

Neuroendocrine Control of Posterior Regeneration in Tropical Earthworm, *Eudrilus eugeniae* (Kinberg)

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Abstract: *Eudrilus eugeniae* (Kinberg), well known vermicomposting earthworms, are often subjected to predator attacks leading to loss of body parts due to their surface living habit. Thus nature has gifted them the power of regeneration of lost body parts. As neurosecretion is the sole source of hormone in oligochaetes, we hypothesize that neurohormone secreted from the neurosecretory cells of the central nervous system (CNS) will control the phenomenon of regeneration in earthworms. In *Eudrilus eugeniae*, appearance of regeneration blastema was noticed within 72 h of posterior amputation. In fact, posterior amputation brought about multiple cytoplasmic alteration in the neurosecretory cells (NSCs) viz. deep stained A cells and moderately stained B cells in cerebral ganglia, deep stained 'U' cells and moderately stained B cells in the sub-esophageal and ventral nerve cord ganglia. Massive depletion followed by marginal accumulation of NSM in the NSCs following 24 h and 48 h of amputation were recorded. Thereafter (72 h and 96 h of amputation) moderate to massive engorgement of NSM in the B cells, coupled with spectacular increase in number of A cells were noticed. Sequential changes involved in the secretory dynamics of NSCs, as well as, NSM accumulation both within and periphery of the ganglia (perineurium) provides evidence for the utilization of materials through repaired vascular systems during posterior regeneration in *E. eugeniae*.

Key words: Neuroendocrine control, neurosecretory cells, regeneration, *Eudrilus eugeniae*, regeneration inhibiting factor, regeneration promoting factor.

1. Introduction

Eudrilus eugeniae (Eudrilidae) is a tropical earthworm species, well known for its protein source and vermicomposting potential. Because of surface dwelling habit (epigeic) they are often subjected to predator attacks leading to loss of body parts. They often show autotomy of posterior body segments following disturbance. Thus nature has gifted them the power of regeneration of lost body parts.

In earthworms, in absence of any definite endocrine gland as found in insects, the highly vascularised central nervous system equipped with abundance of neurosecretory cells (source of neurohormone) acts as neuroendocrine system that controls different physiological activities including regeneration [1, 2]. The importance of the central nervous system in the

phenomenon of oligochaete regeneration has been elucidated by several classical investigators [3, 4]. A brain hormone released soon after segment loss that initiates the process of regeneration in *Eisenia fetida*, *Lumbricus terrestris*, *Allolobophora terrestris* and *Lumbricus rubellus* [5, 6]. In contrast, it was found that brain removal followed by loss of body segments did not prevent posterior regeneration in *Eisenia fetida* [7] and *Allolobophora icterica* [8]. The ventral nerve cord C₃ neurosecretory cells exhibited spectacular cytological response to the loss of anterior segments [9]. An inhibitory effect of the brain and stimulatory effect of the anterior nerve ganglia on regeneration of posterior body segments in earthworm was also reported [10]. Nanda and Chaudhuri [11] recorded cytological response of moderately stained neurosecretory cells (B cells) in the ventral ganglia during anterior regeneration in tropical earthworm, *Metaphire peguana*.

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The remarkable ability of the earthworms to regenerate transected or ablated giant axons [12] and the cerebral ganglia [13-16] has drawn the attention of neuro-scientists seeking convenient models for studies on neural regeneration. Recent studies indicate that free-floating coelomocytes migrate early to the site of wounds in earthworms and are involved in the formation of wound plug and subsequent tissue reorganization by virtue of their phagocyte activities and delivery of nutrients and growth-stimulating molecules.

In spite of importance of regeneration in the ecology of earthworms, little research on earthworm regeneration and its endocrine control has been reported since 1980s. In fact, studies on the neurosecretory involvement in earthworm regeneration are mainly based on temperate earthworms [1]. Information is scanty regarding neuroendocrine involvement in the regeneration in tropical earthworms [11]. So the aim of our present paper is to study the involvement of brain and ventral nerve cord-neurosecretory system in South African tropical earthworm, *Eudrilus eugeniae* during posterior regeneration.

