a number of the factors we studied regarding signal recognition and signal transduction have also been investigated in relation to absence epilepsy. This concerns the possible roles of NMDA and AMPA receptors, PV-mediated Ca\(^{2+}\)-buffering and the role of HVA Ca\(^{2+}\) channels. We will consider the general relevance of these data in some detail. From studies on the GAERS model it appears that NMDA and AMPA receptors can contribute to cortical hyperexcitability and, as a consequence, to SWD generation (Pumain et al. 1992; Avanzini et al. 1996; Destexhe 1999). Moreover, the expression of the \(\gamma(2)\) subunit of voltage-dependent Ca\(^{2+}\) channels, which regulates the targeting of AMPA receptors to the postsynaptic membrane (Chen et al. 2000), is strongly reduced in the stargazer mouse (Letts et al. 1998; Sharp et al. 2001). The leaner mouse seems to have a changed Ca\(^{2+}\) homeostasis in cerebellar Purkinje cells as a result of a reduced PV expression (Dove et al. 2000), but up to now no data were available regarding the Ca\(^{2+}\)-buffering capacity in cortical neurones of
absence epileptic animals. Our studies are the first to demonstrate reduced NMDA and GluR4-rich AMPA receptor densities in the cortical SWD initiation site associated with a loss of PV expression.

In GAERS rats, but especially in mouse models, HVA Ca\(^{2+}\) channels play some role in absence epilepsy (e.g. McEnery et al. 1998; Fletcher and Frankel 1999; Burgess and Noebels 1999, 2000; Lakaya et al. 2002)(see also Chapters 1 and 5). We here demonstrate an increased, presynaptic Ca\(_{v}2.1\) channel expression concomitant with SWD activity, supporting the idea that this specific HVA Ca\(^{2+}\) channel type is important for the maintenance of absence epilepsy (Fletcher et al. 1996; Lorenzon et al. 1998; Wakamori et al. 1998; Burgess et al. 1999; Fletcher and Frankel 1999; Jouveneau et al. 2001; Zhang et al. 2002).

**Perspectives**

An intriguing question is whether the observed differences in the presence of Ca\(_{v}2.1\) channels, PV and glutamate receptors between WAG/Rij compared to ACI rats are reactive, compensatory or causative to absence epilepsy-associated SWD activity. Although some changes are already observed in presymptomatic WAG/Rij rats and others are correlated with the occurrence of SWD, they do not provide insight into the issue ‘cause, compensation or consequence’. Therefore, gene-screening studies, comparing WAG/Rij and ACI rats would be a plausible follow-up. Moreover, in the future it might become possible to study the effect on absence epileptogenesis of genetically engineered, tissue-specific deletion or overexpression of the genes, for the Ca\(_{v}2.1\) gene, the GluR4-rich AMPA receptor gene and the PV gene.

To extend the preliminary model proposed above, more research is necessary. Patch-clamp studies could provide a more detailed insight in the significance of the reduced NMDA receptor expression in the peri-oral somatosensory cortex of the WAG/Rij rat. Similarly, the observation of less spontaneously oscillating rRTN cells in young WAG/Rij rats, suggesting a less strong synaptic connections of the rRTN with the cortex and thalamus, deserves further neurophysiological support, also in adult animals, e.g. on the basis of (immuno)electron microscopy and patch-clamp studies in combination with Ca\(^{2+}\)-imaging.

Immunoelectron microscopy, *in situ* hybridisation and patch-clamp studies can support the found immunocytochemical data regarding GluR4-rich AMPA receptor and Ca\(_{v}2.1\) channel expression in the rRTN. Subcellular location of these receptors and channels, and (receptor) channel translation and kinetics would put our data into a broader perspective. Moreover, patch-clamp experiments allow a more in detail study of the effects of Ca\(^{2+}\)-buffering mechanisms involving PV on cortical neurones.

