

A transmission electron microscopic study of microglia/macrophages in the hippocampal cortex and neocortex following chronic exposure to valproate

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Summary

In chronic administration of sodium valproate to rats, significant disorders of structural integrity of the hippocampal gyrus and the neocortex of the temporal lobe, observed in the last two stages of the experiment (after 9 and 12 months), coexisted with increased number of microglial cells and, especially after 12 months, with intense phagocytic activity within these cells. At the ultrastructural level, phagocyte microglial cells were hypertrophied with several broadened processes. Their cytoplasm contained rich lysosomal apparatus, numerous lipofuscin-like structures, lipid droplets and multilaminated bodies. The nuclei of these cells were characteristic oval or round and sometimes triangle in shape with dense and highly clumped heterochromatin, distinctly accumulated under nuclear envelope, and sparse euchromatin. Microglia/macrophages were frequently present in a close vicinity of changed neuronal somata and also close to the altered elements of the neuropil pyramidal layer of the cortex. Microglial response may, together with abnormalities in neurones, astroglia and blood–brain barrier, play a significant role in the development of experimental valproate encephalopathy.

Keywords:

hippocampal cortex, microglia/macrophages, neocortex, rats, ultrastructure, valproate

The term microglia refers to intrinsic cells of the central nervous system (CNS) of mesodermal origin, characteristic morphology, and antigen phenotype consistent with that of a specialized type of mononuclear phagocyte. These cells are distributed ubiquitously throughout the CNS and comprise up to 20% of the cell population in rodent brain. Microglia are the major components of macrophages found in the brain (Perry & Gordon 1988; Dickson *et al.* 1991; Dickson & Lee 1995).

The morphology of cells with microglia phenotype *in vivo* varies according to the location and to the microenvironmen-

tal factors. This ranges from compact amoeboid cells to widely ramified cells, with considerable variation in the length and complexity of branching of the processes (Giulian & Baker 1986; Perry & Gordon 1988; Dickson & Lee 1995).

Functionally, microglia are an important immune effector cell population in the brain (Perry & Gordon 1988; Streit *et al.* 1988; Graeber & Streit 1990; Gehrman *et al.* 1992; McGeer *et al.* 1993) and have been implicated in the pathogenesis of several human neurological disorders, e.g. Alzheimer's disease, Parkinson's disease, multiple sclerosis, human immunodeficiency virus encephalopathy and amyotrophic lateral

sclerosis (Dickson *et al.* 1991; McGeer *et al.* 1993; Dickson & Lee 1995; Le *et al.* 2001).

According to Banati (Banati 2002; Bonati *et al.* 1993a), the characteristic feature of microglia is their capacity for swift activation in response to neuronal stress and for site-directed phagocytosis. The most common way to study microglia *in vitro* is to use cultures derived from neonatal rat brain.

Under these experimental conditions, their differentiation into a macrophage-like morphology and immunophenotype has been demonstrated (Giulian & Baker 1986; Frei *et al.* 1987; Graeber *et al.* 1989; Banati *et al.* 1991). However, to study the differentiation of microglia into phagocytes *in vivo*, remote lesion models, e.g. facial nerve transection and facial nerve degeneration induced by the injection of toxic ricin (which leave the blood–brain barrier untouched), have been used (Streit *et al.* 1988). The phagocytic capacity of microglial cells has also been studied in other neuropathologies, such as in the deafferented dentate gyrus, which is a model of anterograde degeneration (Gehrmann *et al.* 1991).

In previous studies, we have investigated the effects of chronic valproate exposure on different cellular elements of selected CNS regions, of both neuroectodermal and mesenchymal origin, including the cerebellar cortex, the hippocampal gyrus and the neocortex of the temporal lobe. Long-term administration of the antiepileptic and antipsychotic drug sodium valproate (VPA) to rats caused significant ultrastructural degenerative changes in nerve cell perikarya and their processes, protoplasmic astrocytes and morphology of the blood–brain barrier in all the CNS sites mentioned above (Sendrowski *et al.* 2003; Sobaniec-Lotowska & Sobaniec 1996a; Sobaniec-Lotowska *et al.* 2000, 2001; Sobaniec-Lotowska 2001b, 2002, 2003).

