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The Distribution of MAP-2 Phosphorylation in Cerebral Cortex of Long-Tailed Monkey Fetuses (*Macaca fascicularis*) in the Last Trimester of Gestation

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Abstract

Memories are storage in cholinoceptive cells, the cells which are enriched with microtubule-associated protein 2 (MAP-2) that localized in the neuronal dendrite and the cell bodies. Phosphorylation of MAP-2 may increase memory with reduce stability of dendrite by altered dendrite length and lead new side-branches of neuronal as a neuronal plasticity processes in cerebral cortex. The aim of this research is to study the distribution of MAP-2 phosphorylation neurons in cerebral cortex of long-tailed macaques in the third semester of gestational immunohistochemically using avidin biotin conjugated complex method. Neurons MAP-2 phosphorylation immunoreactive were located in dendrites and cell bodies, mostly in pyramidal neurons of cerebral cortex. Intensity of MAP-2 phosphorylation immunoreactivity in layer V were stronger than another layer and the neurons that very intensely stained were the pyramidal cells in frontal and parietal lobes, that was suggested that neurons in this areas more responsive to neuroplasticity. From the results we concluded that MAP-2 phosphorylation already distributed in the cerebral cortex of long-tailed macaque fetuses at the last trimester of gestation, mostly in the pyramidal cells of layer V that is suggested plays a role for preparation of memory formation.

Keywords: fetus, long-tailed monkey, cerebral cortex, memory, MAP-2 phosphorylation

Introduction

The distribution of protein MAP-2 in the fetal cerebral cortex that suggested play a role in neuronal plasticity for memory formation during prenatal periode was studied immunohistochemically using MAP-2 phosphorylation antibodies

MAP-2 is a protein binding microtubules, consists of a pair of high molecular mass (280kDa) polypeptides, called MAP-2a and MAP-2b, and several low molecular mass (70kDa) protein called MAP-2c (Chung *et al*., 1996). MAP-2s are predominantly expressed in neurons and serve as substrates for most of protein kinases and phosphatases in the neuron (Sánchez *et al*., 2000) which are sensitive to many input for differentiation and plasticity of neurons (Johnson and Jope, 1992). MAP-2 is distributed in dendrite and soma of neurons which is plays a role in neuronal signaling by modulate the stability of microtubule through direct linkages with cytoskeletal element.

Memory is stored in cholinoceptive cells which every cholinoceptive cell is furthermore enriched with abundant amount

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of a labile cytoskeletal protein: microtubule-associated protein-2 (MAP-2). Conversely, 80% of MAP-2 rich cells are cholinoceptive, and 20% of MAP-2 rich cells that are not cholinoceptive that may constitute to another group of memory-related cells that respond exclusively to other neurotransmitters (Woolf, 1993;1998).

The neurobiological studied of memory in animal have several potential advantages. Beauregard and Bachevalier (1996) reported that monkeys with early hippocampectomized in infancy fits remarkably well with that of schizophrenic symptoms, which had subtle changes in social interactions in infancy that resulted in a profound loss of social affiliation when they reached adulthood. They also displayed selective deficits in memory functions and a marked increase in locomotor stereotypies in adulthood. Exploration of animal memory can also provide information about particular memory mechanisms evolved.

From psychology and neuroscience points, there are at least five major memory systems in humans: episodic memory, semantic memory, the perceptual representation system, procedural memory, and working memory. Episodic memory is the explicit recollection of incidents that occurred at a particular time and place in one's personal past. Both prefrontal cortex and medial regions of temporal lobe play important roles in episodic memory. Semantic memory refers to general knowledge of fact and concepts that is not linked to any particular time and place. The anterior and lateral regions of the temporal lobe play important roles in semantic memory system. The perceptual representation system plays in important role in the identification of word and object on the basis of their form and structure. PET scanning studies have revealed that specific regions within the extrastriate occipital cortex are involved in processing and representing the visual form of word, whereas regions within the temporal and frontal cortices are involved with word meaning. The inferior temporal and fusiform gyrus were suggested are involved in representing the global structure of an object. Procedural memory refers to the acquisition of skills and habits that are acquired gradually through repetitive practice. Procedural memory depends critically on a cortical-striata system (Eichenbaum *et al*., 1999). The term of working memory refers to a brain system that provides temporary storage and manipulation of the information necessary for such complex cognitive tasks as language compherension, learning, and reasoning. Working memory has been found to require the simultaneous storage and proceeding of information (Baddeley, 1992). The working memory processed were in the parietal and the prefrontal cortex in combination with widespread cortical area (Eichenbaum *et al*., 1999).