2. Materials and Methods

Seventy clitellate earthworms, *E. eugeniae* (average length 150 mm) were collected from laboratory stock culture (average room temperature 27 °C and RH 60%) and divided into 4 groups. In group I (10 individuals) both anterior 6 segments (including nerve ring in 3-4 segments) and posterior 6 segments were amputated by a sterilised knife. In the group II (10 individuals) where the nerve ring was retained, only posterior most 6 segments were amputated. *E. eugeniae* belonging to group III (10 individuals) and group IV (40 individuals) were considered as control and experimental sets for histological studies on the CNS neurosecretory system. Amputation of posterior tail segments was made by a sterilized blade in group III and group IV individuals. Amputated earthworms were

kept in earthen pots containing decomposed cow dung.

The cerebral, sub-esophageal and ventral nerve cord ganglia of control and experimental earthworms (24, 48, 72, 96 h after amputation) were fixed in Bouin's fluid. Identical sets of ganglionic components were dissected out from un-operated control earthworms. Sections (7 µm thick) were stained with both Gomori's Chrome Alum—Haematoxylin Phloxin [17] and simplified Aldehyde Fuchsin staining [18] technique following acid permanganate oxidation.

The nucleocytoplasmic (NP) ratios of the neurosecretory cells (10 cells of each type) were determined by measuring the maximum diameter of the perikaryon, as well as, the nucleus. Palcovit's formula ($V = \pi/6 \times LD^2$) has been applied for calculation of nucleus and cell volume. The average value of the ratio $\frac{V_n}{V_c - V_n}$ (V_n = nuclear volume, V_c = cell volume) has been considered to assess the neurosecretory activity. Besides these, indices of neurosecretory activity were further correlated with the nature and amount of NSM present within perikarya.

3. Observation

3.1 Control

A majority of the NSCs of the CNS were in various phases of secretion (Fig. 1 a-b) which could be determined on the basis of the staining intensity in descending order to locate the concentration of neurosecretory materials (NSM). Relatively small deep stained, AF positive neurosecretory A cells are located peripherally beneath the perineurium and possess homogeneously stained colloidal cytoplasm without any detectable cytoplasmic vacuoles. The medium to large sized moderately stained AF positive B cells are located in between the peripheral cortical tier of A cells and central fibrous neuropile. The B cells possess variable amounts of secretory inclusions apart from clarity in their cytoarchitecture. Both A and

