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Contrasting Effect of 2 Weeks Exposure of Unpredictable Chronic Mild Stress on the Morphology of Neurons of the Hippocampal Formation in Postnatal Chicks

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Abstract: In birds, hippocampal formation is the narrow-curved strip of tissue in the dorsomedial surface of telencephalon. It is subdivided into parahippocampal area and hippocampus proper that plays important role in learning and memory. In the present study, 5 days old chicks were divided into two groups: non-stressed (group 1) and stressed (group 2- treated with unpredictable chronic mild stress). Chicks were sacrificed after 2 weeks stress exposure for Golgi cox technique. It was observed that chronic stress affects majorly the morphology of neurons by causing significant decrease in various neuronal characteristics such as, spine length, distance of secondary branches from soma and dendritic branches at different radius circles from soma centre in multipolar neurons, the similar decrease in dendritic field, and axonal length was observed in both multipolar as well as stellate neurons. But the significant decrease due to chronic stress was observed in spine number and corrected spine number in all the neuronal classes (multipolar, pyramidal and stellate neurons) as dendritic spines are known for their dynamic nature that acquire the ability to alter their morphology and density under very short duration due to changing external and internal environment. So, it is concluded that all these dendritic remodeling and changes in dendritic spine structure and density occurs due to environmental conditions i.e., chronic stress exposure which initiates neuronal plasticity. Hence, by reorganizing the structures, connections and functions, the nervous system helps the animal to adapt and survive in adverse environmental conditions.

Keywords: Hippocampal formation, Stress, Projection neurons, Spines, Dendritic remodeling

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Introduction

In the avian brain, a narrow, curving strip of tissue that exists on the dorsomedial surface of the telencephalon is the hippocampal formation (HF). The HF is separated from the rest of the cerebral hemisphere by the lateral ventricle and is anatomically subdivided into two major regions, the hippocampus proper (Hp) and the parahippocampal area (APH). The Hp is widest from the dorsal side but tapers near the septum whereas APH is considered the transitional zone between the hippocampus and the adjacent telencephalic areas (Montagnese *et al.*, 1996; Tömböl *et al.*, 2000; Srivastava *et al.*, 2007). Many studies have reported that avian HF is homologous to the mammalian HF in terms of function, topography (Craigie, 1930) and developmental origin (Kuhlenbeck, 1938). The avian hippocampus is situated ventral to APH and is made up of a layered arrangement of closely packed neurons; a clear margin between the two structures is absent. In mammals, HF is overlaid by the neocortex and is recognized as two visible regions of a three-layered structure, the dentate gyrus and hippocampus (Ammon's horn or hippocampus proper) (Bingman *et al.*, 2003). The avian and mammalian HF plays an essential role in learning and memory formation (Krebs *et al.*, 1989; Morris *et al.*, 1989). The avian HF aids in spatial memory, homing, social behaviour, imprinting, emotional, and sexual behaviour (Atoji and Wild, 2006)

The brain is the primary organ of stress and adaptation to physical and social stressors because it recognizes threats, stores memories, and controls physiological as well as behavioural reactions to stressors that may be harmful or protective (McEwen, 1998; McEwen *et al.*, 2015). There are prominent morphological and structural changes that occur in the hippocampal formation due to different environmental changes, aging, and stress conditions (McEwen, 1999). Stress is the transactional process that arises from actual or apparent environmental conditions that can be considered threatening or beneficial, depending on the adaptive and coping abilities of an

organism (McEwen and Gianaros, 2010). Single acute stress can disrupt neuronal morphology by increasing the spine density and anxiety without changing dendritic arborization (Mitra *et al.*, 2005; Kumar *et al.*, 2021a,b). Whereas many scientists have reported that chronic stress affects the structure of neurons (Sapolsky *et al.*, 1986; McEwen and Gianaros 2011) synaptic plasticity, variations in the arborization of the dendritic tree, and dendritic morphology in the hippocampus (Watanabe *et al.*, 1992; Sunanda *et al.*, 1995). Stress is associated with psychopathology and reduced neural plasticity, especially when it is chronic and severe. Unpredictable chronic stress exposure may become maladaptive and make the brain more susceptible to diseases (McEwen and Chattarji, 2004; Karssen *et al.*, 2005; McEwen, 2007). Many diverse effects of chronic stress have been spotted on the central nervous system, including modifications in cellular activity, neurochemistry, and neuronal morphology (Mendelson *et al.*, 1993).