Memory also can be divided into short-term and long term memory. Short term memories which includes memories that last for sec or at most min are stored in the hippocampus unless they are converted into longer-term. Long-term memory which once stored, can be recalled up to years or even a lifetime later, is generally believed to result from actual structural changes, instead of only chemical changes, at the synapses, and these enhance or suppress signal conduction in neocortex (Ganong, 2001).

The transition from short-term memory to long-term memory begin with changes in neuronal activity, then in neurotrophin release that increase the production of cholinergic enzymes in cholinergic cells include choline transferase (ChAT), followed by enhanced acetylcholine (Ach) release, muscarinic response and Ca^{2+} influx. Cholinergic muscarinic activation acting through phosphoinositide-spesific phospholipase C (PI-PLC), which then turn on PKC and Ca^{2+}/calmodulin dependent kinase II (C/CMK), which is leads to increased phosphorylation of MAP-2 protein. Phosphorylation of MAP-2

may reduce cytoskeletal stability (Johnson and Jope, 1992; Woolf, 1998; and Sánchez *et al*., 2000) and play a key role in controlling dendritic elongation and branching (Hely et al., 2001). Breakdown of MAP-2 allows new side-branches of dendrite to form and that this prosess would involve new protein synthesis (Woolf, 1998), thereby favoring dendritic plasticity. MAP-2 phosphorylation is rapidly dephosphorylated by N-methyl-D-aspartate (NMDA) receptor activation (Woolf, 1997).

The cerebral cortex plays a role for integrates sensory signals, initiates motor response, and involve in learning and memory activities (Samuelson, 2007). Based on phylogenetic categories, the cerebral cortex were divided into archicortex, paleocortex, and neocortex cortex (Palomero-Gallagher and Zillers, 2004). The archicortex is the cerebral cortex which earlier develop and the areas which are included in archicortex are paraterminal gyrus, supracallosal gyrus, fasciolar gyrus, and hippocampal formation (dentate gyrus, hippocampus, alveus, fimbria of hippocampus, subiculum) (Martin and Bowden, 1997). The Neocortex is part of cortex cerebral cortex which develop latest. Frontal, parietal, temporal, and occipital cortexs are grouped as neocortex. The paleocortex develop between archicortex and neocortex. Orbirofrontal, agranular insular, perirhinal, cingulated, and retrosplenial cortexs are grouped as paleocortex (Palomero-Gallagher and Zillers, 2004).

Neocortex is devided in 6 layer. The most superficial is layer I or plexiform layer or molecular layer, primarily consist of fiber which horizontal Cajal neurons are scattered. Layer II /external granular layer consist of densely packet of small size stellate cells also small and medium-sized pyramidal cells. Layer III/external pyramidal layer consist of pyramidal cells that increases in size in deeper part of layers. Layer IV/internal granular layer consist of densely packed pyramidal and small size stellate cells. Layer V/ internal pyramidal layer consist of large and medium sized of pyramidal neurons, stellate neurons and cell Martinoti. The deepest layer is layer VI/ multiformis layer which consist of mixture neurons. Archicortex and paleocortex are 3 layered: molecular layer, pyramidal layer and granular layer (Afifi and Bergman, 2005).

Developing brain of human and macaques monkey share similar features and long-tailed macaques (*Macaca fascicularis*) since their closed relationship in anatomy and physiology, for that reason we choose this animal as a human model. Gestation period of long-tailed macaques is approximatly 165 days (Supriatna and Wahyono, 2000), so the last trimester of gestation is begin from 110 days of gestation.

Materials and Methods

Materials

The samples were 121 and 150 days old long-tailed macaque fetuses (*Macaca fascicularis*) as a gift from the Indonesian Primate Research Center, Bogor Agricultural University (Pusat Studi Satwa Primata, LPPM-IPB). The gestational age of 121 days old fetus was estimated by artificial insemination and for the fetus at 150 days old was estimated by timed mating.