The aim of the present study was the ultrastructural analysis of microglia which appear in the cortex of the hippocampal gyrus and in the neocortex of the temporal lobe of rat during the course of long-term VPA treatment. Until now, except for our preliminary studies (Sobaniec-Lotowska 2001a; Sobaniec-Lotowska & Sobaniec 1996b), such investigations have not been performed. This subject is of particular interest in the light of controversies, which surround morphogenesis and function of these cells in various experimental models of CNS damage and because of not yet fully elucidated morphological basis of valproate encephalopathy.

Materials and methods

Two groups of 3-month-old male Wistar rats (initial body mass 160–180 g) were preselected according to the standard pharmacological screening tests and used for the these studies.

The animals were kept in a well sunlit room at 18–20 °C and fed standard granulated rat chow and tap water. All procedures were carried out in strict accordance with Helsinki Convention Guidelines for the care and use of laboratory animals.

Group I consisted of 30 rats receiving sodium valproate as a 20% solution of sodium salt of valproic acid daily in fasting state with an intragastric tube, at the effective dose of 200 mg/kg body weight for 1, 3, 6, 9 and 12 months (six animals in each time subgroup).

Group II consisted of 10 control animals matched in respect of age with experimental animals, receiving physiological saline in the same way as the Group I rats treated with VPA.

The rats of both the groups were subjected to behavioural examinations using Lat's test to evaluate the psychomotor and cognitive activity of the animals. The rats were weighed every 2 weeks to verify the correct dose of the antiepileptic drug.

Serum concentrations of sodium valproate in Group I were measured by gas chromatography and ranged between 60 and 135 µg/ml (mean 111.3333 µg/ml; SD 21.6131) (Sobaniec-Lotowska 2001b).

At the end of the experiment, 24 h after the termination of the final VPA administration, the animals were killed under Nembutal anaesthesia by intravital intracardiac perfusion with 2.5% glutaraldehyde in 0.1-M cacodylate buffer, pH 7.4, at constant pressure of 80 mmHg. Small specimens (six, 1 mm³) cut of the gyrus hippocampal cortex (using a magnifying glass) and neocortex of the temporal lobe were processed routinely for embedding in Epon 812. Semithin sections were stained with Toluidine Blue and examined in the light microscope. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined using an Opton 900 PC transmission electron microscope (Zeiss, Oberkochen, Germany) (Sobaniec-Lotowska & Sobaniec 1996a; Sobaniec-Lotowska 2001b, 2002, 2003).

The material obtained from the cortex of CNS structures examined in the control group was processed using the same techniques as for the VPA-treated animals.

Results

In previous studies of chronic administration of VPA to rats, the first discrete electron microscopic changes in structural elements of the cortex of the hippocampal gyrus and the neocortex of the temporal lobe were observed after 3 months, and these changes intensified with time, being most pronounced after 9 and 12 months. These changes include significant disorders of structural integrity of the CNS regions examined, and at 9 and 12 months, they coexisted with changes in microglial cells (Figures 1 and 2). These were

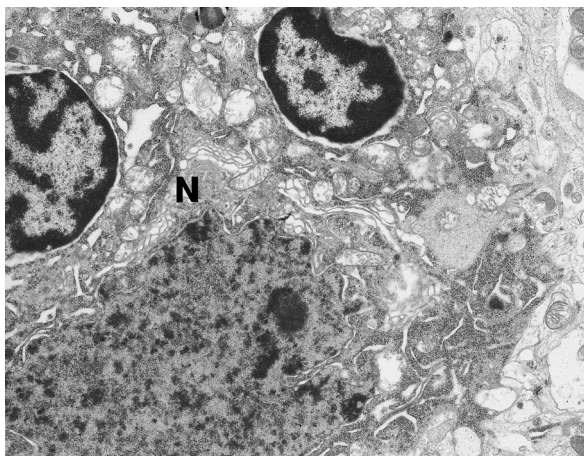


Figure 1 Two microglial cells adhering to dark neurone (N). The nuclei of these cells contain dense heterochromatin distinctly accumulated under the nuclear envelope. Hippocampal cortex. Nine months of valproate administration. Original magnification $\times 4400$.