Another important factor in absence epilepsy is GABAergic neurotransmission (e.g. Luhmann et al. 1995; Luijtelaar and Coenen 1997; Danober et al. 1998; Huntsman et al. 1999; Lin et al. 1999; Sohal et al. 2000; Princivalle et al. 2003). In this light it is interes-
ting that we recently obtained preliminary results indicating that in the WAG/Rij rat SWD
initiation in the peri-oral somatosensory cortex is concomitant with a lowered expression
of both glutamic acid decarboxylases GAD65 and GAD67, two distinct isoforms of the
GABA-synthesising enzyme (Feldblum et al. 1993).
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Summary

Human absence epilepsy, also known as ‘petit mal’, is a typical childhood disease occurring in children of school age (onset between 5-10 years). During an absence epileptic attack consciousness is abruptly impaired and the child interrupts ongoing activities, stares and appears dazed. There is a blank facial expression, with the eyes drifting upwards, accompanied by mild facial muscle contractions. The attack lasts about 5-20 secs and afterwards the child resumes ongoing behaviour like nothing happened. In the cortical electroencephalogram (EEG) the absence seizure is characterised by generalised, bilaterally, synchronous, regular, stereotyped and symmetrical 3 Hz spike-wave discharges (SWD). The neuronal basis of SWD and, hence, absence epilepsy lies within the thalamocortical network. This network consists of three major parts, mutually connected by elaborate fibre pathways: the cortex, the thalamic nuclei and the reticular thalamic nucleus (RTN). SWD activity can only be generated when the thalamocortical network is anatomically and functionally intact. Within the network especially the cortex and the RTN are key players in the initiation and maintenance, respectively, of SWD.

In this thesis, the WAG/Rij rat model for absence epilepsy has been used to obtain insight in the possible cellular and molecular determinants responsible for the initiation and maintenance of the neuronal driving force for SWD activity by the peri-oral somatosensory cortex and the rostral part of the RTN (the rRTN), respectively. The WAG/Rij rat is a well-characterised animal model for research on the mechanisms underlying human absence epilepsy. From an age of 3 months, WAG/Rij rats develop SWD activity and at the age of 6 months they display hundreds of absence epileptic seizures per day, concomitant with minor behavioural characteristics as seen in humans. For WAG/Rij rats it has been established that SWD activity starts in the peri-oral region of the somatosensory cortex and that it is maintained by the pacemaking activity of (rRTN).
After the general introduction presented in Chapter 1, Chapter 2 describes the RTN of the WAG/Rij rat at the ultrastructural level. In Chapter 3 the various signal inputs to and within the rRTN are ultrastructurally characterised and classified. It is demonstrated that an intense glutamatergic cortical synaptic input acts on rRTN neurones, which outnumbers both the (intrinsic) GABAergic input and the glutamatergic synaptic input from the thalamus. This synaptic organisation is in line with the role of the rRTN as a pacemaker that maintains SWD in the extensive corticothalamic network. However, it does not obviously differ from the synaptic organisation of the rRTN in non-epileptic ACI rats. Therefore it is concluded that the role of the rRTN in the maintenance of absence epilepsy-associated SWD does not depend on its synaptic organisation. The results presented in Chapter 4 suggest that in the peri-oral somatosensory cortex of the WAG/Rij rat, SWD start as a result of a reduction of glutamatergic stimulation mediated by NMDA and GluR4-rich AMPA receptors, the latter presumably located on cortical GABAergic interneurones. SWD may be maintained in the rRTN by an increase in excitation by glutamate via an increased (postsynaptic) AMPA-GluR4 receptor presence. Moreover, our data demonstrate a loss of the Ca\(^{2+}\)-buffering protein parvalbumin (PV) in GABAergic neurones in the peri-oral somatosensory cortex (Chapter 6) and an increased expression of presynaptic Ca\(_{\text{v}}\)2.1 channel-related \(\alpha_{12.1}\) subunits in the rRTN (Chapter 5), findings that may be related to SWD initiation and maintenance, respectively. Chapter 7 describes that young, postnatal WAG/Rij rats have less rRTN cells with spontaneous oscillations in their Ca\(^{2+}\)-concentration compared to non-epileptic rats, this observation suggests weak synaptic connections of the rRTN with the cortex and the thalamus.