Sample collection and tissue processing

To obtain the fetal specimens, pregnant dams were anasthelized to deep surgical level with intramuscular pentobartital (20mg/kg bw). The fetuses were delivered by midline laparotomy and anasthelized with pentobarbital (6mg/kg bw) intraumbically. The fetuses were perfused transcardially with 0,2% paraformaldehyde in 0,01 M phosphate buffer (PB) pH 7.4 at 37°C with peristaltic pump at rate 20 ml/min. After blood removal, perfusion was switched to cold 2% paraformaldehyde in PB for 20-30 min, and then the brains were removed from cranium and past-fixed by immersion in cold 2% paraformaldehyde for 24 h.

The brain were processed for paraffin embedding and cut in coronal section 12 µm thickness. Two serial section of each sample which corresponding to ac-8 and ac-24 at Template Atlas of The Primate Brain (Martin and Bowden, 1997) were selected for cresyl violet and MAP-2 phosphorylation immunohistochemistry staining. The method for collecting sample was approved by Institutional Animal Care and Use Committee (IACUC) Primate Research Center, Bogor Agricultural University (Pusat Studi Satwa Primata-LPPM IPB) No. 0200301R.

Cresyl-violed staining

Selected sections for cresyl-violet staining were deparaffinized in 3 changes xylene and rehydrate in ethanol from absolute-95%-80% to 70% and finally in distilled water, five min each. The sections were stained with cresyl-violet solution (Chroma, 1A 396) for 30 min at 37°C and dehydrate in graded ethanol from 70% to absolute, then clearing in 3 changes xylene, after that cover slipping using kanada balsam (Merck, Art. 1691).

Immunohistochemistry staining

After deparafinized and rehydration, the selected sections were washed in 0,01 M phosphate buffer saline (PBS) pH 7.4, three changes 10 min each and bloked for endogenous peroxidase using 3% H_2O_2 in distilled water solution for 30 min at room temperature, then pretreated by an antigen-retrieval technique used sodium citrate buffer solutions (10 mM sodium citrate 0,05% tween, pH 6,0) by heating in a microwave oven. For unspesific protein in sections were bloked in 10% normal goat serum (Vector Laboratories) for 1 h at room temperature, then incubated with mouse-monoclonal antibody MAP2a,b,c-primary antibody (1:100; Clone AP18, Cat.#MS-250-R7, Thermo Scientific) in a humidified chamber overnight at 4°C. After an overnight incubation, the sections were washed in PBS and incubated with biotinylated goat anti-secondary antibody IgG (1:100; Vector Laboratories) for 1 h at room temperature, then washed in PBS and incubated with peroxidase labeled avidin biotin conjugated complex (Vector Laboratories) for 1 h at room temperature. The immunoreactive product of antibodies were visualized using 3,3' diaminobenzidine-H_2O_2 as the chromogen, after that, the sections were counterstained with Mayer hematoxylin (Sigma Aldrich), and cover slipping using kanada balsam (Merck, Art. 1691).

Results

The archicortex, paleocortex, and neocortex already exist at Fd 121 and 150 days old. The archicortex as examined in hippocampal formation with was visible by appearance of cornu ammonis. The paleocortex was examined in posterior cingulate gyrus and insula. The neocortex was observed in frontal, parietal, temporal, occipital lobes and parahippocampus gyrus. The frontal lobe was examined in precental gyrus. The parietal lobe was examined in postcentral gyrus, superior parietal lobule, supramarginal gyrus, angular gyrus, and precuneus. The temporal lobe was examined in superior temporal gyrus, middle temporal gyrus, inferior temporal gyrus and fusiform gyrus. The occipital lobe was examined in occipital gyrus, inferior occipital gyrus, cuneus, and lingual gyrus (Figure 1).

Hippocampal formation is devided into dentate gyrus, cornu ammonis 1 (CA1), cornu ammonis 2 (CA2), cornu ammonis 3 (CA3), and subiculum. Dentate gyrus characterized by high density of small neurons in the granule layer, and the cornu ammonis and subiculum are dominated by pyramidal cell layer.