increased in number especially after 12 months. These numerous, round or oval cells with features of microglial and macrophage cells were most frequently seen in the pyramidal layer of the cortex of the CNS regions examined, in close vicinity of damaged neuronal perikarya (identifiable as ischaemic neurones or sometimes as fragments of cells) (Figures 3a, 5 and 6) or injured neuropil elements (Figure 4a). As macrophages, these cells showed similar ultrastructural change, irrespective of location in CNS. These findings about the cells found in the cortex of the hippocampal gyrus and in the neocortex of the temporal lobe are presented together.

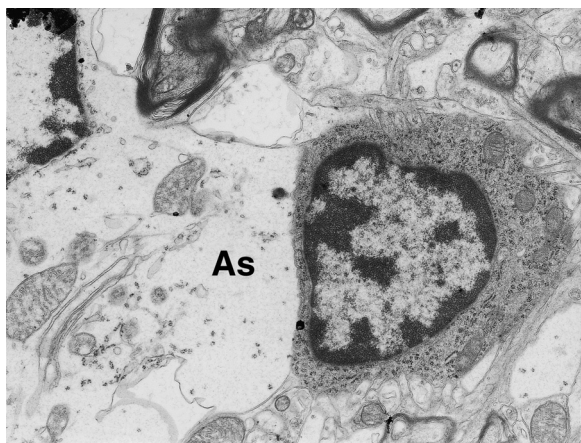


Figure 2 A well-preserved microglial cell with a triangle-shaped nucleus in a close vicinity of swollen protoplasmic astrocyte (As). Neocortex. Nine months of valproate administration. Original magnification $\times 7000$.

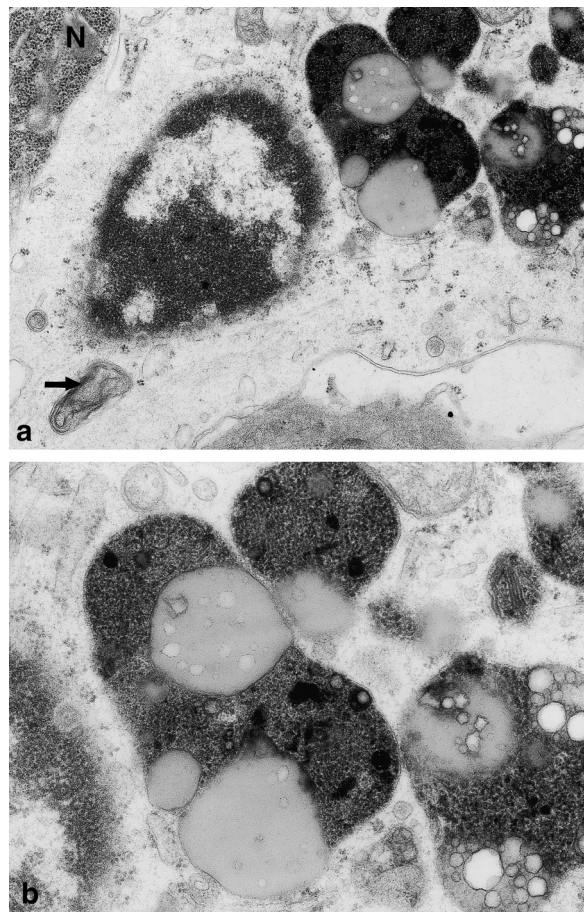


Figure 3 (a) Fragment of microglial cell perikaryon showing features of marked phagocytic activity; the nucleus contains dense, highly clumped heterochromatin; and under nucleus visible multilaminated body (\rightarrow). N - small fragment of dark neurone. Hippocampal cortex. Nine months of valproate administration. Original magnification $\times 12,000$. (b) Higher magnification ($\times 20,000$) of microglial cell perikaryon, showing lipofuscin-like structures and frothy lipophagosomes of phagocytic microglial cell.

