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IMMUNOCYTOCHEMICAL LOCALIZATION OF UBIQUITIN IN DEVELOPING OCCIPITAL AND PREFRONTAL CORTEX OF HUMAN FETAL BRAIN DURING SECOND TRIMESTER

T. S. KHEDKAR, Y. A. GADHIKAR*[1] AND S. A. KOUSHIK[2]
Department of Zoology, Nabira Mahavidyalaya, Katol, Nagpur – 441 302, (M. S.)
[1]Department of Zoology, Government Vidarbha Institute of Science and Humanities, Amravati - 444 606, (M. S.)
[2]Department of Zoology, Institute of Science, Nagpur – 440 00, (M. S.)
E-mail: trupti_khedkar2003@yahoo.com

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***Corresponding author**

ABSTRACT
Ubiquitin immunoreactive profiles were studied in developing human fetal occipital cortex and the prefrontal cortex during the second trimester. We employed immunolabelling techniques on seven human fetal occipital cortices and prefrontal cortices ranging from 12 weeks gestation (w.g.) to 25w.g. by using ubiquitin antibody. At the end of first trimester, immunoreactive profiles were relatively meager in both the areas. In 12w.g. moderate to intense ubiquitin immunoreactivity was detected in the marginal zone (MZ) and ventricular zone (VZ), where as fibrous layer at this stage showed diffuse nonspecific immunoreactivity. At 17 weeks occipital and prefrontal cortex, staining was restricted to the cells while at 18, 19, 20w.g. immunoreactive product was observed in the fibrous network. At 22w.g. ubiquitin immunoreactivity was detected in the fibrous network of occipital cortex while wide spaces were detected in the fibrous layer of prefrontal cortex. With the increase in fetal age *i.e.* 25w.g, the ubiquitin reactivity was almost absent in both the areas viz. prefrontal cortex and occipital cortex. We suggest that expression of ubiquitin in the occipital and prefrontal cortex may be of transient occurrence during the second trimester.

INTRODUCTION

Ubiquitin is a heat stable, 76 amino acid proteins with a molecular weight of 8565 Daltons. Its distribution in nature is ubiquitous, hence its name. Ubiquitin remains highly conserved throughout evolution. Ubiquitin has been implicated in pathogenesis of several disease processes because of its role as a cell stress protein (Lowe *et al.*, 1995). Several investigators have demonstrated that the level of ubiquitin rises in stress condition (Bond *et al.,* 1988; Ryan *et al.,* 1995) Ubiquitin has also been implicated in neurodegenerative disorders including Alzheimer's and Parkinson's diseases (Swaab *et al.,* 1992; Wang *et al.,* 1991). It also functions as heat shock protein. Several investigators studied distribution of ubiquitin in normal and diseased human brain. Ubiquitin immunoreactive structures were demonstrated in normal brain ranging from 2 months to 91 years with light and electron microscopy (Dickson *et al.,* 1992). Ubiquitin was demonstrated in neurofibrillary tangles and also in dystrophic neuritis in cerebeller senile plague. Ubiquitin and 70 KD heat shock proteins were studied in the cerebellum and caudate nucleus of 24 adult human brains. Abnormal ubiquitinated deposits related to aging were examined in normal human brain (Pappollo *et al.,* 1989). Ubiquitin in one of the first gene to be up regulated during apoptosis or programmed cell death and apoptosis plays a major role during development. It is involved in cell cycle regulation, DNA repairs, lysosomal a n d non-lysosomal protein degradation. Ubiquitin is thus a multifunctional protein that maintains a delicate balance between cell proliferation, differentiation and cell death. In spite of several studies in normal and diseased brain there is no significant information on the ontogeny of this peptide in developing human brain. However, ubiquitin conjugates and its immunoreactivity has been demonstrated in developing rat brain (Flann *et al.,* 1997). The present study was undertaken for two reasons. Firstly, because the cerebral cortex has been shown to be the main foci of ubiquitination in developing rat brains and also to be the main cause of dendritic pattern formation and we expected the occurrence of a similar mechanism in the human fetal brain. Secondly, both these areas Viz. the occipital and prefrontal cortex are usually obtained intact and are unambiguously identified in all the fetal samples collected less than 1-3 hrs postmortem time interval. Further these areas are functionally important. The occipital lobe contains the primary visual cortex and visual association areas. The surface area of the human occipital lobe is approximately 12% of the total surface area of the neocortex of the brain. Damage to the occipital lobe results in complete or partial blindness. The prefrontal cortex is associated with somatic and visceral activity and the medial part is concerned with auditory and visual functions. In the present study, therefore, we investigated the distribution of ubiquitin immunoreactive profiles in developing occipital and prefrontal cortex of humans during the second trimester.