MAP-2 phosphorylation immunoreactive (MAP-2 IR) neurons were bipolar or multipolar in morphology, with the immunoreactivity were located in the cell bodies and dendrites. The MAP-2 IR neurons were mostly labeled the pyramidal cells and few number of

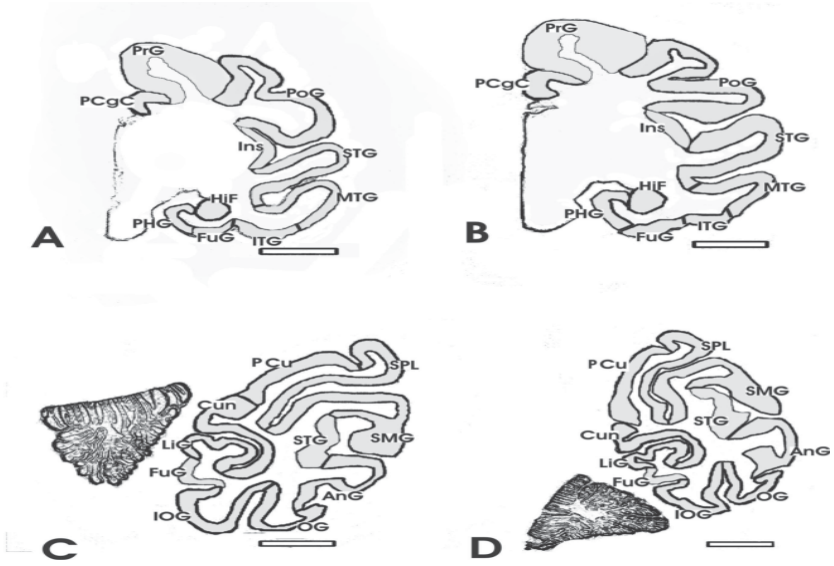

Figure 1. Area of cerebral cortex of long-tailed monkey fetuses at 121 (A,C) and 150 days old (C,D) examined was corresponding approximately to ac-8 and ac-24 based on Template Atlas of The Primate Brain (Martin and Bowden, 1997). Area of archicortex that examined was hippocampal formation (HiF). Area of paleocortex that examined was posterior cingulat gyrus (PCgC) and insula (Ins), and the neocortex was the frontal lobe: precental gyrus (PrG); the parietal lobe postcentral gyrus (PoG), superior parietal lobule (SPL), supramarginal gyrus (SMG), angular gyrus (AnG), and precuneus (PCu); the temporal lobe: superior temporal gyrus (STG), middle temporal gyrus (MTG), inferior temporal gyrus (ITG) and fusiform gyrus (FuG); the occipital lobe: occipital gyrus (OG), inferior occipital gyrus (IOG), cuneus (Cun), and lingual gyrus (LiG); and the parahippocampus gyrus (PHG). Scale bar, 5mm.

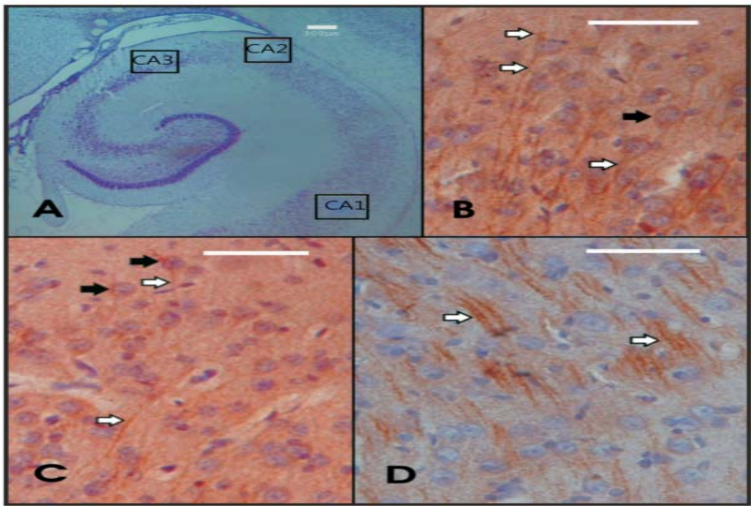

Figure 2. MAP-2 phosphorylation immunolabelling of hippocampal formation of long-tailed monkey fetus at 121 days old as an archicortex, in coronal section. (**A**) Cresyl echt violet-stained section 8 mm posterior to the anterior commisura, ac-8 in template atlas of the primates brain (Martin and Bowden, 1997) showed the compartments of the hippocampus: cornu ammonis 1 (**CA1**); cornu ammonis 2 (**CA2**); cornu ammonis 3 (**CA3**). (**B**) MAP-2 phosphorylation immunolabelling of cornu ammonis 3 (**CA3**). (**C**) MAP-2 phosphorylation immunolabelling of cornu ammonis 2 (**CA2**). (**D**) MAP-2 phosphorylation immunolabelling of cornu ammonis 1 (**CA1**). MAP-2 phosphorylation was labeled the dendrites (white arrows) and cell bodies (black arrows) in pyramidal layers of CA2 and CA3. Contrary to CA2 and CA3, MAP-2 phosphorylation in CA1, which labeled in a large number of dendrites, many of which were not obviously connected with cell bodies. Scale bar: **A** = 300μm; **B,C,D** = 50μm.

