NEURONAL PARVALBUMIN AND ABSENCE EPILEPSY IN WAG/Rij RATS

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ABSTRACT

In the WAG/Rij rat, a model for human absence epilepsy, spike-wave discharges and absence epileptic behaviour are fully symptomatic at an age of 6 months. The hypothesis is tested that the brain disorder underlying absence epilepsy is related to a changed distribution of neuronal parvalbumin (PV). Quantitative immunocytochemistry of PV shows that, in comparison to non-epileptic ACI control rats, the number of PV-containing neurons in WAG/Rij rats is considerably lower in the somatosensory cortex (parietal cortex area 1 and the forelimb area) and in the olfactory tubercle, but similar in the rostral reticular thalamic nucleus. The possible significance of the low amount of PV in the cortical areas in relation to absence epilepsy 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 human 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 month-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 SWD in the rRTN depends on the interaction between low- and high-threshold calcium currents that are mediated by T- and L-type voltage-operated calcium currents, respectively, and on a calcium-activated potassium conductance (McCormick and Bal, 1997). In WAG/Rij

rats, manipulation of T-, L- and P/Q-type calcium currents regulates the number of SWD (van Luijtelaar *et al.*, 2000). Recent quantitative immunocytochemical studies indicate the involvement of P/Q-type channels in absence epileptogenesis in the rRTN of the WAG/Rij rat (M.C. van de Bovenkamp-Janssen *et al.*, in press.). The calcium-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 GABA-ergic neurons (Celio *et al.* 1990) where it protects neurons from neurotoxic calcium 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 calcium 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 GABA-ergic neurons (Houser *et al.*, 1980; Celio *et al.* 1990). In the light of the important role of both voltage-activated calcium channels and calcium-activated potassium 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 rRTN, the caudal reticular thalamic nucleus (cRTN) and in several cortical areas, comparing absence epileptic WAG/Rij rats with age-matched, non-epileptic ACI control rats (Inoue *et al.*, 1990).

MATERIALS AND 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 PREPERATION

Animals were anaesthetized by intraperitoneal administration of 40 mg sodium pentobarbital (Sanofi Sante, Maassluis, The Netherlands) and intracardially perfused with 4% paraformaldehyde in PB (72 mM Na_2HPO_4, 27.5 mM NaH_2PO_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_2HPO_4, 22 mM NaH_2PO_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_2O_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 mm^2, at the level Bregma 0.7 mm and Interaural 9.7 mm, in 5 cortical regions, viz. cingulate cortex area 1 (Cg1), parietal cortex area 1 (Par1), olfactory tubercle (Tu), 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 cRTN, 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 neurons 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). Both ACI and WAG/Rij rats show

some particular phenomena. Within certain brain structures, areas are poor in PV-positive cells and some structures even reveal regions that hardly or not show not devoid of neurons. Moreover, with PV-immunoreactive cells (e.g. Par 1; Fig. 3a,b). CV staining reveals that these regions are not devoid of neurons. Moreover, with cytodeath staining no apoptotic cells are visible, neither in WAG/Rij nor ACI rats which 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 neurons 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 observation 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.

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 neurons, determination of their numerical density was focussed on the rRTN, cRTN and on the cerebral cortex, with the hippocampal CA3 area, which is not known to be involved in absence epilepsy, as a reference (Inoue *et al.*, 1993). No statistically significant differences in density between WAG/Rij and ACI rats were found in the rRTN, cRTN, the Gu, the Cgl and the CA3 area, although for all cortical areas the WAG/Rij rat tends to contain less PV-immunoreactive cells compared to similar regions in the ACI rat. However, clear differences appear in the Par1 and in the FL, where ACI rats show about 2 times as many PV-positive cells as WAG/Rij rats.

The most prominent difference was seen in the Tu, where ACI rats demonstrate a substantial number of PV-positive neurones but WAG/Rij rats almost completely lack such cells (Fig. 4).