MATERIALS AND METHODS

Tissue acquisition and approval: Fetal specimens for this study were obtained from the Government Medical College, Nagpur.

The clearance from the local ethical committee and written consent of the parents were also obtained. Gestational age was determined by measuring the crown rump length. (O'Rahilly and Muller, 2006) 12 fetal specimens were collected and after dissection, brains of only 7 specimens that had minimal post-mortem changes as apparent with haematoxylin and Pischinger's methylene blue staining for Nissl substance were used for the present study, Table 1 lists the specimens, their age and postmortem time interval, used for histological and immunocytochemical studies.

Tissue for Immunocytochemistry: The prefrontal area and occipital area were dissected and immersed in cold 4% paraformaldehyde for 48h. The tissues were subjected to several washes of cold phosphate buffered saline during the next 24h and transferred to polyvinyl pyrolidon (K30) for 24h (4OC). Tissue blocks of medial frontal gyrus containing brodman areas 17, 18, 19 (visual area) and 46 and 9 were cryosectioned at 10um, fixed in chilled acetone and stored at -200C for ubiquitin immunolabelling. Some sections at regular intervals were stained with haematoxylin and Pischinger's methylene blue (PMB) for histological examination. Those sections having cell bodies were demarcated and near adjacent sections were used for ubiquitin immunohistochemistry.

Immunolabelling: Sections were washed in cold PBS, Subjected to endogenous peroxides blocking for 20min. in 0.5% hydrogen peroxide in 20% methanol in Tris buffered saline at pH- 7.4. The sections were treated with ubiquitin antibody (Sigma) at 1:50 dilution for 2h at room temperature or overnight at 40C. Goat, anti-rabbit IgG conjugated to biotin was used as the secondary antibody.Sections were treated with streptavidin peroxidase (Sigma, U.S.A.) for 45 minutes followed by 3-3' diaminobenzidine tetra hydrochloride (Sigma) in 0.05m Tris buffer with 0.01% hydrogen peroxide to produce a brown immunoreaction product. Slides were dehydrated, cover slipped and photomicrographs were taken using Zeiss Microscope.

Table 1: Gestational age in weeks (w.g.), post-mortem time interval, sex and status at birth of human fetal specimens used for histological, immunocytochemical studies

Age in w.g	Post-mortem time interval(Hrs & Min.)	Sex	Sex Status at birth and causeof death
12	0.45	F	Abortion
17	1	M	Abortion
18	1.30	F	Abortion
19	1.30	F	Abortion
20	1.15	M	Abortion
22	1.25	F	Pre term, new born
25	2.15	M	Pre term, still born, low weight

RESULTS

We focused on the prefrontal cortex and occipital cortex area of the developing human fetal brain. The period of growth and development was between 12 through 25 weeks of gestation represents a transient phase during which the neuronal circuitries are built up which are essential for subsequent development of mature projections.

Histological studies

At the end of the first trimester, the 12 weeks human fetal brain has a distinct marginal zone (MZ) and a ventricular layer(vl) but the bulk of tissue is made up of fibrous layer with nuclei intermingled with the fibers (Fig. 1A). At this gestational stage the granular cell population occurs at the MZ of occipital and the prefrontal cortex which forms the sub-pial granular layer. The cells of this layer are mostly stained with haematoxylin; below this layer are cortical plate and a deep axonal fibrous field. The bulk of these fibers which are mainly oriented rostro-caudally extend from ventricular layer to marginal layer. The fibers were mostly stained with eosin while the cells with haematoxylin intermingled between them around mid - gestation (17-19). Nissl stained preparations of occipital and prefrontal cortex revealed MZ with densely packed cells followed by extensive cytoplasmic connections. At 20w.g. occipital cortex has thin marginal layer followed by cortical plate, subplate and intermediate zone with extensive fibrous connections stained with haematoxylin (Fig.1B). Further, Nissl stained preparations at 21w.g. has a thick cortical plate followed by subplate (Fig.1C).