The phagocytic microglial cells were enlarged (with a high nuclear to cytoplasmic ratio) with expanded cytoplasm and broad processes. They showed prominent features of phagocytic activity, such as abundant lysosomes with characteristic, well-developed, highly polymorphic, frothy lipophagosomes, numerous deposits of lipofuscin-like material, lipid droplets and multilaminated bodies (Figures 3–6).

The nuclei were characteristic oval or round and sometimes triangular in shape with dense and highly clumped heterochromatin, accumulated distinctly under the nuclear envelope. The euchromatin was sparse (Figures 3 and 4a,b).

The perinuclear region showed few conspicuous organelles, usually including a few strands of granular endoplasmic

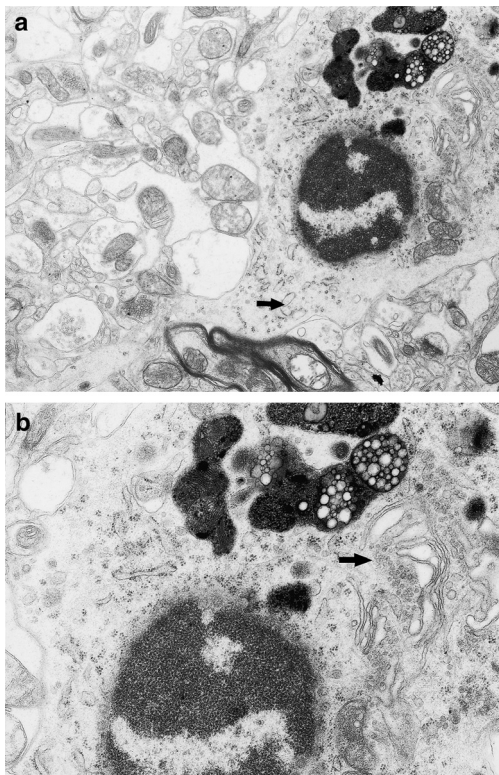


Figure 4 (a) Phagocytic microglial cell in a close vicinity of swollen neuropil elements. The nucleus of this cell is round and contains dense heterochromatin, highly clumped under nuclear envelope and sparse euchromatin; in the swollen cytoplasm visible profiles of granular endoplasmic reticulum (→). Neocortex. Nine months of valproate administration. Original magnification $\times 4000$. (b) Higher magnification ($\times 12,000$) of phagocytic microglial cell. Well-developed frothy lipofuscin-like bodies and dilated Golgi apparatus (→).

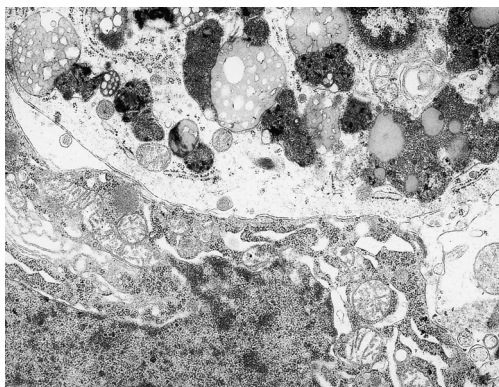


Figure 5 Phagocytic microglial cell adhering to the pericaryon of damaged pyramidal neurone – channels of granular endoplasmic reticulum within neuronal cytoplasm markedly broadened. Hippocampal cortex. Twelve months of valproate administration. Original magnification $\times 7000$.

