Journal of Global Biosciences
Peer Reviewed, Refereed, Open-Access Journal
ISSN 2320-1355
Volume 11, Number 4, 2022, pp. 9266-9280
Website: www.mutagens.co.in
URL: www.mutagens.co.in/jgb/vol.11/110402.pdf

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Research Paper

CYTOMORPHOMETRIC ANALYSIS AND PROTEIN EXPRESSION PROFILE OF CEREBRAL NEUROSECRETORY SYSTEM WITH NOTES ON NEUROSECRETION PATTERN IN THE ENDOGEIC EARTHWORM *Metaphire posthuma* (VAILLANT) UNDER CONTROL CONDITION

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Abstract

The cytomorphometric characteristics of cerebral neurosecretory system of endogeic earthworm *Metaphire posthuma* revealed basically two types of neurosecretory cells (NSCs)-A cells and B cells. On the basis of shape, size and location A- cells are further classified into A1, A2 and A3 types. Notably the B cells were less in number than A cells. Number of NSCs gradually declined from the dorsal to the ventral part of cerebral ganglia.Dorso-lateral part of the cerebral ganglion showed thick and compact arrangement of NSCs in contrast to its mid dorsal part. A1-cells preferably discharged neurosecretory materials towards the perineurium through the process of axonal transport. However, discharge of secretory materials through axonal transport from A2 cells at the margin of fibrous neuropile was also noticed. When the cerebral ganglia was solubilised and subjected to SDS-PAGE analysis, several protein bands were visualized. The molecular weight of these proteins ranged from 12-165 KD.

Key words: Neurosecretory system, protein expression, Cerebra ganglia, neurosecretion, neuropile, *Metaphire posthuma*, accumulation zone.

INTRODUCTION

The neurosecretory systems in annelids are of classical interest as they represent the first class of coelomates exhibiting significant pattern of neurosecretory activity. Annelids do not show the possession of epithelial endocrine glands. So any hormonal control over development seemed to be due to neurosecretion[1]. This is characterised by the synthesis of proteinaceous 'glandular' products- the neurosecretory granules (neurohormones) in the versatile neurons. The granule itself has the active principle

tagged with an inactive protein carrier that possesses specific staining properties and as such bears ultrastructural characteristics.Transport of neurosecretory elaborations is monitored through axoplasmic flow and reach at the axon terminals where from the neurohormones are released generally through exocytosis and thereby diffused into the blood stream or terminate to the surrounding tissues. Neurosecretory cells have been reported throughout the central nervous system (CNS) of the three major classes of annelids. Furthermore, most studies on annelids have substantiated the status of the CNS as an endocrine organ which produces neurohormones as well as neurotransmitters [2]. Neurosecretory cells within the CNS of earthworms are regarded as the sourse of hormones controlling different vital physiological functions such as growth, osmoregulation [3], reproduction [4] and thermal acclimation[5].

The morphology of neurosecretory system of annelids has been well studied [4,6,7]. Inspite of this, there is lack of details information on cytomorphology of neurosecretory cells of cerebral ganglia of earthworm *Metaphre posthuma*. Therefore, the present study aims to gives a clear account of cytomorphometric nature of cerebral ganglia revealed by light microscopic observation along with its protein expression pattern analysed by SDS-PAGE.

MATERIALS AND METHODS

Specimen Collection and culture:

The fully matured clitellate adults endogeic earthworm, *Metaphire posthuma* (Megascolecidae) (average length 120 mm and breadth 6 mm) were collected by digging and hand sorting process from moist, sandy loam soils from Khowai, Amarpur, Agartala region of Tripura. The live earthworms were brought to the laboratory where they were acclimated in moist sandy loam soils kept in earthen pots (average temperature 28^{o}C andRH-80%). Periodic sprinkling of water to the soil was made to keep the soil bed moisturised.

Light Microscopic study:

After anesthetization, cerebral ganglia were quickly dissected out and fixed in 2% Ca-Bouin's fluid for 18 hours. Tissues were subsequently dehydrated, cleared in xylol and embedded in paraffin wax (MP: 58^{0} C-60^{0}C) as per routine histological techniques. Serial frontal sections of 7µ thickness were stained with Gomari's Chrome- Alum Haematoxylin-Phlixin(CAHP) as modified by Bargmann and simplified Adehyde Fuchsin(AF) staining technique[8,9].