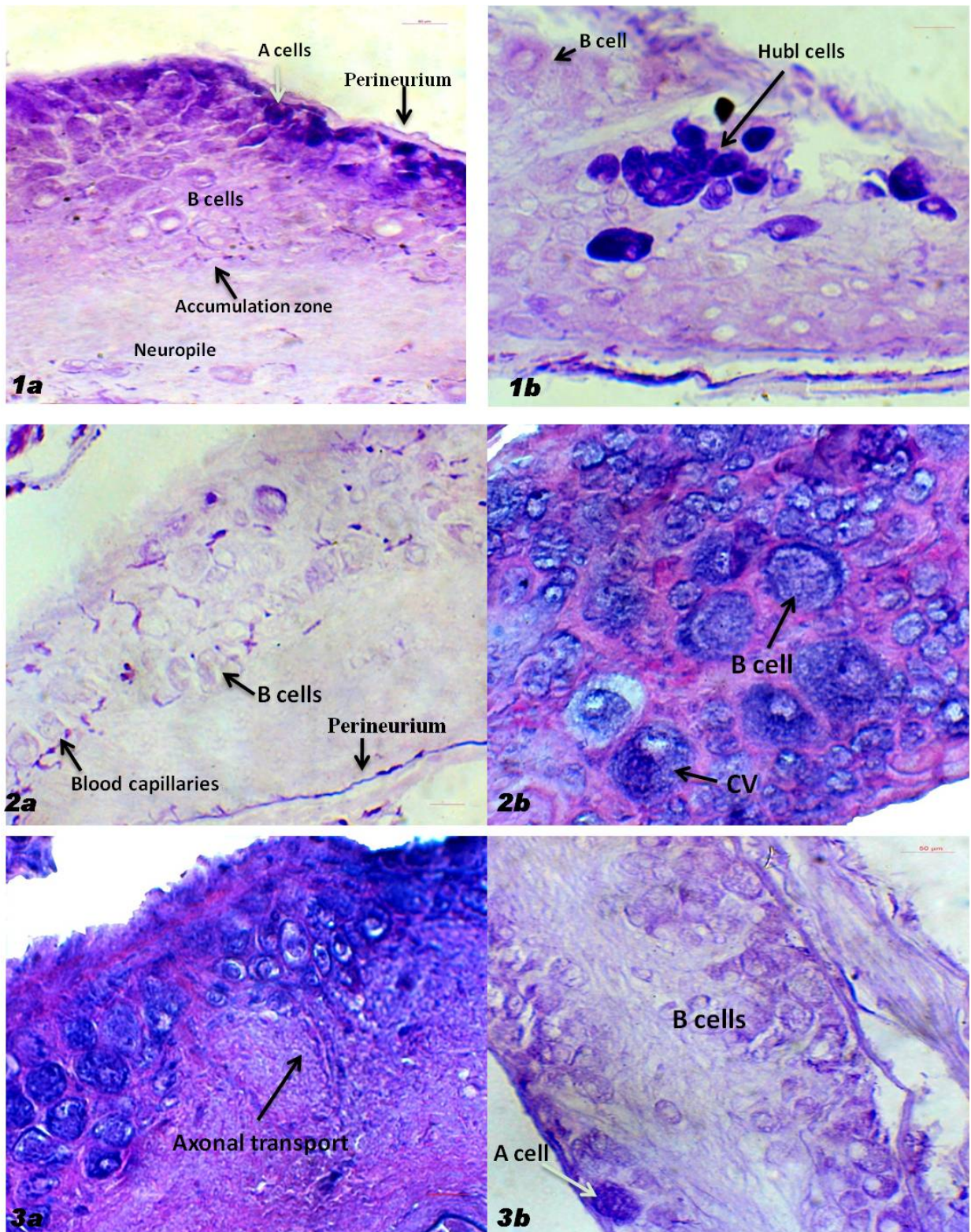


Fig. (1a-3b) Neurosecretory cells in the cerebral, sub esophageal and ventral ganglia of *Eudrilus eugeniae* at different hours interval after posterior amputation during regeneration.1. Control a. cerebral ganglia b. Sub esophageal ganglia; 2. 24 hours after amputation a. cerebral ganglia b. Ventral nerve cord ganglia. 3. 48 hours after amputation, a. cerebral ganglia b. Ventral ganglia.(Figs. 1a, b, 2a, 3b-AF stains; Figs. 2b, 3a-CAHP stains).

B cells are distributed throughout the ganglia of CNS i.e. cerebral, sub-esophageal and ventral ganglia. A well defined group of AF positive deeply stained neurosecretory cells, called Hubl cells (after the name of Hubl) or 'U' cells or 'S' cells lie at the base of circum-esophageal connective of the sub-esophageal ganglia.

3.2 Experimental

In group I earthworms, where both anterior and posterior most 6 segments were simultaneously amputated, a regeneration blastema at the anterior part of the body was formed one day earlier i.e. after 48 h of post amputation compared to that of the posterior part (72 h). In the group II individuals with intact brain, were only 6 posterior most body segments were amputated, regeneration blastema at the posterior end of the worm was formed within 72-96 h. On the 10th day after amputation, in group I worms, 5 anterior and 5 posterior body segments (of lesser width than original size) were restored. But group II earthworms (with brain) regenerated only 4 smaller posterior segments.