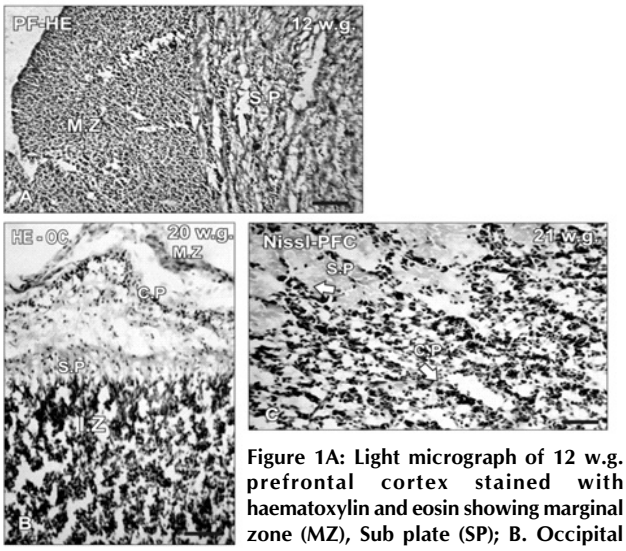

Figure 1A: Light micrograph of 12 w.g. prefrontal cortex stained with haematoxylin and eosin showing marginal zone (MZ), Sub plate (SP); B. Occipital cortex of 20wg. Stained with haematoxylin – eosin showing marginal zone (MZ), cortical plate (CP), Subplate (SP) and intermediate zone (IZ); C. 21W.g. prefrontal cortex stained with methylene blue showing subplate (SP) and cortical plate (C.P.) (Scale bar = 50m)

Immunocytochemistry

Occipital Cortex (Oc) and Prefrontal Cortex (Pfc)

12w.g. fetal brain: At 12w.g. the occipital and prefrontal cortex of human fetal brain has a distinct marginal Zone (MZ) which display sub pial (Sl) granular layer which is followed by extensive fibrous layer (SP) and a ventricular layer (vl). The fibrous layer is 2½ times as thick as the cortical plate (Cp) with small cells intermingled with the fibers. This fibrous layer shows diffuse non specific ubiquitin immunoreactivity and moderate to intense ubiquitin immunoreactivity was observed in the marginal zone and ventricular layer. (Fig. 2A)

17 and 18 w.g. fetal brain: The 17 and 18 w.g. occipital and prefrontal cortex has extensive fibrous connections which were seen extending from ventricular layer towards the sub plate. At 17 w.g. occipital and prefrontal cortex numerous intensely stained ubiquitin immunoreactive cells were seen intermingled

