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EXPRESSION OF 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHODIESTERASE IN THE AMOEBOID MICROGLIAL CELLS IN THE DEVELOPING RAT BRAIN

C. Y. WU,[a,b] J. LU,[c] Q. CAO,[a] C. H. GUO,[a] Q. GAO[a] AND E.-A. LING[a]*

[a]*Department of Anatomy, Yong Loo Lin School of Medicine, Block MD 10, 4 Medical Drive, National University of Singapore, Singapore 117597*

[b]*Department of Histology and Embryology, Kunming Medical College, 191 West Renmin Road, Kunming, PR China 650031*

[c]*Defence Medical and Environmental Research Institute, DSO National Laboratories, 27 Medical Drive, Singapore 117510*

Abstract—Expression of 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) in amoeboid microglial cells (AMC) in developing rat brain from prenatal day 18 (E18) to postnatal day 10 (P10) was demonstrated by immunohistochemistry/immunofluorescence and immunoelectron microscopy both *in vivo* and *in vitro*, respectively. Furthermore, real time-polymerase chain reaction (PCR) was performed to determine the expression of CNPase at mRNA level in cultured microglial cells in control conditions and following lipopolysaccharide stimulation. CNPase immunoreactive amoeboid microglia occurred in large numbers in the corpus callosum, subventricular zone and cavum septum pellucidum at P0 but were progressively reduced with age and were undetectable at P14. By immunoelectron microscopy, immunoreaction product was associated primarily with the plasma membrane, filopodial projections and mitochondria in AMC. Real time-PCR analysis revealed that CNPase mRNA was expressed by cultured amoeboid microglia and was significantly up-regulated in microglial activation induced *in vitro* by lipopolysaccharide. The functional role of CNPase in AMC remains speculative. Given its expression in AMC transiently occurring in the perinatal brain and that it is markedly elevated in activated microglia, it is suggested that the enzyme may be linked to the major functions of the cell type such as release of chemokines and cytokines. In relation to this, CNPase may play a key role associated with transportation of cytoplasmic materials.

Key words: CNPase, amoeboid microglial cells, development, rat, lipopolysaccharide.

It has been reported that the enzyme, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) is more concentrated in the nervous system than in other non-neural tissues (Weissbarth et al., 1981). The enzyme is a myelin-associated enzyme, and in the CNS and peripheral nervous system, it is expressed at high levels in the two cell types that elaborate myelin, the oligodendrocytes and the Schwann cells, respectively (Sprinkle, 1989). By virtue of this cell-specific expression, CNPase is recognized as a characteristic marker for these two myelin-producing glial cell types (Kim et al., 1984; Sprinkle, 1989). CNPase is involved in the very rapid growth of myelin membrane during early oligodendrocyte membrane biogenesis and possibly maintenance (Sprinkle, 1989), and has a role in oligodendrocytes as part of the mechanism giving rise to process extension in early stage myelinogenesis (Santambrogio et al., 2001; Zhang et al., 2005). The physiological roles of CNPase are still under investigation, although it has been implicated in regulation of tubulin polymerization/distribution (Bifulco et al., 2002) and myelin-axon interaction (Zhang et al., 2005).

Oligodendrocytes arise from precursor cells derived from the subventricular zones during early development of the rat brain (Paterson et al., 1973; Privat, 1975; see also review by Baumann and Pham-Dinsh, 2001). The onset of CNPase expression occurs before that of either myelin basic protein (MBP) or proteolipid protein (PLP). CNPase mRNA can be found as early as embryonic day (E) 15, whereas MBP and PLP mRNAs cannot be detected until after birth in different rat brain regions (Kanfer et al., 1989). There is evidence suggesting that CNPase is expressed in both oligodendrocyte precursors and myelinating oligodendrocytes (Yu et al., 1994; Scherer et al., 1994). Other than oligodendrocyte precursors, mature oligodendrocytes and glioblasts (glial precursors) also expressed CNPase as described by Braun et al. (1988). As far as can be ascertained, there is no available information if other glial cells express CNPase.