Study of Protein Expression Profile:

Sodium dodecyl sulphate- Poly Acrylamide gel electrophoresis was performed in the presence of SDS for analyzing the polypeptide diversity following the method of Laemli [10]. Electrophoresis was carried out with solubilised cerebral neurosecretory system. For this method first the protein solution was reduced and ligated with SDS by heating the protein samples in a water bath for 5minutes along with1:2 volume of sample buffer. The resulting solution contained SDS bound polypeptides in open form and this was applied on to the casted gel lane. Approximate molecular weight of the separated polypeptides were to be determined by this method as Biorad molecular weight marker having known molecular weight polypeptides which were loaded in the extreme left end side of the gels. For performing SDS-PAGE two stage gel system was employed. First for separating the resolving gel was casted containing 15% total acrylamide concentration in the separating gel buffer containing SDS. Above the separating gel stacking gel was laid in stacking gel buffer. In this gel total acrylamide concentration was 5%. The electrophoresis was performed with electrode buffer containing 0.025M Tris, 0.192M glycine, P^H 8.3 and 0.1% w/v SDS. The electrophoresis was conducted at 16mA constant current for 2 hours. When the tracking dye reached the bottom electrophoresis was stopped. The separated gel was immersed in the staining solution for overnight and then distained by destainer. Photographed were taken with Chemidoc. The molecular size of the protein was determined using the standardized marker of Biorad with MW range of 10-250 KD.

RESULTS AND DISCUSSION

In the bilobed cerebral ganglia of earthworms, neurosecretory cells (NSCs) formed several layered thick tier beneath the peripheral connective tissue sheath perineurium above which was the non-cellular neural lamella (Fig 1). Number of NSCs gradually declined from the dorsal to the ventral part of cerebral ganglia. Dorso- lateral part of the cerebral ganglion showed thick and compact arrangement of NSCs in contrast to its mid dorsal part (Fig 1&2). Beneath the cortical tier of NSCs was the fibrous and vascularised neuropile (Fig 2). Distribution of neurosecretory materials (NSM) at the margin of neuropile was visualised (Fig 2)

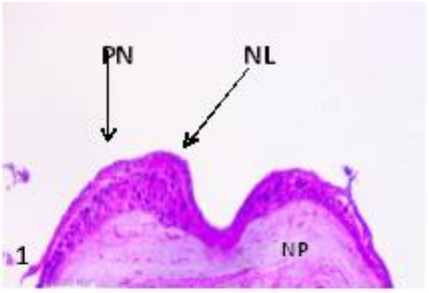

Fig1. Frontal section showing disposition of CAHP-positive A1, A2 and A3 NSCs in the dorso-median part of the bilobed cerebral ganglion of *Metaphire posthuma*. 100X

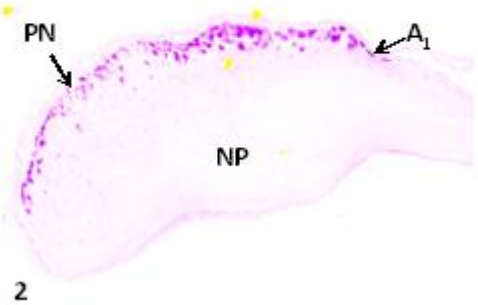

Fig2. Frontal section showing thick cortical tier of AF positive A1 cells and A2 cells through the dorsal-lateral part of the cerebral ganglion.100X

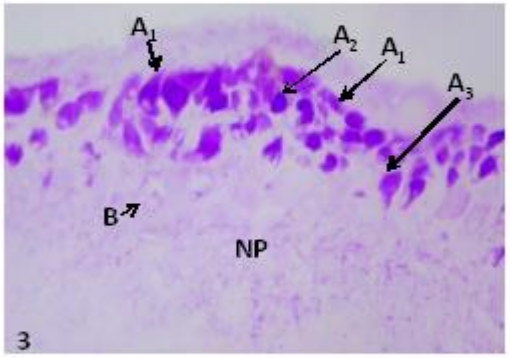

Fig3. Frontal section showing rich distribution of AF positive NSCs at the dorso-median part of cerebral ganglion. Note distribution of A3-NSCs at the median part of the ganglion.Note monopolar axonal transport from the A3 NSCs towards zone of accumulation.400X