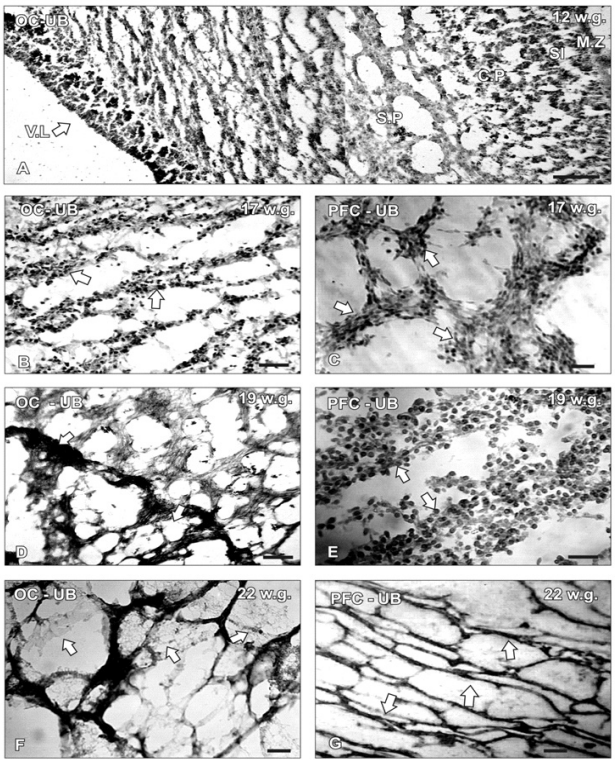

Figure 2(A–G): Ubiquitin immunolabelling in human Fetal brain at four different gestational ages (12, 17, 19 and 22 w.g.). A) Arrow indicates ubiquitin immunoreactivity in the ventricular zone (VZ) and marginal zone (MZ) in 12 w.g. occipital cortex (Scale bar = 100m); B) Arrow indicates ubiquitin immunoreactivity in cells of occipital cortex in 17w.g.; C) Cluster of cells (arrow) showing ubiquitin immunoreactivity in 17w.g. Prefrontalcortex; D) Intense immunoreactivity (arrows) observed in dendritic fibres of 19 w.g. occipitalcortex; E) Arrow indicates ubiquitin immunoreactivity in cells in 19 w.g. prefrontal cortex; F) Arrow indicates ubiquitin positive dendritic fibres in 22w.g. occipital cortex; G) Dendritic fibres forming wide spaces (arrow) in 22 w.g. prefrontal cortex.(B – G scale bar = 50 m)

between fibers. The ubiquitin immunoreactive cells of the occipital cortex were small, rounded and mostly aggregated in the fibrous layer of the sub plate (Fig. 2B). Whereas ubiquitin immunoreactive cells of prefrontal cortex were mostly rounded to ovoid and were seen in clusters in the sub plate (Fig. 2C). In the 18w.g. Occipital and prefrontal cortex the outer granular layer is followed by extensive fibrous connections that show diffuse ubiquitin immunoreactivity. The territories occupied by some intensely stained ubiquitin immunoreactive structures extend along the rostro-caudal axis of the fibrous layer of the subplate along the marginal zone.

19 w.g. fetal brain: With further development, Ubiquitin immunoreactivity was seen at some locales in the subplate but mostly accumulated in the intermediate zone. At 19 w.g. occipital cortex extensive fibrous connections extend from posterior margins towards the anterior margins. These fibrous connections show intensely stained ubiquitin positive structures mainly located in the intermediate zone (Fig. 2D). While at 19 w.g. prefrontal cortex robust immunoreactivity was detected in the cells located along the rostro-caudal axis in the sub plate and intermediate zone (Fig. 2E).

20 - 22 w.g. fetal brain: An important feature of ubiquitin immunoreactivity in the developing occipital and prefrontal cortex was observed from 20 through 2 w.g. At 20 w.g. occipital cortex some ubiquitin positive structures silhouetted by fibrous connections were seen in the marginal area at some locales. At 22 w.g. occipital and prefrontal cortex have extensive fibrous connections. Some of these fibrous connections exhibited intense reactivity with wide spaces between them. These are mainly located at the marginal zone. The prefrontal cortex also showed ubiquitin immunoreactive fibers extending antero-posteriorly from the margins of subplate towords the marginal zone (Fig. 2G).

25 w.g. fetal brain: The 25-w.g. occipital cortex and prefrontal cortex of human fetal brain reveals a complete absence of ubiquitin reactivity in the marginal granular layer and ventricular layer. Dendritic immunorectivity is also lost with the growing age of fetus.

DISCUSSION

Ubiquitin is a multifunctional protein. Since it is known to be a stress protein we conjectured that the human fetus being in maximum physical stress during delivery, the brain regions would have ubiquitin immunoreactive structures. Hence, the present study has undertaken to demonstrate ubiquitin immunoreactive structures in the occipital cortex and the prefrontal cortex of the human fetal brain. However, we were intrigued to find that the areas under study viz. the occipital cortex and prefrontal cortex did not show extensive ubiquitination throughout the fetal age. Instead at 12 w.g. ubiquitination was observed in the occipital cortex and prefrontal cortex, and with progression in development, there is a gradual decrease in ubiquitination. The period of growth and development of human fetal brain in the present study was between 12 through 25 w.g. This second trimester period represented a transitional phase during which the neuronal circuitry undergoes drastic changes and these changes are essential for subsequent development of mature projections (Chan *et al.,* 2002) As development progresses, *i.e.* at 17-18 w.g. intense ubiquitin positive structures were detected only along the marginal zone and subplate. With further growth, by 19 w.g. there is a shifting of the ubiquitin immunoreactive areas into the intermediate zone of the occipital and prefrontal cortex. In older fetuses there is a drastic change in the morphology and distribution pattern of ubiquitin immunoreactive areas and by 25 weeks it is negligible. During the second trimester important developmental events such as migration and differentiation of neurons occur and these events shape the overall architecture of the human brain (Hayaran *et al.,* 1992, Aquino *et al.,* 1996 and Chan *et al.,* 2002). Maturation and development of neurons in the human cerebellar dentate nucleus was shown to commence at 14-15w.g. when bipolar neurons have been observed. At 19-20w.g. bipolar, hemispheric and pyriform neurons and by 24-25w.g. multipolar and nuclear boundary neurons have been observed. All these five cell types were shown to be persistent during subsequent development. Our findings to substantiate the fact that the 12 through 25w.g. period represents a critical window in human fetal brain development. In the present context, intense ubiquitin activity in the highly