For many years, the entire morphology of neurons and neural circuits of the avian HF were examined by the Golgi method which revealed that there are 3 to 6 types of neurons present in birds (Franzoni *et al.*, 1984; Molla *et al.*, 1986a; Montagnese *et al.*, 1996; Tömböl *et al.*, 2000; Srivastava and Singh, 2012). The main feature of neuronal dendrites is the dendritic spine. The increase or decrease in the spine density of the hippocampus was directly associated with the formation of excitatory synapses, learning, and memory (Mahmmoud *et al.*, 2015; Ojha and Singh, 2021). The main objective of the present study was to evaluate the effect of unpredictable chronic mild stress (UCMS) on the neurons of hippocampal formation after 2 weeks exposure to *Gallus gallus domesticus*.

Materials and Methods

Animals:

Total 8 chicks (*Gallus gallus domesticus*) of both sexes of 5 days old were purchased from Pahari

Poultry House, Hawalbag, Almora, Uttarakhand, India. The chicks were kept in animal cages of 0.90×0.60×0.60 m³ dimensions with food and water *ad libitum*. The animal house was maintained with 12/12 h light/dark cycle with a moderate temperature of 22-26°C.

Unpredictable Chronic Mild Stress (UCMS):

Chicks were divided into two groups: Group 1 (4 chicks) was the control or non-stressed (NS) group whereas Group 2 (4 chicks) was the experimental or stressed group. The control group reared without any disturbance but the experimental group was treated randomly with different UCMS namely darkness, isolation, food deprivation, and cold temperature (12-16°C) daily for 4 h 10:00 am to 2:00 pm for 2 weeks. All the experimental procedures were carried out according to the Institutional Animal Ethical Committee (IAEC) guidelines of Kumaun University, Nainital (Protocol no. KUDOPS/157).

Body length and weight of chicks:

During the present study, the length (l) and weight (w) of all chicks (control and experimental) were measured thrice. Then the average length and weight were calculated from the first day till 2 weeks of stress exposure.

Golgi Cox Staining Method:

After 2 weeks, chicks from groups 1 (non-stressed) and group 2 (stressed) were anaesthetized with ketamine (Thermo Fisher Scientific, Mumbai, India) and sacrificed for Golgi cox technique. The brain was immediately removed from the skull, immersed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) and kept for 30 min at room temperature (RT). Later, the brain was transferred into the Golgi Cox solution (Levine *et al.*, 2013) and stored in dark for 24 h at RT. The next day, the brain was immersed in fresh Golgi Cox solution and stored in the dark for 14 days at RT. After impregnation, the brain was immersed in 1% potassium dichromate (Thermo Fisher Scientific, Mumbai India) solution (K₂Cr₂O₇ for 24 h), washed 2-3 times with distilled water (15 min), and

dehydrated in ascending grades of alcohol (Thermo Fisher Scientific, Mumbai India): 30%, 50%, 70%, 90%, and 100% (30 min in each). The brain was cleared in xylene (15 min) (Thermo Fisher Scientific, Mumbai, India) and embedded in paraffin wax (Molychem, Mumbai, India) for sectioning. The brain sections of 120 µm were cut by rotary microtome (Spencer), deparaffinized in xylene (10 min), rehydrated in descending grades of alcohol (100%, 90%, 70%, 50%, and 30% - 5 min in each). Sections were then placed in 1% K₂Cr₂O₇ solution, 28% ammonia solution (Molychem, Mumbai, India), 1% sodium thiosulfate (Na₂S₂O₃.5H₂O) solution (Thermo Fisher Scientific, India), dehydrated in 100% alcohol, cleared in xylene (5 min in each) and mounted in D.P.X. (Molychem, Mumbai, India) (Kumar *et al.*, 2021a,b).