On the basis of staining intensity two major categories of NSCs were observed in the postero-dorsal part of the cerebral ganglion in the studied earthworm species. These were deeply stained A cells and light or moderately stained B cells. All the cells irrespective of their types were CAHP positive but only A cells particularly the A1 and A2 cells stained very deeply with AF stains(Fig 4).CAHP positive small neuroglial cells, undifferentiated nerve cells and ordinary neurons were also present in the cortical cell tier.

Shape, size, distribution, staining characteristics and morphometric measurements of cerebral NSC types are given in Table 1 and 2.

1. A-Cells:

   A-cells were the predominating NSCs distributed beneath the perineurium as a thick cortical tier of 5-10 layers at the postero-dorsal part of the brain (Fig 1). They often showed distinct secretory cycles with different phases of secretion and cytoplasmic vacuoles (Fig 3&4). Their numbers were maximum at the postero-lateral, moderate at the postero-median and minimum at the anterior and ventral part of the cerebral ganglia (Fig 7). These cells were oval and pear shaped with eccentric oval or spherical nuclei. Cytoplasm was charged with

deeply AF-stained colloidal secretory materials in the axon .On the basis of shape, size and distribution A-cells were further categorized into following sub-types:

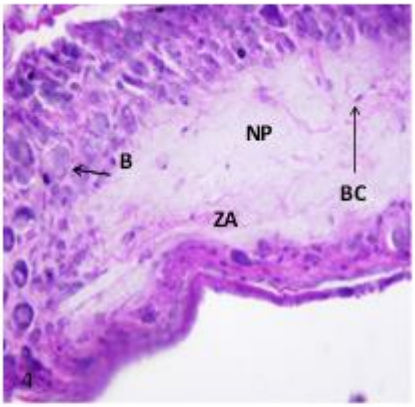

Fig4. Frontal section showing CAHP-positive A_1,A_2,B NSCs were maximum at the dorso-lateral, moderate at the dorso-median and minimum at the ventral part of the cerebral ganglion.note 'zone of accumulation'(ZA)and blood capillaries (BC)charged with NSM.400 X.

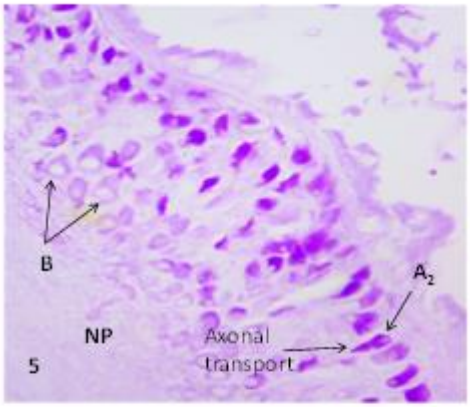

Fig5.Frontal section showing thick cortical tier of different types of AF positive NSCs and 'accumulation zone' of NSM at the margin of the neuropile. Note theB NSCs in the mode of axonal transport towards margin of the neuropile from B cells with axon oriented voluminous nuclei.400X

i)**A1-Cells**: Smallest unipolar or multipolar A1-NSCs with more or less oval shapes, homogeneously deep stained cytoplasm and ill defined nuclei formed superficial single or double layered cells just beneath the perineurium (Fig 2&3). Generally axons of these cells were very small and directed towards the perineurium (Fig 2).

ii) **A2-Cells**: They were the predominating among A-cell types that formed thick cortical tier beneath theA1 cells. Generally the A2 cells are provided with axons which directed towards the neuropile (Fig 2). Rarely these cells had axonal discharge towards the perineurium. A2 cells possess distinct central or axon oriented nuclei. The A2-cells showed distinct secretory cycle ie cells were in different phases of secretion (NSM).

iii**) A3-Cells**: Generally they formed a very small group isolated of CAHP positive NSCs at the dorso-median part of the brain. A3 cells are provided with long axons penetrating through the neuropile.