intertwined fibers appears to modify the prefrontal and occipital neuronal circuitry from 12 through 25w.g. As development proceeds, *i.e.* at mid gestation immunolabelling is restricted to nuclei of cells while the intertwined bands of fibers lose their identity and instead clusters of ubiquitin positive cells appear, until finally at 25w.g. Immunolabelling is negligible, thereby suggesting completion of the process. Ubiquitinated deposits have been studied in normal human brain related to aging (Dickson *et al.,* 1992**).** Ubiquitin levels have been shown to increase many fold in homogenates of cerebrum and cerebellum of Alzheimer's patients and the increase correlated strongly with degree of neurofibrillary change in tissue (Wang *et al.,* 1991). Several workers have demonstrated ubiquitin in Alzheimer's disease brain (Swaab *et al.,* 1992). Ubiquitin immunoreactivity was observed evenly distributed throughout the white matter characterized by dot like structures in cases of Alzheimer's disease and in less severe form of normal aging. The neurofibrillary tangles in the locus coeruleus in the human brain has been shown to express ubiquitin with aging in patients of Alzheimer's disease and Parkinsonism (Kida *et al.,* 1992). Intense staining of neuronal perikarya, nuclei and dendrites in early postnatal cerebral cortex and hippocampus has been observed in developing rat brain (Flann *et al.,* 1997). The main foci of ubiquitination were the pyramidal cells of cerebral cortex and hippocampus and Purkinje cells in the cerebellum. They observed a striking decrease in ubiquitin staining after post-natal day 14 and stated that most of the ubiquitin activity is lost in the adult. They postulated that protein ubiquitination is more active in neuronal processes which are growing and arborizing, and that the activity of the ubiquitin system decreases as dendritic stabilization occurs. Based on our observations and those of other workers, we are of the opinion that ubiquitination and arborization bear an inverse relationship in the developing human brain and the same probably holds true for other vertebrates. Further, in the present study, there is a gradual descent in the ubiquitin immunoreactive structures. Ubiquitin has been shown to be down regulated between 12- 25w.g. during maturation of the occipital and prefrontal cortex. Maturation of these areas of the human fetal brain during 14 through 22w.g. has also been reported (Aquino *et al.,* 1996). The laternoted that during the second trimester, important events such as the migration andidifferentiation of neurons and astrocytes occur to shape the overall cytoarchitecture of the human brain. Based on quantitative immunolabelling they demonstrated a gradient of glial fibrillary acidic protein levels along the caudo-rostal axis *i.e.* highest GFAP in the occipital lobe, medium in the parietal lobe and lowest in the prefrontal cortex at 22 w.g.

In contrast, NF-66 content was uniform among the three cortices. These two proteins are cell type specific and were hence chosen. They studied action as a general 'housekeeping' protein against which to monitor other proteins. They expected action to show a pattern of up regulation during development since it is involved in cells proliferation and motility and neurite extension. However, they found a sharp decrease in actin content during 16- 18w.g. Based on these observations *i.e.* down regulation of actin during 14w.g through 22w.g. and our present findings viz. down regulation of ubiquitin immunoreactivity in the zones of the occipital and prefrontal cortex during 22-25w.g.of development we believe that actin ubiquitination might be employed as a mechanism for maturation of the neuronal circuitry. We conclude that the appearance of ubiquitin system in the occipital cortex and prefrontal cortex may be important for the development of these cortical areas during 12 -22w.g. in the development of human fetal brain, The decrease ubiquitination in these areas suggest dendritic stabilization might have occurred in the occipital and prefrontal cortex.

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