3.2.1 24 h after amputation

Most of the NSCs irrespective of their types had intense depletion of NSM. There were dramatic fall in the number of A cells throughout the CNS and 'U' cells in the sub-esophageal ganglia. The A and B NSCs in general, were characterised by the hypertrophic condition, voluminous nuclei, abundance of cytoplasmic vacuoles and meagre secretory inclusions (Fig. 2a-b, Table 1). Intra ganglionic blood capillaries in the cortical part of the cerebral ganglia and perineurium had rich distribution of NSM (Fig. 2a).

There were significant increase ($P < 0.05$) in the nucleocytoplasmic indices of NSCs, irrespective of cell types throughout the CNS (Table 2).

3.2.2 48 h after amputation

In the cerebral ganglia, A cells were in the state of depletion. The B cells in the postero dorsal part of the

cerebral ganglia exhibited cytoplasmic vacuoles and axonal transport of NSM (Fig. 3a). There were reappearance of a few deep stained 'U' cells and A cells with colloidal secretions and voluminous nuclei in the sub-esophageal and ventral ganglia (Fig. 3b). The B cells throughout the CNS increased their staining intensity with marginal accumulation of NSM beneath the cell membrane besides possession of voluminous nuclei and cytoplasmic vacuoles (Fig. 3a-b, Table 1).

There was significant increase in the NP indices ($P < 0.05$) of B cells throughout the ganglionic complements of CNS (Table 2). The NP indices of 'U' cells in the sub-esophageal ganglia, following its initial decline at 24 h of amputation, increased significantly ($P < 0.05$) after 48 h of amputation (Table 2).

3.2.3 72 h after amputation

The A cells reappeared with darkly stained AF positive colloidal secretion following 72 h of amputation. Their axons had also deposition of AF positive material (Fig. 4a). Majority of the B cells, however, had axon oriented voluminous nuclei, fine to coarse AF positive secretion and abundance of cytoplasmic vacuoles (Fig. 4a). The 'U' cells in the sub esophageal ganglia showed vacuolated cytoplasm with intense depletion (Fig 4b). Hypertrophic conditions of the NSCs were recorded throughout the CNS.

Although there were no changes in the NP indices of A cells of cerebral ganglia, the same in the sub-esophageal and ventral ganglia decreased significantly ($P < 0.05$) compared to those of control and 48 h of amputation. The NP indices of B cells in the cerebral and sub-esophageal ganglia increased significantly ($P < 0.05$) compared to 24 h and 48 h of posterior amputation (Table 1). Similarly the 'U' cells in the sub-esophageal ganglia also increased their NP indices ($P < 0.05$).

3.2.4 96 h after amputation

In the cerebral ganglia there were thick cortical tier

Table 1 A, B and U cell volume in the central nervous system of *E. eugeniae* at different time interval after posterior amputation.

CNS	Cell types	Control		24 h		48 h		72 h		96 h	
		CD±SD	ND±SD	CD±SD	ND±SD	CD±SD	ND±SD	CD±SD	ND±SD	CD±SD	ND±SD
Cerebral ganglia	A cell	6.70±0.14	3.80±0.29	7.10±0.35	4.60±0.59	10.42±1.3	5.10±0.61	9.30±0.3	4.70±0.4	7.38±0.57	4.20±0.56
	B cell	14.20±0.56	5.59±0.27	14.68±0.80	6.13±0.26	15.86±0.81	6.90±0.26	16.20±0.8	7.70±0.43	15.50±0.50	6.14±0.29
Sub esophageal ganglia	A cell	11.45±0.65	4.00±0.52	11.75±0.56	4.40±0.32	10.90±0.79	5.50±0.36	13.40±0.89	5.70±0.19	11.93±0.70	4.80±0.23
	B cell	16.79±1.3	8.70±0.35	16.25±0.9	7.30±0.44	18.15±0.20	8.20±0.46	21.12±0.70	9.9 ±0.10	17.6±0.85	8.9±0.46
	U cell	14.61±0.91	6.70±0.24	12.47±0.70	5.50±0.46	13.44±0.41	5.90±0.11	13.78±0.57	6.20±0.30	14.47±0.46	6.6±0.32
Ventral Nerve cord ganglia	A cell	11.31±0.74	4.7±0.21	13.6±0.7	6.8±0.31	12.9±0.7	5.8±0.22	12.2±0.9	5.68±0.27	11.56±0.72	5±0.3
	B cell	14.01±0.9	7.18±0.58	15.74±0.61	11.7±0.38	16.9±0.7	9.8±0.87	18.85±0.79	9.7±0.56	16.32±0.87	8.12±0.62