Microscopic analysis:

The neurons in the Golgi-stained sections were observed under the light microscope. By using the computer-aided microscope (Leica-DMIL) at 400X (40X × 10X) magnification selected neurons and dendritic segment containing spines were photographed. Drawings of the selected neurons were drawn from the prepared slides by Camera Lucida attached to a light microscope at 400X (40X × 10X) primary magnification. The drawings of the neurons were scanned, whereas all photomicrographs, as well as their drawings were labelled and photo plates were made by Adobe Photoshop 7.0.

Neuronal Data Analysis:

The total number of neurons (x) was counted from Golgi impregnated slides under the light microscope at 400X (40X × 10X) primary magnification, and then their percentage was calculated. Various neuronal morphological features viz., dendritic field, soma diameter, dendritic diameter, spine head diameter, spine length, spine number, the distance of secondary dendrite from the soma, axonal length, and projection were calculated with the help of a computer-aided microscope (Leica-DMIL) at 400X

(40X × 10X) magnification. The number of dendritic branches at 25, 50, 75 and 100 µm radius circles from the soma centre were counted directly from Camera Lucida's drawing of the neurons. To calculate spine density for each type of neuron, number of dendritic spines (n) were counted per 25 µm of a dendritic segment. The dendritic diameter was measured three times from different points, and their average was calculated. The dendritic radius (Dr) was calculated as half of the dendritic diameter. The perpendicular linear distance from the surface of dendritic shaft to the tip of the dendritic spine was taken as spine length (Sl), and the mean of three spine head diameters (Sd) was also measured. The total number of spines is represented as spine density 1 (Horner and Arbuthnott, 1991), whereas spine density 2 provides a more accurate spine density because it also includes those spines that are present on the other side of the dendritic circumference (Srivastava *et al.*, 2014). Spine density 1 = n/Dl and spine density 2 = N/Dl, N the corrected spine numbers, and Dl the dendritic length over which spines were counted. Spine density (N) was calculated by the following equation (Feldman and Peters, 1979):

$$N = \frac{n\pi[(Dr + Sl)^2 - (Dr + Sd)^2]}{[\frac{\theta}{90}\pi(Dr + Sl)^2] - 2[(Dr + Sd)\sin\theta(Dr + Sd)]}$$

Where N is the corrected spine number, n is the number of visible dendritic spines, Dr is the radius of the dendrite, Sd is the spine head diameter, Sl is the spine length, and θ (Theta) is the central angle.

$$\cos \theta = \frac{Dr + Sd}{Dr + Sl}$$

Statistical analysis:

The mean value of various neuronal morphological features was analysed by using an unpaired t-test with Welch's correction. A minimum criterion of probability level of *P<0.05, **P<0.01 and ***P<0.001 were accepted as a significant difference. Results were presented as mean ± standard error. All the statistical analysis of neurons was performed using Microsoft Excel and Graph Pad Prism 9.0.

Results

Body length and weight of chicks:

On the first day of stress protocol the average length was 14.36 cm (NS); 14.95 cm (UCMS) and an average weight was 76.55 g (NS); 76.45 g (UCMS), whereas, after 2 weeks the average length of the chicks was 21.83 cm (NS); 22.83 cm (UCMS) and an average weight observed was 210.00 g (NS); 180.22 g (UCMS). According to this, the stressed chicks showed approximately similar length as the non-stressed chicks, but the weight of the stressed chicks decreased as compared to non-stressed chicks which clearly depicted that chronic stress affects the normal growth of chicks.

Neuronal morphological analysis of chicks:

In the present study, three neuronal classes i.e., multipolar (MP), pyramidal (PY) and stellate (ST) neurons in the HF were observed in chicks (*Gallus gallus domesticus*). Additionally, the Golgi technique also revealed percentage of neurons, dendritic field, soma diameter, the axons, axon collaterals, secondary branching, four types of spines on the dendritic segment of neurons, spine head diameter, spine length, spine number, corrected spine number, and dendritic branches at different (at 25, 50, 75, and 100 µm) radius circles from soma centre.