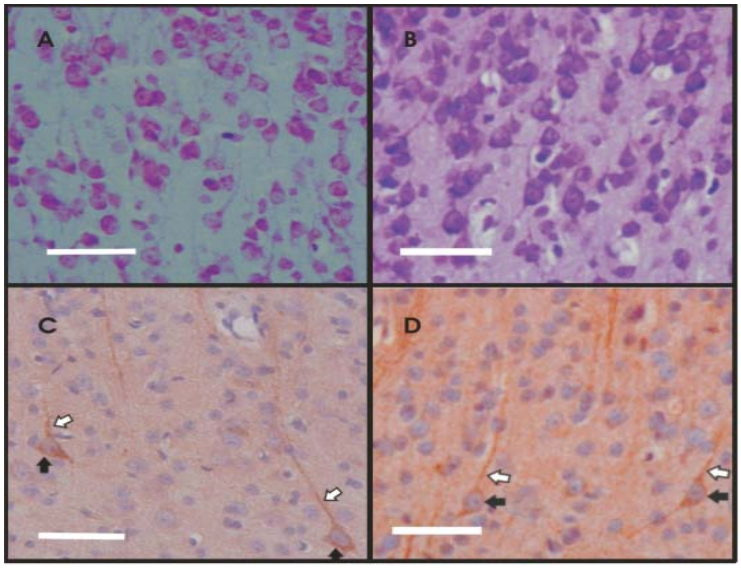

Figure 3. MAP-2 phosphorylation immunolabelling of layer V in posterior cingulate gyrus of long-tailed monkey fetuses as a paleocortex, in coronal section. Cresyl echt violet-staining of pyramidal layer at fetus 121 days old (**A**) and 150 days old (**B**). MAP-2 phosphorylation immunolabelling at layer V at fetus 121 days old (**C**) and 150 days old (**D**). MAP-2 phosphorylation is highly concentrated in the cell bodies and dendrites of pyramidal neurons. MAP-2 phosphorylation was labeled in neuronal dendrites (white arrows) and cell bodies (black arrows). Scale bar in A,B,C,D = 50µm.