1. **B-Cells**:

These were median and large sized NSCs with variable shapes and sizes (Fig 5). These cells stained lightly or moderately with AF stain but moderates with CAHP. B neurosecretory cells were distributed in the inner cortical cell tier in between layer of A2 cells and the central fibrous neuropile. The B cells exhibited clear cyto-architecture

with centrally placed, abaxonal or axon oriented phloxinophilic nuclei, fine to coarse secretory granules scattered throughout the cytoplasm and detectable cytoplasmic vacuoles besides traceable axonal processes. Notably the B cells were less in number than A cells in the brain. Moreover their numbers were more in the antero-lateral and ventral, than in the postero median part of brain.

Neurosecretion and storage of NSM:

Neurosecretion elaborated in different NSCs were discharged through the surface of the perikaryon or through the axons into the blood capillaries or tissue spaces. During the activity period (June to October) of the tropical earthworm species, neurosecretory granules (NSM) were found in the axon hillocks and axons of the NCSs irrespective of their types. The discharge of NSCs from the B cells was accomplished either through the perikarya or axonal transport or both. There was a rich blood supply to the cerebral ganglion including its outer sheath, perineurium. A1-cells preferably discharged NSM towards the perineurium through the process of axonal transport (Fig 2&3). However,discharge of secretory materials through axonal transport from A2 cells at the margin of fibrous neuropile were also noticed. This vascular zone because of having appreciable amount of AF-positive NSM has been considered as 'elementary' or rudimentary neurohaemal organ. NSM deposited in the extracellular spaces or 'zone of accumulation' (Fig 2) or beneath the perineurium (Fig 7) found their ways through the posterior or sub neural blood vessels.

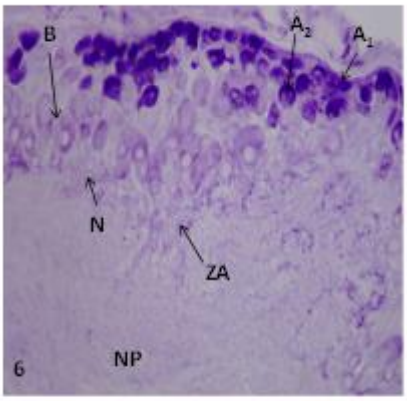

Fig6. Frontal section showing secretory cycle in the AF positive NSCs(A&B). Note abundance of cytoplasmic vacuoles in both A and B type NSCs with fluctuating NSM content.400X

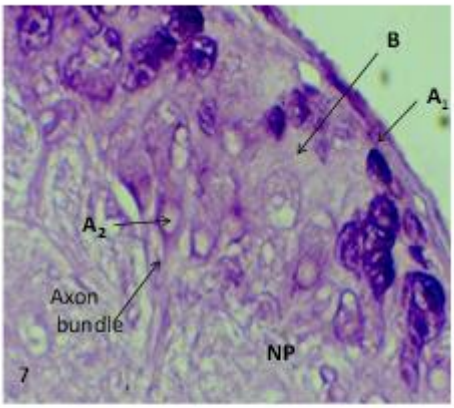

Fig7.Frontal section showing series of long axon bundles emerging from the CAHP positive A_1 cells and penetrating through the neuropile.400X

Secretory cycle of the NSCs:

The secretory cycle of the NSCs includes the process of elaboration of NSM by the perikaryon, the accumulation of the secretory granules, and finally its discharge and transportation. During late summer and monsoon when earthworms were active, their NSCs irrespective of their types showed distinct secretory cycles with different phases of secretion (Fig 3-6). In the first phase, the NSCs were nearly devoid of any detectable NSM. However their nuclear membrane showed great affinity to AF and CAHP stains. The nucleus was highly phloxinophilic, the chromatin granules are staine deep blue. This suggests that during this early phase of secretory cycle the nucleus played an important role in the initiation of the neurosecretory activity. The second phase was marked with the appearance of the chromophilic neurosecretory granules which formed aggregates that were spread over a large area of the cell. The third and final phase of neurosecretory cycle differed among A and B type cells. In this phase the A cells were stained deeply purple and blackish violet or blackish blue AF and CAHP respectively. This homogeneity of the staining was due to the compact arrangement of the neurosecretory granules in the perikaryon. The NSM was discharged through the axons either into the tissue spaces or beneath the peripheral vascularised sheath i.e the perineurium or to the storage zone at the margin of the neuropile (Fig 2). In B cells, during the final phase of secretion, the secretory granules aggregated to form large granules which became most abundant in abaxonal region. The cytoplasm was moderately stained and several cytoplasmic vacuoles appeared inside the perikaryon. The NSM was discharged either through the perikaryon or the axons to the richly vascularised ventral part of the ganglion for their final dispatch.