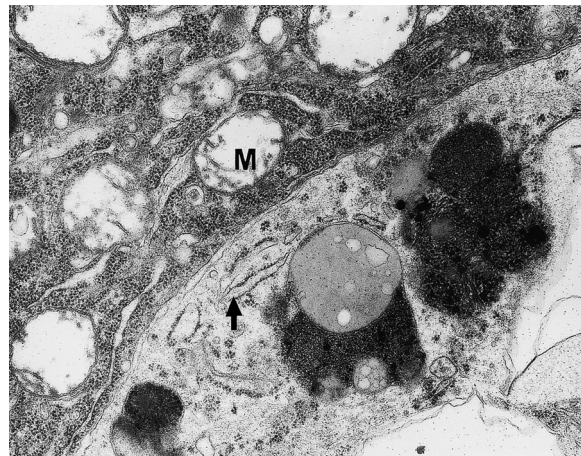


Figure 6 Fragment of phagocytic microglial cell in a close vicinity of injured pyramidal neurone. The cytoplasm of phagocyte contains channels of granular endoplasmic reticulum (→). M – altered neuronal mitochondria. Neocortex. Twelve months of valproate administration. Original magnification $\times 12,000$.

reticulum and dilated Golgi apparatus (Figures 4a,b and 6). Some mitochondria were normal, but others were swollen with degenerative changes.

At 12 months, the phagocytic cells showed similar general features, but some cells contained more phagolysosomes than were seen at 9 months, and also showed features suggesting degradation of ingested material.

Discussion

At the end of chronic VPA administration, i.e. after 9 and 12 months, significant abnormalities in the structural elements of the cortex of the hippocampal gyrus and the neocortex of the temporal lobe were accompanied by increased number of microglial cells and by evidence suggesting intense phagocytic activity within these cells.

Microglia/macrophages were present frequently in a close vicinity of damaged neuronal cells and in the neighbourhood of altered elements of the neuropil pyramidal layer of the cortex of all areas studied.

The morphological features of microglial reaction described in the present study were not specific for VPA. Similar ultrastructure of microglial and macrophagic cells *in vivo* in various regions of CNS has been observed under other conditions, e.g. in the arcuate nucleus after neurotoxic injury induced by a single subcutaneous injection of monosodium glutamate in neonatal rats (Pelaez *et al.* 1999); in the lizard medial cortex, after intraperitoneal injection of the neurotoxin 3-acetylpyridine (Nacher *et al.* 1999); in the transgenic mouse model for CNS primary demyelination and motor disease, where the

cytokine interleukin-3 is expressed in astrocytes (Chiang *et al.* 1996; Powell *et al.* 1999); in the developing cerebellum of rats exposed to X-ray irradiation (Das 1976); and in the cerebellar cortex of rats following an episode of total ischaemia (Walski & Borowicz 1995).

The differentiation of microglia into brain macrophages in response to neuronal stress is accompanied by the release of several secretory products. They include lysosomal proteinases, such as cathepsin B/L (Banati *et al.* 1993b); cytokines (e.g. interleukin-1, interleukin-6, neurokinins, tumour necrosis factor- α ; Griffin *et al.* 1989; Dickson *et al.* 1991; Banati 2002); colony-stimulating factors (CSFs), particularly macrophage CSF and granulocyte macrophage CSF (Giulian & Ingeman 1988; Suzumura *et al.* 1990); and excitatory amino acids like glutamate (Giulian *et al.* 1990; Piani *et al.* 1991).

Thus, the microglia/macrophages found in the CNS in the present study following chronic administration of VPA and/or its metabolites participate in the removal/phagocytosis of injured nervous tissue. Together with abnormalities in neurones, astroglia and blood-brain barrier, cells must play a role in the development of experimental valproate encephalopathy, and with the toxic effects of VPA in humans treated with these drugs. However, the ultrastructural findings favour a reactive phagocytic role, rather than a secretory role, for these cells during the development of the neuropathology.

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