In Chapter 8 the results obtained in the thesis research are put into a broader perspective, leading to a (preliminary) model describing the cellular and molecular mechanisms underlying SWD initiation and maintenance. In short, the model predicts that, as a result of reduced GluR4-rich AMPA receptor-mediated glutamatergic stimulation of cortical GABAergic interneurones together with prolonged Ca\(^{2+}\)-activated K\(^+\) channel activity in these interneurones, caused by a loss of PV-mediated Ca\(^{2+}\)-buffering, cortical inhibition is reduced leading to hyperexcitability within the peri-oral somatosensory cortex. Consequently, SWD activity is initiated, spreads throughout the different cortical areas and is imposed on the thalamic nuclei, including the rRTN. A strong cortical drive upon the GABAergic rRTN neurones enables the rRTN to act as a pacemaker that maintains SWD activity, namely by providing a strong disynaptic inhibition of the thalamus that overcomes the monosynaptic thalamocortical excitation. This hyperpolarises the thalamic neurones, making them prone to produce SWD. We demonstrate that in the absence epileptic WAG/Rij rat this strong rRTN excitation is provided by an increased (postsynaptic) GluR4-rich AMPA receptor-mediated excitation together with an intense glutamatergic neurotransmission that is mediated by presynaptic Ca\(_{\text{v}}\)2.1 channels.
Humane absence epilepsie is een ziekte die ook wel bekend staat als ‘petit-mal’ epilepsie. Het is een typische kinderziekte die begint op een leeftijd van ongeveer 5-10 jaar. Een absence aanval wordt gekenmerkt door een abrupte verstoring van het bewustzijn, waarbij het kind zijn bezigheden onderbreekt, een starende blik heeft en een versufte indruk maakt. Er is een nietszeggende gelaatsuitdrukking, waarbij de ogen omhoog draaien en het gezicht kleine spiertrekkingen vertoont. Zo’n aanval duurt 5 à 20 seconden, waarna het kind weer verder gaat met zijn bezigheden zonder dat het beseft een aanval te hebben gehad. Bij het maken van een corticale electroencephalogram (EEG) wordt zo’n aanval geregistreerd als bilaterale, synchrone, regelmatige, stereotype en symmetrische 3 Hz piek-golf complexen (Eng: spike-wave discharges, SWD). De neurale basis van deze SWD, en dus van absence epilepsie, ligt in het zogenaamde thalamocorticale netwerk dat bestaat uit de cortex, de thalamus en de reticulaire thalame kern (Eng: reticular thalamic nucleus, RTN). Alleen een thalamocorticale netwerk dat volledig anatomisch en functioneel intact is, kan SWD opwekken. Vooral de cortex en de RTN spelen een prominente rol in respectievelijk het initiëren en het handhaven van SWD.

De WAG/Rij rat is een zeer goed diermodel om onderzoek te doen naar humane absence epilepsie. In het onderzoek dat in dit proefschrift wordt beschreven, is dit model gebruikt om inzicht te verkrijgen in de mogelijke cellulaire en moleculaire aspecten die verantwoordelijk zijn voor deinitiatie en het in stand houden van SWD door respectievelijk de peri-orale somatosensorische cortex en het rostrale gedeelte van de RTN (rRTN). WAG/Rij ratten ontwikkelen SWD vanaf een leeftijd van 3 maanden. Een rat van 6 maanden vertoont honderden absence aanvallen per dag, die samengaan met kleine gedragsstoornissen, welke vergelijkbaar zijn met die bij de mens. Het is bekend dat SWD in deze ratten beginnen in de peri-orale somatosensorische cortex en in gang gehouden worden door de rRTN.