Classification of NSCs in the cerebral ganglion of earthworms has been made chiefly on the basis of cytomorphological characteristics, their distribution and staining characteristics [11,12, 13, 14]. Some of these observations agree with one another but many others differ. Thus it seems difficult to compare present observations with the previous ones concerning the cell types (Table 2). It is even more difficult to establish their homology since the cells of same kind may remain in different phases of secretory activity. The present cytomorphometric studies on the cerebral NSCs reveal four types of NSCs viz.A1, A2, A3 and B cells belonging to two major categories of cells i,e deeply stained A cells and lightly stained B cells. Available literature indicates that A1, A2, A3 cells (together comprise A cells) occupying the postero-dorsal part of the cerebral

ganglion comprise the B-DSCs of Chaudhuri et.al.[15], 'dark stained cells' of Datta and Banerjee[16] and 'a' or 'A' neurosecretory cells of various investigators[11,17,18,19,20]. According to Al-Yousuf [2] A cells with its different types (A1- A5) are peptide secreting NSCs. Chaudhuri et.al[21] recorded intense phosphatase activity in the cerebral AF positive deep stained NSCs (corresponding to A cells) of *E.gammiei*. Earlier Teichmann et.al[22] have classified A cells into A1, A2 and A3 cells in the cerebral ganglia of *Lumbricus herculeus* and *Eisenia fetida* on the basis of histochemical characteristics. According to Herlant-Meewis[23] type A1 and A2 cells are possibly the source of somatotrophic and gonadotrophic hormone respectively. Absence of secretory cycle in A1 cells under adult condition indicates that these cells probably remain active during growth phase of the earthworm. Chaudhuri et. al [24] and Chaudhuri and Chaudhuri [25] reported drastic reduction in the number of deep stained BDSCs (homologous to A cells) with acute depletion of NSM in cerebra ganglion of *E. gammiei* following dehydration and hyperthermic conditions. Hypertrophy with increased synthetic activity of A1 cells in cerebral ganglion of *E. fetida* following light stress was reported by Banovacki and Matavulj [26]..

The B cells in the cerebral ganglion of the species under study correspond to large and middle sized neurons[9], the 'B' or 'b' neurosecretory cells of several investigators [11,17] and moderately stained cells or MSCs [15,16]. Role of moderately stained cells (corresponding to B cells in the present investigation) in regulation of food intake in *M.peguana* and *E.gammiei* was reported [27]. Distinct secretory cycle with voluminous nuclei and axonal transport of NSM from the moderately stained NSCs (Bcells) in the ventral ganglia of *M.peguana* during anterior regeneration was reported by Nanda and Chaudhuri[13]. According to Golding and Whittle [28] A and B types NSCs in the CNS of earthworms have ultrastructural and histochemical features of peptide secreting and amine secreting cells respectively. By light microscopy, fluorescence and ultramicroscopic studies, both peptide and amine secreting NSCs have been identified in the central nervous system of earthworms, *Octolasium complanatum* and *E.fetida* [29,30].According to Bianchi[29] amine secretion appears to be associated with the perikaryon and is being discharged directly into the blood stream. Thus in the present study prevalence of cytoplasmic vacuoles in the B cells, indicating direct discharge of NSM from the perikarya [31] may have bearing with amine secreting characteristics. However, vacuole rich B cells with axonal processes terminating either to the neuropile

or in the vicinity of blood vessels are seldom. Thus both perikarya and axonal discharge from different or even the same cells are quite possible. Ultrastructural studies on the NSCs in the cerebral ganglia of five different species of oligochaeta, *Lumbricus terrestris*, *E. fetida*, *Octolasium cyaneum*, *Dendrobaena subbrubicunda* and *Allolobophora longa* by Al-Yousuf [32] indicate the possibility of secretion of hormone from their cell bodies and release of modulators/transmitters from their axons.Discrete axonal bundle, as found in insects [33] and chelicerate arthropods are not seldom in the A_2 and B NSCs(Fig 8). Such conditions are very likely due to occurrence of 'pool' of these NSCs with functional importance.