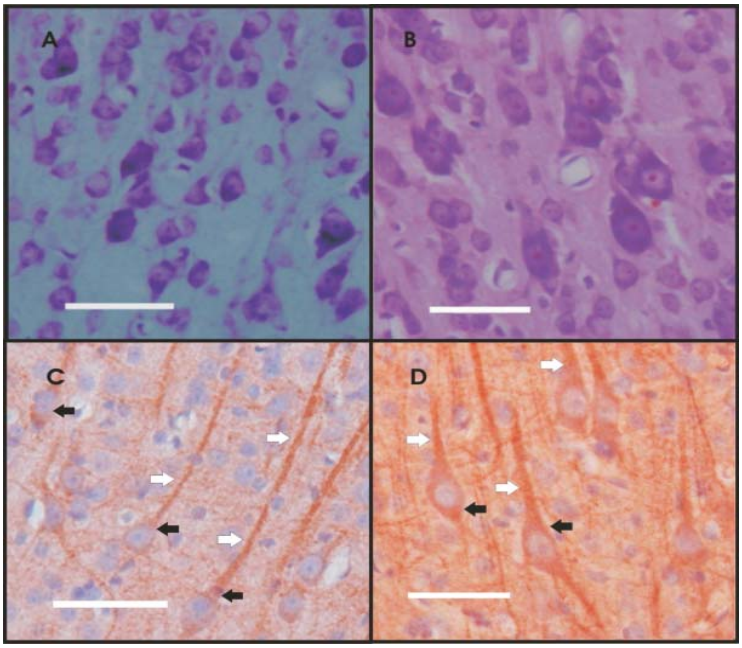

Figure 4. Neurons in layer V in precentral gyrus of long-tailed monkey fetuses frontal cortex, in coronal section. Cresyl echt violet-staining at layer V at fetus 121 days old (**A**) and 150 days old (**B**). MAP-2 phosphorylation immunolabelling at layer V at fetus 121 days old (**C**) and 150 days old (**D**). MAP-2 phosphorylation is highly concentrated in the cell bodies and dendrites of pyramidal neurons in layer V. MAP-2 phosphorylation was labeled the neuronal dendrites (white arrows) and cell bodies (black arrows). Scale bar, 50µm.

neuron besides of pyramidal cells were MAP-2 IR.

Examination in hippocampus showed that MAP-2 IR neurons were pyramidal layer neurons in the cornu ammonis 2 (CA2) and 3 (CA3). Contrary to CA2 and CA3, the dendrites of pyramidal neurons in CA1 more MAP-2 labeled that the cell bodies (Figure 2).

The MAP-2 IR neurons of paleocortex and neocortex are mostly pyramidal neurons. In neocortex, the MAP-2 IR neurons showed a pattern of intense staining: the most intense staining neurons is the layer V compare to the other layer (Figure 3 and 4). Large pyramidal neurons in the frontal and parietal lobes are more intensely MAP-2 phosphorylation staining compare with other area of cortex cerebri.

Discussion

The distribution of MAP-2 phosphorylation in the dendrites and cell bodies of neuron of cortex cerebri in the last trimester of gestation is indicated that this protein already expressed to abtain the neuronal plasticity. This founding was suggested that the MAP-2 IR neurons have attained a certain level of plasticity around birth. Gordon *et al*. (2006) reported that synaptic plasticity rules widely determines how cortical network develop and store information. Plasticity compartmentalization of basal dendrites expands the networks plasticity rules and may support different learning and developmental functions.

Our results showed that MAP-2 phosphorylation neurons were distributed in pyramidal layer in cortex cerebral. The pyramidal cells in layer V of neocortex are larger than the pyramidal cells in layer III, this is why the layer V more intensely staining. Woolf (1998) reported that pyramidal neurons represent the main neuronal type in the neocortex, and their MAP-2 expression is influenced by acetylcholine and other neurotransmitter. MAP-2-enriched cholinoceptive cells were in a module of the cerebral cortex which is innervated by the cholinergic cells (Woolf, 1997). Woolf (1998) reported that the function of MAP-2 phosphorylation to encode memory is influenced by Ach which the release of Ach could alter cortical circuitry and thereby permanent encode memory. In cortical pyramidal cells, excitatory synaptic response to muscarinic reseptor activation are slow, with latencies around 250 msec (McCormick and Price, 1986), and during this latency period, MAP-2 phosphorylation is predicted promoting and prolonging an event such microtubular coherence (Woolf, 1997). Need to study further are the MAP-2 IR neurons also cholinoceptive cells using marker for cholinoceptive cells.

The large pyramidal neurons in frontal lobe and parietal lobe are strongly MAP-2 IR, which indicated that they contain high concentration of MAP-2 phosphorylation compare to other area of cortex cerebral. This finding is in accordance with Kaufmann *et al*. (2000) that the frontal motor or postcentral somatosensory cortex are the major cholinoceptive cells. As reported by Sims *et al*. (1988) that in general, the intensity of MAP-2 immunoreactivity in cell bodies and dendrites correlated with the degree of neuronal differentiation, but the pattern of intracellular staining also varied as a function of laminar position, and presumably cell type.

The neurons immunoreactive for MAP-2 phosphorylation may prepared for memory storage in cerebral cortex. Joseph (2000) was reported that the fetus is capable of considerable behavioral complexity. These complex actions appear to be mediated and governed by brainstem with minimal forebrain participation. The fetus is incapable of thinking, reasoning, understanding, comprehending, or experiencing or generating "true" emotion or any higher order to mediated cognitive activity as the cortex cerebri functions. In contrast, the other studies reported that the fetus show the ability

to discriminate sounds and voices. The ability of human fetuses to recognize their own mother's voice was also examined. Fetal heart rate increased in response to mother's voice and decreased in responsed to the stranger's (Kisilevsky *et al*., 2003). In the last trimester fetuses become familiar with recurrent maternal speech sounds. The prenatal exposure to the mother's tongue can enhance fetal reaction to linguistically important speech sound and thus could promote language-relevant perceptual tuning before birth (DeCasper *et al*., 1994). The capable of learning, the increasingly complex behaviors demonstrated by fetus and neonate, including head tuning, eye movement, startle reaction, crying, screaming, and rudimentary smilling are probably described as brainstem reflexes. Smilling, as well as screaming and crying in neonate can be produced from brainstem stimulation even with complete forebrain transaction or destruction (Joseph, 2000).