SD-Standard deviation, CD-cell diameter, ND-nuclear diameter.

Table 2 Nucleo-Cytoplasmic indices of A- and B nscs in the CNS of *E. eugeniae* after posterior amputation.

CNS	*Cell types	Neucleocytoplasmic Indices (NPI)				
		Control	24 h	48 h	72 h	96 h
Cerebral ganglia	A cell	0.06–0.22 0.10±0.01 ^a	0.29–0.10 0.22±0.02 ^b	0.045–0.19 0.10±0.01 ^{ac}	0.07–0.15 0.10±0.009 ^{ac}	0.06–0.27 0.13±0.021 ^{ac}
	B cell	0.01–0.06 0.04±0.002 ^a	0.02–0.07 0.05±0.003 ^{ac}	0.038–0.108 0.07±0.003 ^{bc}	0.076–0.241 0.11±0.009 ^d	0.04–0.20 0.07±0.001 ^{bc}
Sub esophageal ganglia	A cell	0.01–0.04 0.03±0.003 ^a	0.029–0.057 0.04±0.003 ^b	0.06–0.09 0.08±0.004 ^c	0.05–0.081 0.06±0.003 ^d	0.032–0.057 0.04±0.003 ^b
	B cell	0.07–0.18 0.12±0.011 ^a	0.05–0.1 0.07±0.006 ^b	0.06–0.1 0.09±0.004 ^{cb}	0.07–0.17 0.103±0.012 ^{ac}	0.1–0.16 0.134±0.006 ^{ad}
	U cell	0.06–0.09 0.07±0.004 ^a	0.03–0.08 0.05±0.004 ^b	0.05–0.07 0.06±0.002 ^c	0.05–0.07 0.07±0.003 ^{ac}	0.06–0.08 0.07±0.002 ^{ac}
Ventral Nerve cord ganglia	A cell	0.04–0.06 0.05±0.003 ^a	0.1–0.15 0.12±0.003 ^b	0.06–0.14 0.09±0.008 ^c	0.05–0.09 0.06±0.004 ^d	0.05–0.06 0.058±0.002 ^{ad}
	B cell	0.05–0.1 0.10±0.007 ^a	0.17–0.22 0.2±0.006 ^b	0.14–0.23 0.18±0.014 ^{bc}	0.10–0.13 0.11±0.006 ^a	0.05–0.17 0.10±0.01 ^a

Values represent Range & Mean±SE; Dissimilar letters indicating significant difference at 5% level of significance. * One way ANOVA.