Axonal transport with eventual discharge of NSM within the ganglia is evident when their termination to the'accumulation zone' lies between the cortical cell layers and the fibrous neuropile and probably combine the function of neuroendocrine control in relation to the storage and release of NSM [28]. Both perikarial and axonal discharge of NSM within the ganglia of the CNS probably necessitate rich vascularisation of the neuropile for efficient disposal of hormones in absence of any discrete neurohaemal organ. Scanning electron microscopic studies on the cerebral ganglia of earthworm, *E. gammiei* revealed rich distribution of blood capillaries within the neuropile[34]. Thus margin of the vascularised neuropile that received axon terminals from the NSCs groups may serve the purpose of 'plexiform neurohaemal complex' [28], rudimentary[20], incipient[16] or elementary [31] neurohaemal organ. According to Tombes [33] the neural lamella acts as an acellular diffusion medium and a possible reserver for neurosecretory products. In almost all species, neurohaemal areas are not formed within the neural ganglia but on their surface or further away (outer peripheral sheath above nervous tissues in molluscs, corpus cardiac in insects and neurohypophysis in vertebrates). The existence of extra-cerebral "neurohaemal organ'' in the earthworm too, would better fit this general scheme then the intra-cerebral location [35].Thus in annelids that possess a well differentiated neurohaemal structure situated it in the periphery of the brain where coelomic sinus and blood vessels are present, secretion from neurosecretory axon terminals above or below the brain floor diffuse through the neural lamellae and reach the blood stream very easily. The coelomic fluid may be an additional medium for the distribution of neurosecretion [28].

Table 1: Characteristic features of NSC types in the cerebral ganglion of *Metaphire posthuma*

Cell types	Cell shape	Arrangement and location	Cytoplasmic characteristics	Staining affinity of NSCs	
				CAHP	AF
A_1	Ovoid with more than one axon. Generally one axon directed towards the perineurium	One layered just beneath the perineurium, dorsal and postero-lateral in position	Colloidal with ill defined nucleus. No cytoplasmic vacuole, no secretory cycle	Very stronge affinity, dark blue	Very stronge affinity, dark purple
A_2	Typical pear shaped unipolar or bipolar. Axon generally terminates towards neuropile	Multilayered(3-6) below A_1 cells. Outnumber all types of NSCs	Colloidal with well defined nucleus and occasional cytoplasmic vacuoles. Distinct secretory cycle	Strong affinity,dark blue	Stronge affinity, dark purple
A_3	Fusiform shape, strictly monopolar with termination towards neuropile; larger than A_1 and A_2	Few in number, located at the dorso-median line of the cerebral ganglion	Granular or coarse cytoplasm with cytoplasmic vacuoles	Moderate affinity, light blue	Moderate affinity, light purple
B	Variable in shape. Apolar, unipolar or	Dorso-lateral, as well as ventral in location. Number less than A type	Fine granular cytoplasm with abundance of cytoplasmic	Light to moderate affinity,light blue	Light to moderate affinity,light purple

	bipolar. Axons may penetrate deep inside the neuropile	cells. Inter-mingled with A2NSCs and close to the neuropile	vacuoles. Distinct secretory cycle		

Table 2: Cytomorphometric measurements of cerebral ganglion of *Metaphire posthuma*

Cell type	Cell shape	Cell size(µm), n=20		
		Cell length(±SD)	Cell diameter(±SD)	Nucleus diameter(±SD)
A1	Ovoid with more than one axon.Generally one axon directed towards the perineurium	16.14±13	6.25±54	ill defined
A2	Typically pear shaped unipolar or bipolar. Axon generally terminates towards neuropile	16.68±04	6.76±23	5.41±12
A3	Fusiform shape, strictly monopolar with termination towards neuropile, larger than A1 and A2.	24.21±14	8.13±03	7.26±54
B	Variable in shape. Apolar, unipolar or bipolar. Axon may penetrate deep inside the neropile.	19.60±34	7.87±17	6.19±08

n= number of cell considered; SD= standard deviation

When the solubilised cerebral ganglia was subjected to SDS-PAGE analysis several polypeptide bands became apparent (Fig. 8). For analysis of the molecular weight of the resolved polypeptides parallel run of molecular weight marker protein were given.