Na de algemene inleiding (Hoofdstuk 1) wordt in Hoofdstuk 2 een beschrijving gegeven van de ultrastructuur van de RTN van de WAG/Rij rat, terwijl in Hoofdstuk 3 de
verschillende input-signalen naar en binnenin de rRTN ultrastructureel worden geclasseficeerd. Aangetoond wordt dat de rRTN een zeer omvangrijke glutamaterge input ontvagt vanuit de cortex. Deze input is sterker dan zowel de glutamaterge input vanuit de thalamus als de GABAerge inputs van binnen en buiten de rRTN. Anatomisch gezien is de rRTN door deze synaptische organisatie uitstekend in staat om de SWD activiteit binnen het thalamocorticale systeem te laten voortduren. Echter, in de rRTN van een niet-epileptische controle rat (ACI rat) werd dezelfde synaptische organisatie aangetroffen, waaruit geconstateerd mag worden dat de rol die de rRTN speelt in het in stand houden van SWD niet berust op deze synaptische organisatie. Waarschijnlijk ligt de basis voor SWD activiteit in veranderingen op moleculair en/of cellulair niveau die niet tot uiting komen op het door ons bestudeerde morfologische niveau. Hoofdstuk 4 mondt uit in het idee dat in de peri-orale somatosensorische cortex SWD activiteit geïnitieerd wordt als een gevolg van een verminderde glutamaterge stimulatie door zowel NMDA als GluR4-AMPA receptoren. Deze laatste receptoren bevinden zich naar alle waarschijnlijkheid op postsynaptische elementen van corticale, GABAerge interneuronen. Een verhoogde glutamaterge GluR4-AMPA receptor-excitatie van de rRTN stelt deze kern vervolgens in staat om SWD activiteit te handhaven. Voorts laten de resultaten zien dat in de GABAerge interneuronen van de peri-orale somatosensorische cortex van de WAG/Rij rat minder Ca\textsuperscript{2+}.bufferend parvalbumine (PV) voorkomt dan in de controle ratten (Hoofdstuk 6), terwijl de rRTN een verhoogde expressie van het presynaptische Ca\textsubscript{v}2.1 kanaal laat zien (Hoofdstuk 5). Deze twee bevindingen zijn waarschijnlijk gerelateerd aan respectievelijk de initiatie en het in stand houden van SWD. Tenslotte wordt in Hoofdstuk 7 beschreven dat de rRTN van jonge, postnatale WAG/Rij ratten minder neuronen bevat die spontane oscillaties van de interne Ca\textsuperscript{2+}.concentratie laten zien, dan rRTN neuronen van niet-epileptische ratten. Dit resultaat doet vermoeden dat in WAG/Rij ratten een verminderde synaptische connectiviteit bestaat tussen de rRTN en de cortex en/of thalamus.

In Hoofdstuk 8 worden de gevonden data, beschreven in dit proefschrift, samengevoegd in een breed perspectief hetgeen leidt tot een (preliminair) model dat de cellulaire en moleculaire mechanismen beschrijft die ten grondslag liggen aan SWD initiatie en handhaving. Dit model voorspelt dat SWD ontstaat als gevolg van een hyperexcitatie in de cortex. Een hyperexcitabele peri-orale somatosensorische cortex wordt veroorzaakt door een gereduceerde glutamaterge GluR4-AMPA receptor stimulatie van GABAerge interneuronen en een verlengde hyperpolariserende actie van het Ca\textsuperscript{2+}.geactiveerde K\textsuperscript{+} kanaal als het gevolg van een afgenomen PV Ca\textsuperscript{2+}.buffering in deze interneuronen. Hyperexcitatie in de peri-orale somatosensorische cortex initieert SWD activiteit, die zich snel verspreidt door het gehele thalamocorticale systeem, inclusief de rRTN. De GABAerge neuronen van de rRTN kunnen SWD activiteit binnen het thalamocorticale sytem laten voortduren, doordat ze vanwege een sterke glutamaterge corticale input in staat zijn de neuronen van de thalamus te hyperpolariseren, waardoor deze SWD activiteit kunnen produceren. In dit proefschrift wordt beschreven dat deze sterke corticale stimulatie van de rRTN wordt veroorzaakt door zowel een verhoogde (postsynaptische) GluR4-AMPA receptor als een verstekte glutamaterge neurotransmissie verzorgd door presynapische Ca\textsubscript{v}2.1 kanalen.
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Maartje van de Bovenkamp-Janssen
Curriculum Vitae

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Absence epilepsy-associated spike-wave discharges are initiated in the peri-oral somatosensory cortex and maintained by the rostral reticular thalamic nucleus. To elucidate mechanisms underlying initiation and maintenance of spike-wave discharges, a number of factors potentially determining neuronal signal input, signal recognition and signal transduction in thalamocortical brain centres have been studied using the WAG/Rij rat model for human absence epilepsy. The results lead to a model which predicts that spike-wave discharge activity is initiated in the peri-oral somatosensory cortex, as a result of a reduced GluR4-rich AMPA receptor-mediated glutamatergic stimulation of cortical GABAergic interneurones together with a prolonged Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activity caused by a loss of PV-mediated Ca\textsuperscript{2+}-buffering. The model furthermore indicates that the rostral reticular thalamic nucleus can maintain spike-wave discharge activity due to an increased postsynaptic GluR4-rich AMPA receptor-mediated excitation together with an intense glutamatergic neurotransmission that is mediated by presynaptic Ca\textsubscript{v}2.1 channels.