The multipolar neurons appeared to be the main subtype of projection neurons in the HF of chicks (Figs. 1A,B, 2A, B). The neuronal percentage observed in 3 weeks old chicks was 43.60% in NS and 46% in UCMS. These neurons have soma of different shapes like oval, spherical, rectangular or irregular. Many dendritic branches dispersed towards all the possible directions originating from the medium-sized soma. It was observed that there are about 8-10 spinous dendrites arise from the soma. The main primary dendrites gave rise to many side branches like secondary, tertiary and quaternary dendritic branches. The pyramidal projection neurons were evenly distributed in the HF of chicks (Figs. 1C, D; 2C, D). In 3 weeks old chicks, the neuronal percentage of pyramidal neurons was 21.09% in NS and 22.40% in UCMS.

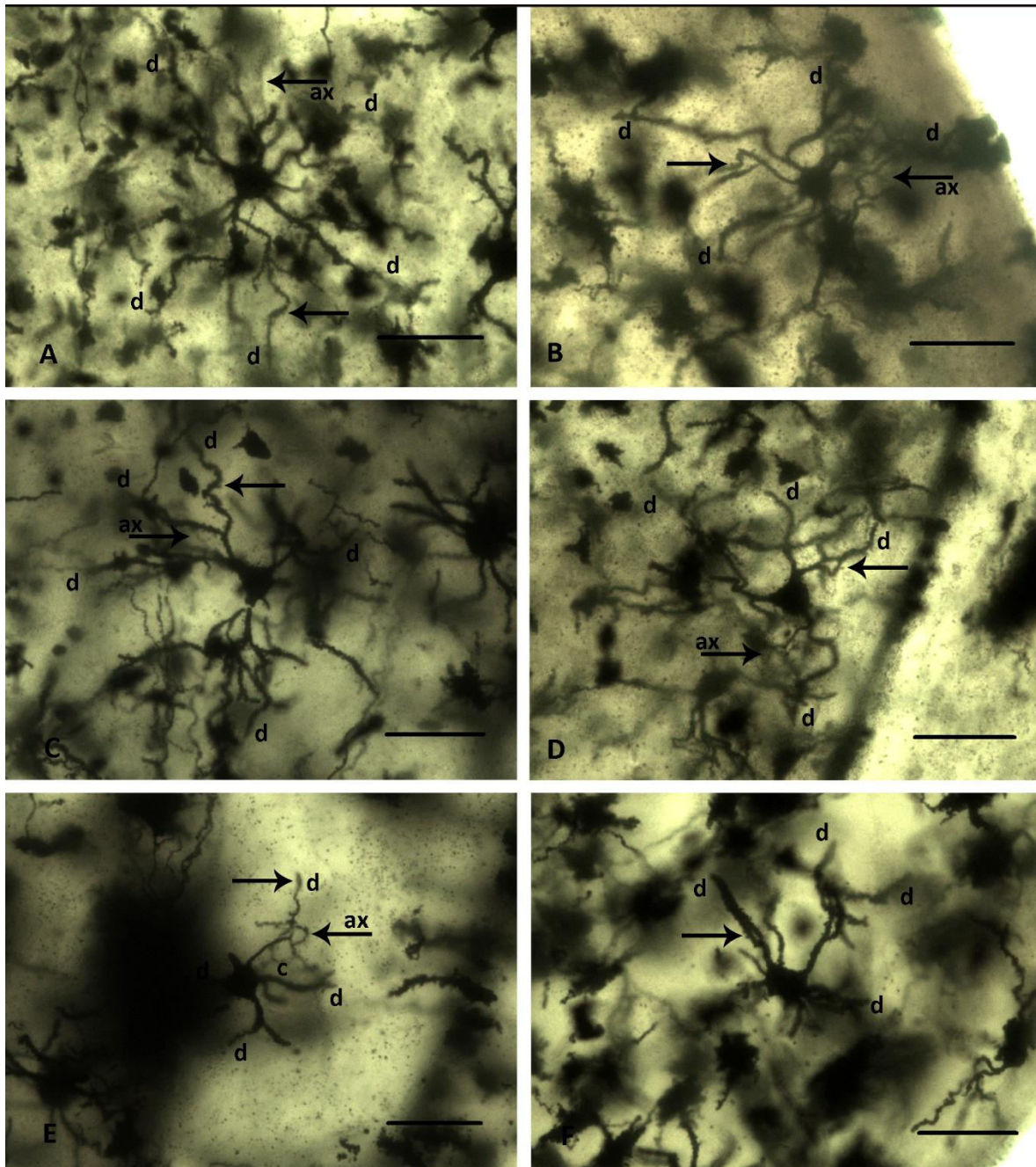


Fig. 1: Photomicrographs depicts three neuronal types i.e., multipolar (A- NS; B- UCMS), pyramidal (C- NS; D- UCMS) and stellate (E- NS; F- UCMS) neurons in the hippocampal formation of 3 weeks old chicks (*Gallus gallus domesticus*). d- dendrites, ax- axon, c- axon collaterals, arrow- spines, NS- Non-stressed chicks, UCMS- Unpredictable chronic mild stressed chicks. Scale bar- 50 μ m.

These neurons have thick apical dendrites, and finer basal dendrite, which is dispersed in all possible directions and originated from the soma. It was observed that there are about 7-9 spinous dendrites arise from the soma. These neurons possess medium-sized somas of triangular or

pyramidal like. The apical and basal dendrites gave rise to many side branches i.e., secondary, tertiary, and, quaternary dendritic branches. The stellate neurons were unevenly distributed in the HF of the avian brain (Figs. 1E, F, 2E, F). The percentage of stellate neurons observed in 3