The neurons immunoreactive for MAP-2 phosphorylation in cerebral cortex probably have been beginning their function for learning and memory since prenatal period, serving in important fuction in early mammalian development. Well and Hepper (2006) reported the prenatal olfactory learning in the domestic dog with examined how prenatal exposure to a chemosensory stimulus via mother's diet affected to chemosensory preferences of neonatal pups. The results indicate that prenatal chemosensory learning is present in the carnivore and suggest that such learning may be present in all mammals. Heteran *et al*. (2000) reported about the fetal learning and memory. They used fetal habituation to repeated vibroacoustic stimulation to assess fetal memory after the initial stimulus. They assessed the fetuses 10 min later and again after 24 h, 16 of 19 fetuses habituated rapidly to stimuli at 10 min and 24 h. The results indicate that fetuses are able to learn, they have a short term memory of at least 10 min, and long term memory at least 24 h.

The distribution of MAP-2 phosphorylation in pyramidal layers in this study was suggested that the immunoreactive cells prepared for memory storage in cerebral cortex and begin the true function for strorage memory from act of knowing like learning, memory, attention, creating, perception, problem solving, and use language at post natal period.

Storage of memory is in cholinoceptive cells, which every cholinoceptive cell is enriched with MAP-2 (Woolf, 1993). MAP-2 rich cells throughout the cerebral cortex correspond almost exactly with cortical cells containing muscarinic reseptors. Many of cholinoceptive, MAP-2 rich cells are large pyramidal cell type, but some are also small pyramidal cells and nonpyramidal types. Cholinergic afferents are module-specific. The tapering apical dendrites of pyramidal cells are proposed as primary sites for cholinergic mediated of linkages between MAP-2 and microtubule because especially high amount of MAP-2 are found here (Woolf, 1997). Need to study futher are the MAP-2 phosphorylation immunoreactive cells also immunorective to Ach neurotransmitter. Previous studies have reported the correlation between MAP-2 and memory in postnatal periods. Woolf *et al*. (1999) reported the alterations in MAP-2 may reflect dendritic remodeling related to contextual memory. The alteration in hippocampus (in pyramidal cell of CA1 and CA2) microtubule-associated protein-2 appear to be highly correlated with contextual memory as measured by significantly heightened fear responses. Compared to naive controls, rats trained in novel context showed significantly increased immunostaining for the high molecular weight MAP-2a/b. Training-related increases in immunohistochemical staining for MAP-2 suggested that there was an increase in overall intact protein, an increase in imunoreactive breakdown products, or changes in protein compartemetalization.

There may also be a correlation between cognitive impairment in post natal and

disturbance of MAP-2 phosphorylation function during fetal periods. In the adult brain, cholinergic markers and the number of pyramidal cell perikarya correlate with severity of dementia, suggesting that loss cholinergic is the major contributor to the cognitive impairments of Alzheimer's disease (Palmer, 1996) and the cytoskeletal distruption is a key pathological feature of Alzheimer's disease. In regions of high neurofibrillary tanged density, MAP-2 histochemical features of remaining nonneurotangled neurons included apical dendrite degeneration with proliferation of basal dendrites (McKee *et al*., 1989). Normally, MAP-2 may contribute to support of neuronal prosesses by making the microtubule they contain longer, more stable and stiffer than those in non-neuronal cells (Matus, 1994).

As a conclusion, MAP-2 phosphorylation immunoreactive neurons are distributed in the cell bodies and dendrites, mostly in pyramidal neurons of long-tailed macaque fetuses in the last trimester of gestation, suggested that this neuron facilitates a response to neuroplasticity for memory processes in fetal cerebral cortex around birth. The existancy and distribution of MAP-2 phosphorylation in the cortex cerebri may prepare for memory storages.

Acknowledgments

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