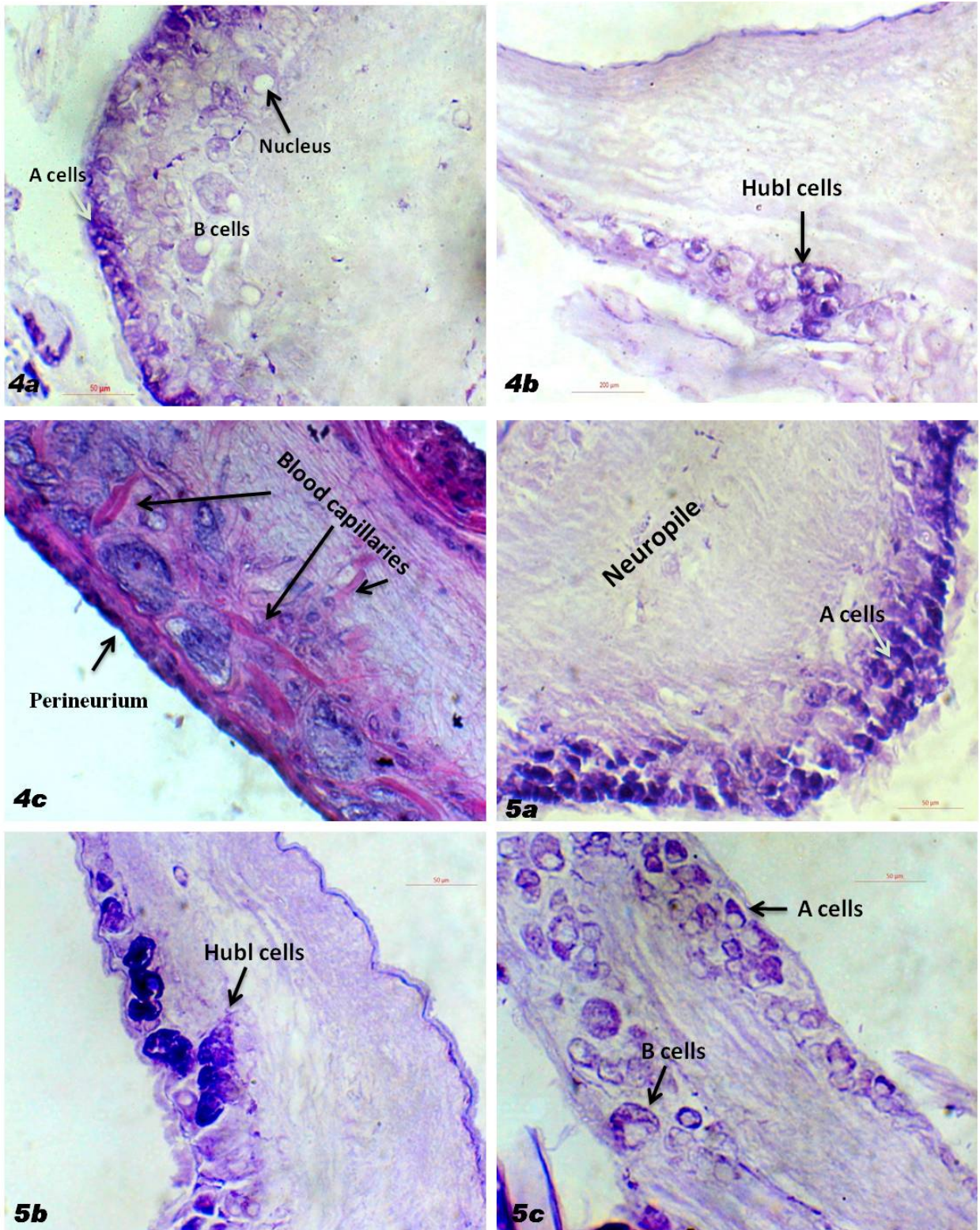


Fig. (4a-5c) Neurosecretory cells in the cerebral, sub esophageal and ventral ganglia of *Eudrilus eugeniae* at different hours intervals after posterior amputation during regeneration. 4. 72 hours after amputation. a. cerebral ganglia, b. sub-esophageal ganglia, c. ventral nerve cord ganglia; 5. 96 hours after amputation. a. cerebral ganglia b. sub-esophageal ganglia c. ventral nerve cord ganglia. (Figs. 4a, b, 5a,b,c-AF stains; Fig. 4c-CAHP stains).

of deep stained A cells loaded with NSM. Cytomorphology of the cerebral neurosecretory systems simulate that of control (Figs. 1a, 5a). The 'U' cells in the sub-esophageal ganglia also attained deep stainability with colloidal secretory inclusions. The B cells especially in the ganglia of the ventral nerve cord exhibited distinct secretory cycle with different levels of coarse secretory inclusions (NSM) and abundance of cytoplasmic vacuoles. While the NP indices in A cells of the cerebral ganglia remained unaltered, that of the sub-esophageal and ventral ganglia declined significantly ($P < 0.05$). The NP indices of B cells in the cerebral ganglia declined and increased in sub-esophageal and ventral ganglia.