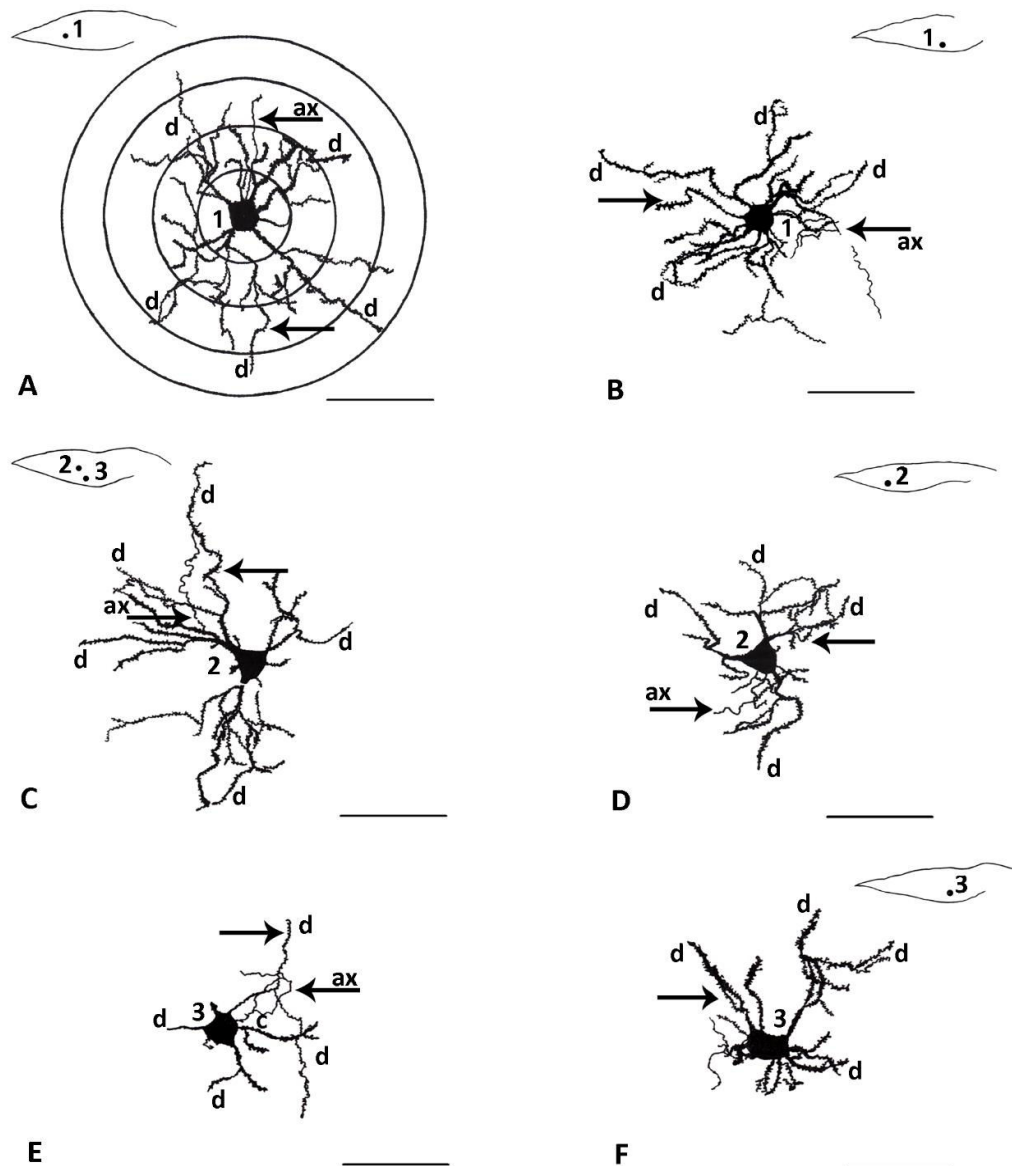


Fig. 2: Camera Lucida drawings of the multipolar (A- NS; B- UCMS), pyramidal (C- NS; D- UCMS) and stellate (E- NS; F- UCMS) neurons in the hippocampal formation of 3 weeks old chicks (*Gallus gallus domesticus*). Inset shows the respective position of these cells (1- MP; 2- PY; 3- ST). A is the circle diagram of multipolar neuron in which circles were drawn of 25, 50, 75 and 100 μ m radius from the soma centre, to calculate the number of dendritic branches. d- dendrites, ax- axon, c- axon collaterals, arrow- spines, NS- Non-stressed chicks, UCMS- Unpredictable chronic mild stressed chicks, MP- Multipolar, PY- Pyramidal, ST- Stellate neurons. Scale bar- 50 μ m.

weeks old chicks was 35.31% in NS and 31.60% in UCMS. The stellate neurons are featured with small, multiangular and ovoid soma that possess small 6-9 dendrites which further divide into secondary and tertiary branches. The dendrites of the stellate neurons were sparsely spinous as compared to other classes of neurons.

In all three neuronal classes the dendrites that originated from the soma possessed small protrusions called spines that are of four types- filopodia, thin, stubby and mushroom-shaped spines observed in multipolar, pyramidal, and stellate neurons of 3 weeks old NS and UCMS chicks (Fig. 3). Besides dendrites, the neurons also