From plotting of logarithm of molecular weight of the standard protein and their Rm values a linear standard curve was prepared. On the basis of that curve the molecular weights of the cerebral ganglion polypeptides of *M. posthuma* were determined range between 12-165 KD and most intense band were observed in between 26-56 KD.Two bands with close molecular mass in the region 37-50 KD are well detected.This is the first study on any cerebral ganglion proteins of an earthworm species. The present report shows the presence of relatively high number of polypeptides in the cerebral ganglion of *M. posthuma* along with its structural asymmetry.

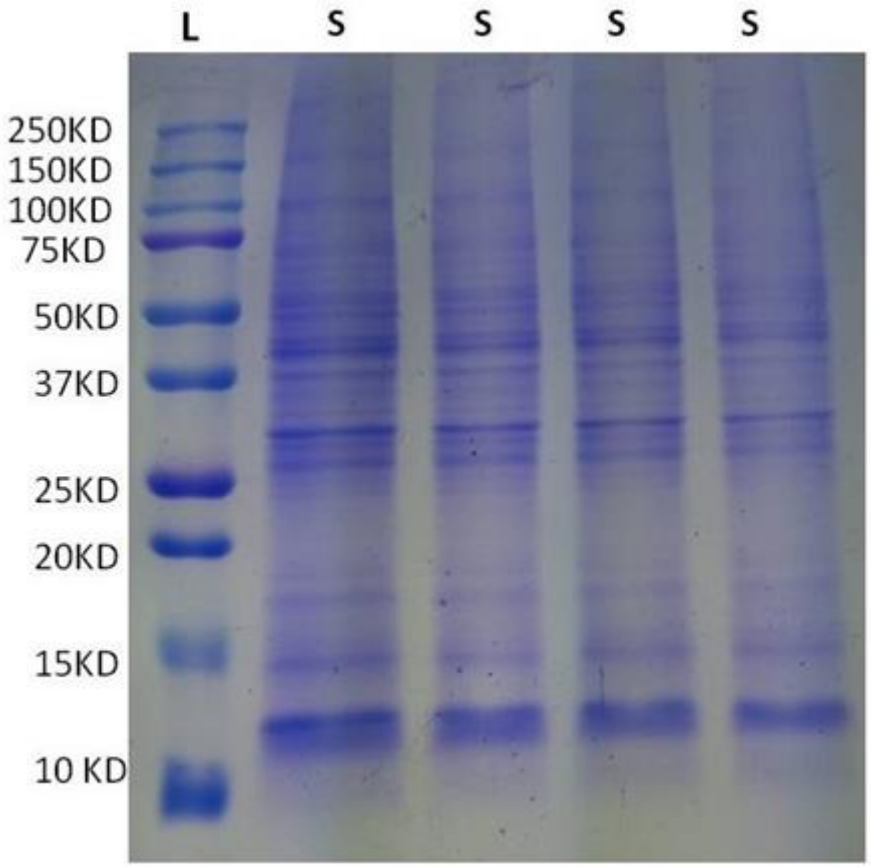

Fig 8. Photograph of the SDS-PAGE electrophorogram of solubilised cerebral proteins of *Metaphire posthuma*. Note- all well is loaded with same sample.

ACKNOWLEDGEMENT

The author thankfully acknowledge the University Grants Commission, New Delhi, India and its North East Regional Office (NERO, Guwahati) [Project No.F.5-467/2014-15/MRP/NERO/2336] for providing the financial support. The author is grateful to the Principal, Dasaratha Deb Memorial College, Khowai, Tripura and Coordinator DBT SBT Hub, Tripura University for providing necessary infrastructure and support to carry out

research work. The author is also grateful to Ex- Prof. Durgadas Ghosh and Ex-Prof Priyasankar Choudhuri, Department of Zoology, Tripura University for encouragement and intellectual input throughout the study.

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