Microglia are a different cell population that arises from a hematopoietic lineage (Ling and Wong, 1993; Eglitis and Mezey, 1997; Corti et al., 2002). The nascent form referred to as the amoeboid microglial cells (AMC) occurs transiently in large numbers in the supraventricular area of the corpus callosum, subventricular zones of the lateral ventricle, and the cavum septum pellucidum in the developing rat brain (see review by Ling et al., 2001). AMC function as active brain macrophages in the perinatal period. With advancing age, AMC transformed into microglia that are ramified, hence, referred to as ramified microglia that persist through the adulthood (Ling et al., 2001). When bone marrow is transplanted, bone marrow–derived cells that are indistinguishable from microglial cells are found in the

*Corresponding author. Tel: +65-65163200; fax: +65-67787643.
E-mail address: antlea@nus.edu.sg (E.-A. Ling).

Abbreviations: AMC, amoeboid microglial cells; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CNP-IR, CNPase-immunoreactive; DAB, 3–3′ diaminobenzidine tetrahydrochloride; DMEM, Dulbecco's modified Eagle's medium; E, embryonic day; FBS, fetal bovine serum; LPS, lipopolysaccharide; MBP, myelin basic protein; P, postnatal day; PB, phosphate buffer; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PLP, proteolipid protein.

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brain (Priller et al., 2001; Corti et al., 2002). Goolsby et al. (2003) reported that bone marrow cells expressed the oligodendroglial gene encoding CNPase. Indeed, our recent finding has revealed that CNPase is expressed in bone marrow stromal cells in culture (Ling et al., unpublished observations). In co-culture, microglia stimulated the synthesis of sulfatide, a myelin-specific galactolipid, in oligodendrocytes (Hamilton and Rome, 1994). Very interestingly, Yokoyama et al. (2004) reported that rat primary microglial cells expressed nestin, A2B5, and O4 antigens, which are markers for oligodendrocyte precursor cells, suggesting that microglia can express antigens shared by oligodendrocytes.

Arising from these finding, this study sought to ascertain if AMC express CNPase by immunohistochemistry, immunofluorescence double-labeling, immunoelectron microscopy and real time-polymerase chain reaction (PCR) both *in vivo* and *in vitro*.

EXPERIMENTAL PROCEDURES

In the handling and care of all rats (Sprague–Dawley strain), the International Guiding Principles for Animals Research, as stipulated by the Council for International Organizations of Medical Science (CIOMS) (1985) and as adopted by the Laboratory Animals Centre of the National University of Singapore, were followed. All efforts were made to minimize the number of rats used and their suffering.

CNPase immunoexpression in normal developmental rat brain

Prenatal rats at E18 and E20, derived from three pregnant Sprague–Dawley female rats, respectively, to postnatal days (P) 0–P14 from three litters were divided into eight groups: E18, E20, P0, P3, P5, P7, P10, and P14 (P0–P14, $n=3$ for each time point). At the respective time points, the rats were anesthetized with 3.5% chloral hydrate or Nembutal (100 mg/kg) injected intraperitoneally and perfused transcardially with Ringer's solution, followed by fixation with 2% periodate-lysine-paraformaldehyde. The brain was removed, post-fixed for 3 h in the same fixative, then cryoprotected in 30% sucrose for 24 h. Frozen sections at 30 μm were cut coronally through the forebrain with a cryostat (Leica CM 3050) and mounted onto gelatin-coated slides and stored at −20 °C until use.

Immunohistochemistry

Immunohistochemistry for CNPase was carried out using the avidin-biotinylated peroxidase complex (ABC) method. Briefly, the sections were washed 3×10 min in 0.1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS) pH 7.4, then incubated in 0.3% H_2O_2 for 15 min to block the endogenous peroxidase. The sections were preincubated with 5% normal horse serum and 0.1% Triton X-100 in 0.1 M PBS for 1 h, followed by incubation overnight with mouse anti-CNPase monoclonal primary antibody (1:300, Chemicon, Temecula, CA, USA) at room temperature. The secondary antibody (biotin-conjugated anti-mouse IgG, 1:200, Vector Laboratories, Burlingame, CA, USA) was applied for 1 h at room temperature followed by avidin–biotinylated horseradish peroxidase (Vectastain ABC kit, 1:100, Vector Laboratories, USA) for 1 h at room temperature. The reaction product was revealed with 3–3′ diaminobenzidine tetrahydrochloride (DAB). The tissue sections were counterstained with 0.25% Methyl Green in 0.1 M acetate buffer pH 4.8 and coverslipped with Permount after dehydration and clearing. For controls, the tissue sections were incubated in the incubation medium without the primary antibody or the medium was replaced with normal horse serum.