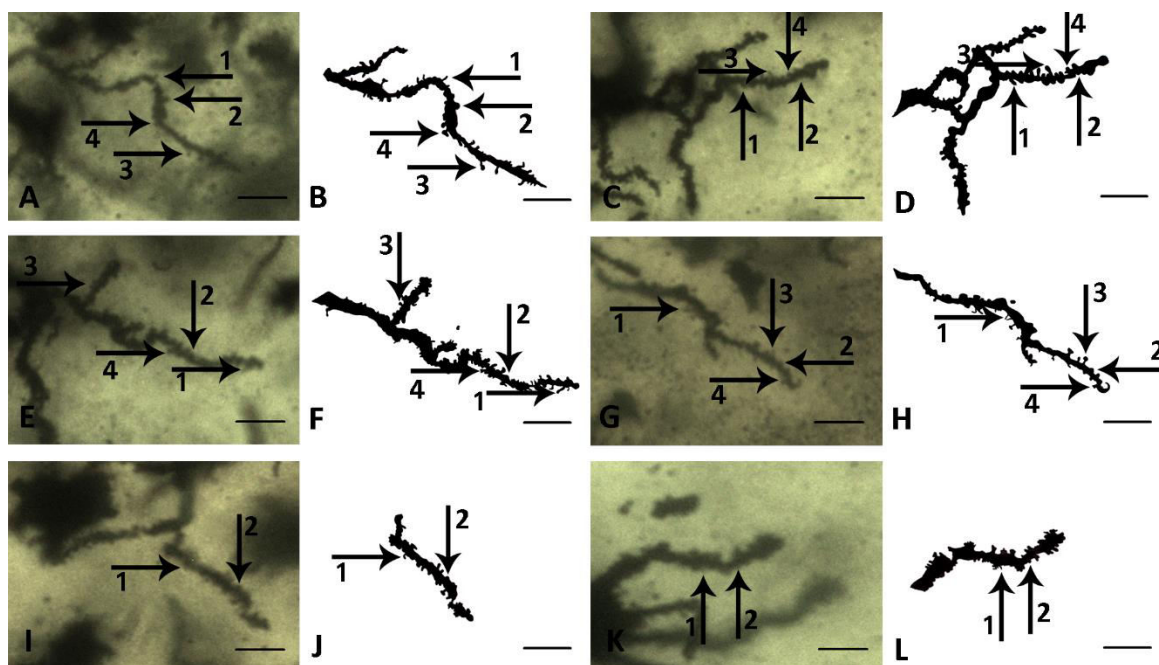


Fig. 3: Photomicrographs showing four types of dendritic spines present in 25µm dendritic segment of multipolar, pyramidal, and stellate neurons in the hippocampal formation of 3 weeks old chicks. The microphotographs A-B (NS) and C-D (UCMS) are the dendritic spines of Multipolar neurons and their respective camera lucida drawings. E-F (NS), G-H (UCMS) are the microphotographs of dendritic spines of Pyramidal neurons and their camera lucida drawings. I-J (NS), K-L (UCMS) are the microphotographs and camera lucida drawings of stellate neurons of 3 weeks old chicks. 1-filopodia, 2-stubby, 3-thin, 4-mushroom shaped spines, NS- Non-stressed chicks, UCMS- Unpredictable chronic mild stressed chicks. Scale bars = 20 µm.

possessed a single axon that originated from the soma, after a short course, sometimes the axon bifurcates into two side branches and forms axon collateral (c) which run up to the dendrites of the same neuron or the dendrites of neighbouring neurons as it was observed in multipolar and pyramidal neurons. The axon of stellate neurons arised from the soma, travels only a shorter distance and sometimes axon collaterals were hardly formed by stellate neurons. The axon and axon collaterals move in all possible directions. The axonal projection was observed in APH, Hp, local, dorsal side and ventral side in the HF of 3 weeks old chicks.

In the present study, statistical data analysis was conducted by Unpaired t-test with Welch's correction to compare the neuronal morphology of 3 weeks old NS with UCMS chicks by analyzing several neuronal parameters.

A significant decrease in the dendritic field

(Table 1, Fig. 4A) was observed in multipolar and stellate neurons whereas, no significant difference was observed in pyramidal neurons. The spine length (Table 1, Fig. 4E) and distance of secondary branches from soma (Table 1, Fig. 4H) in multipolar neurons showed significant decrease but no significant difference was observed in case of pyramidal and stellate neurons. The main neuronal characteristic that was negatively affected by UCMS was the spine number as well as corrected spine number. The significant decrease in the spine number (Table 1; Fig. 4F) and corrected spine number (Table 1, Fig. 4G) was observed in all the neuronal classes. The axonal length (Table 1, Fig. 4I) showed significant decrease in the multipolar and stellate neurons but no significant difference was observed in pyramidal neurons. The number of dendritic branches at 50, 75, and 100 µm radius circles from soma centre significantly decreased due to stress but no significant difference was observed at 25