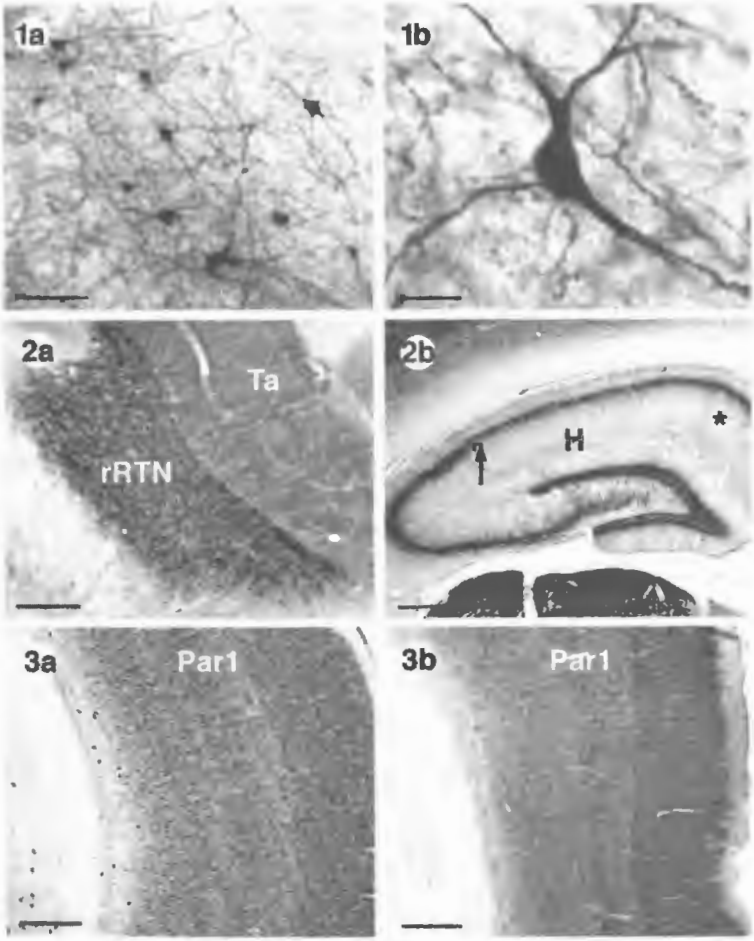

Fig. 1. PV-immunoreactive neurons in piriform cortex of ACI rat. (a) Bar: 50 μm. (b) Detail. Bar: 10μm. Fig. 2. PV staining of WAG/Rij rat brain slice. (a) Rostral reticular thalamic nucleus (rRTN) and adjacent thalamic nuclei (Ta). (b) Hippocampus (H). Arrow indicates the pyramidal cell layers of the hippocampus, asterisks indicates the molecular hippocampal layers. Bars: 500 μm. Fig. 3. Parietal cortex area 1 (Par 1). PV-positive neurons are numerous in ACI rat (a) but scarce in WAG/Rij rat (b). Bar: 400 μm.

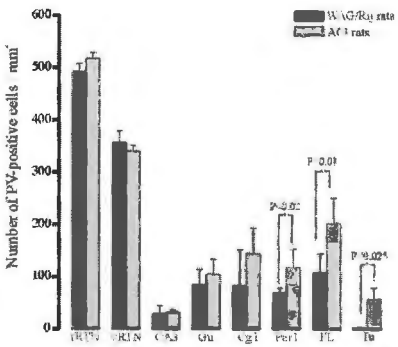

Fig. 4. Number of PV-positive neurons / mm^2 in various brain areas of WAG/Rij (n=5) and ACI (n=5) rats. CA3: CA3 region of hippocampus, Cg1: cingulate cortex area 1, cRTN: caudal reticular thalamic nucleus, FL: forelimb area, Gu: gustatory cortex, Par1: parietal cortex area 1, rRTN: rostral reticular thalamic nucleus, Tu: olfactory tubercle. Asterisks and P-values indicate statistical difference.

The presence of PV-positive cells in the RTN is in line with the GABA-ergic 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 an involvement of the rRTN in SWD does not depend on a changed amount of neuronal PV. On the other hand, in three distinct brain areas, viz. Par1, FL and Tu, 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 may be 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. In neurons devoid of PV, a too high Ca^{2+} concentration might negatively affect such processes. For example, prolonged Ca^{2+}-activated K^+ channel activity would hyperpolarize the GABAergic neuron. Reduced GABAergic inhibition has been demonstrated in the cortex of the WAG/Rij rats (Luhmann *et al.* 1995). If neurons in the cortical focus of absence epilepsy, the Par1, would be affected in such a way,

they might be 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.

The absence of PV in the Tu of WAG/Rij rats can not be readily related to SWD activity since there are no indications that this cortical area is involved in absence epilepsy. However, there are indications that the Tu has a role in adaptation to stressful challenges (Fuxe *et al.*, 1975; Piekarzewska *et al.*, 2000) and it has been demonstrated that WAG/Rij rats deal with stress in a different way compared to non-epileptic ACI rats (de Bruin *et al.*, 2001).

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