4. Discussion

Transaction of posterior body segments in the South African vermicomposting earthworm, *Eudrilus eugeniae* had altered secretory activities in the NSCs of the CNS when the volume of the cells and nuclei, concentration of NSM in the cells and their transport down the axons were considered. Induction of such stimulation was probably propagated through the ventral nerve cord [18]. Neuroendocrine manipulation through ablation of brain in the tail amputated earthworms promotes the process of posterior regeneration compared to those having intact brain. In earthworms having intact brain, regeneration of posterior body segments was delayed. This probably indicates that posterior regeneration in *E. eugeniae* is under the joint influence of regeneration inhibiting factors secreted by brain and regeneration promoting factors released from ventral nerve cord.

In fact cytomorphological alterations in the NSCs are most conspicuous in the cerebral ganglia (brain) following 24 h of amputation. Changes like acute depletion of NSM in the A cells coupled with high nucleo-cytoplasmic indices and rich distribution of AF positive material in the blood capillaries suggest an efferent release of brain neurohormone in response to the afferent stimulus impinged through the loss of

segment. Such acute depletion of NSM in the central NSCs was probably related to metabolic disturbance due to cut stress. Depletion of NSM with reduction in the number of AF positive cells in the brain of earthworm following segment loss has also been reported by several investigators [19-21]. Hypertrophy of A and B cells throughout the CNS following 48 h of amputation are probably due to hyperactivity of these NSCs to tide over "generalised stress action" [22]. Increase in the staining intensity, marginal accumulation of NSM, presence of axonal transport, voluminous nuclei and high nucleo-cytoplasmic indices in the B cells of the CNS are the indication of an accentuation in the functional activity of the NSCs following 48 h of amputation.

With the appearance of regeneration blastema in the 3 days amputees, the secretory profile of the NSCs of CNS becomes entirely different. Facts like marginal accumulation of NSM in the A cells, sign of hypertrophy, depletion of NSM with appearance of cytoplasmic vacuoles, axon oriented voluminous nuclei and high nucleo-cytoplasmic indices in the B cells and 'U' cells of the sub-esophageal ganglia, proliferation of blood capillaries with distribution of NSM especially in the ventral ganglia indicate utilization of material (NSM) through the repaired circulatory systems and also functional involvement of ventral nerve cord neurosecretory cells in the posterior regeneration in *E. eugeniae*.

Facts like reappearance of thick cortical tiers of A cells in the cerebral ganglia, 'U' cells in the sub-esophageal ganglia charged with colloidal AF positive secretory inclusions and declined in nucleo-cytoplasmic indices suggests cessation of secretory activity of these cells following 96 h of amputation in contrast with their functional attribute during the initial stage of post amputation (*i.e.* 24 h).

Indeed dramatic decline in the number of A cells at the initial stage (24 h) of amputation with their reappearance following 72 h and 96 h of amputation and maintenance of high NP indices with distinct

secretory cycle in the B cells at late post amputation period indicate functional change over of A cells and B cells during regeneration. Relatively rich accumulation of NSM around the intra-ganglionic blood capillaries and the perineurium at 24 h and 48 h of experimentation and subsequent exhaustion of the same at 72 h followed by their massive accumulation at 96 h of amputation suggests fluctuation in the secretory dynamics of the NSCs.

In fine, adverse physiological stress condition in the form of injury possibly release cellular products that act an adjunct to stimulate the neurosecretory neurons of earthworms for possible release of regeneration promoting factor from the ventral nerve cord and regeneration inhibiting factor from the cerebral ganglia, the interaction among which probably leads to segment proliferation in *E. eugeniae*.

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