For double immunofluorescence labeling, a mixture of mouse anti-CNPase primary antibody and isolectin B_4 (15 μg/ml, Vector Laboratories) was applied to the sections overnight at room temperature. Isolectin B_4 is a selective cell marker for microglial cells. On the following day, the tissue sections were incubated with fluorescein-conjugated goat anti-mouse (Gt×MS IgG^+ IgM CY_3, Chemicon) antibody and fluorescein avidin DN (15 μg/ml, Vector Laboratories) for 2 h. They were washed, mounted with fluorescent mounting medium (Dako Cytomation, Glostrop, Denmark), and examined under a confocal laser scanning microscope (Olympus FV1000, Olympus, Tokyo, Japan). Primary antiserum omission control as well as normal goat serum control was used to further confirm the specificity of the immunofluorescence labeling.

Immunoelectron microscopy

Two P0∼1 rats were anesthetized with Nembutal (100 mg/kg) and then perfused transcardially with Ringer's solution and then 2.5% paraformaldehyde containing 0.25% glutaraldehyde in 0.1 M phosphate buffer (PB). The brain was carefully removed and post-fixed for an additional 2 h in the same fixative. Coronal sections of the brain were prepared at 100 μm using a vibratome. The sections were processed for CNPase immunohistochemistry using free-floating sections according to the method described above for light microscopy with modifications. Specifically, the reaction product was visualized using DAB containing 0.1% ammonium nickel sulfate. After DAB reaction, the sections were postfixed in 1% osmium tetroxide in 0.1 M PB, pH 7.4, for 1 h at room temperature and dehydrated in a graded series of ethanol. Tissue blocks were embedded in araldite mixture. Ultrathin sections were stained in lead citrate only and viewed in an EM 208S electron microscope.

Along with the above, two P0-1 rats were anesthetized with Nembutal (100 mg/kg) and perfused with 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M PB for routine electron microscopy. Tissue sections were prepared without immunoreaction. Ultrathin sections were double stained in uranyl acetate and lead citrate.

Microglial cell culture

Mixed glial cultures were prepared from 1∼2-day old postnatal rat brain by the method of Giulian and Baker (1986) with some modifications. Briefly, the meninges were stripped off from the hemispheres, minced and dissociated in 0.05% trypsin and deoxyribonuclease (20 μ/ml) in Dulbecco's modified Eagle's medium (DMEM). Cells were plated in a 75-cm^2 flask at a density of 1.0×10^6 cells/ml in DMEM with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (Sigma, St. Louis, MO, USA), 0.1 mM non-essential amino acid (Invitrogen Life Technologies, Carlsbad, CA, USA), 0.001% insulin (Sigma) (complete medium) and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was replaced after 24 h and then every 3∼4 days. After 2 weeks, confluence of cells was achieved. Microglial cells could be isolated by mild trypsinization method (Saura et al., 2003). In brief, confluent mixed glial cultures were subjected to a trypsin solution (0.25% trypsin, 1 mM EDTA in DMEM) diluted 1:3 in DMEM for 10 min at 37 °C, resulted in the detachment of an upper layer of cells in one piece, whereas a number of cells remaining attached to the bottom of the flask were microglia. DMEM with 10% FBS was then added for trypsin inactivation. The medium containing the layer of detached cells was then aspirated and then replaced with complete medium for 24 h. At this juncture, total RNA was extracted for real time-PCR analysis. The adherent microglial cells could also be recovered by a 5 min incubation with trypsin 0.25% with vigorous pipetting and replated at 2.0×10^5 cell/ml in a 24-well plate with polylysine-coated coverslips for immunofluorescence, or in a chambered coverglass for immunoelectron microscopy. The cells were allowed to adhere for 40 min