Table 1: Depicts various neuronal characteristics such as, mean dendritic field, soma diameter, dendritic diameter, spine length, spine head diameter, spine number, corrected spine number, distance of secondary branches from soma, and axonal length of multipolar, pyramidal and stellate neurons present in the hippocampal formation of 3 weeks old non-stressed (NS) and unpredictable chronic mild stressed (UCMS) chicks, *Gallus gallus domesticus*. Unpaired t-test with Welch's correction show significant decrease in the dendritic field and axonal length of multipolar and stellate neurons due to UCMS in 3 weeks old chicks. Similarly, spine length and distance of secondary branches from soma shows significant decrease only in multipolar neurons. Soma diameter, dendritic diameter, and spine head diameter shows no significant difference. The spine number and corrected spine number shows significant decrease in all the neuronal classes of 3 weeks old chicks. Data is significantly different at level: *P < 0.05; ** P < 0.01; *** P < 0.001. Here, MP- multipolar; PY- pyramidal; ST- stellate neurons.

Neuronal characters	Neuronal Classes	NS	UCMS	Unpaired t-test (with Welch's correction) at P < 0.05		
				Degree of freedom	t _{calculated} value	P value
Dendritic field (Mean ± SEM)	MP	174.60 ± 5.17	145.90 ± 5.27	77	3.889	0.0002***
	PY	155.60 ± 6.42	147.80 ± 6.41	61	0.8639	0.391
	ST	102.10 ± 2.49	92.94 ± 3.19	42	2.273	0.0282*
Soma Diameter (Mean ± SEM)	MP	19.11 ± 0.33	18.28 ± 0.40	68	1.603	0.1136
	PY	19.33 ± 0.41	18.18 ± 0.45	59	1.891	0.0635
	ST	18.11 ± 0.41	17.62 ± 0.41	52	0.8351	0.4075
Dendritic Diameter (Mean ± SEM)	MP	1.29 ± 0.02	1.28 ± 0.02	75	0.4528	0.652
	PY	1.28 ± 0.01	1.25 ± 0.01	62	1.621	0.110
	ST	1.30 ± 0.02	1.26 ± 0.02	43	1.513	0.1376
Spine Length (Mean ± SEM)	MP	1.70 ± 0.01	1.65 ± 0.01	82	2.370	0.0201*
	PY	1.67 ± 0.02	1.68 ± 0.02	63	0.4077	0.6848
	ST	1.66 ± 0.02	1.63 ± 0.02	58	1.177	0.2441
Spine Head Diameter (Mean ± SEM)	MP	1.22 ± 0.01	1.20 ± 0.01	74	1.872	0.070
	PY	1.23 ± 0.01	1.23 ± 0.01	63	0.2770	0.7827
	ST	1.24 ± 0.01	1.21 ± 0.01	55	1.672	0.1002
Spine Number (Mean ± SEM)	MP	13.56 ± 0.32	12.06 ± 0.31	79	3.352	0.0012**
	PY	12.97 ± 0.43	11.74 ± 0.29	61	2.381	0.0204*
	ST	10.61 ± 0.31	9.35 ± 0.39	42	2.568	0.0139*
Corrected Spine number (Mean ± SEM)	MP	47.00 ± 0.98	42.45 ± 1.30	62	2.802	0.010**
	PY	48.43 ± 1.96	41.8 ± 1.32	61	2.808	0.0067**
	ST	40.16 ± 1.48	34.57 ± 1.68	47	2.499	0.016*
Distance of secondary branches from soma (Mean ± SEM)	MP	13.78 ± 0.45	12.19 ± 0.49	74	2.395	0.0191*
	PY	13.41 ± 0.60	12.58 ± 0.82	51	0.815	0.4188
	ST	11.95 ± 0.54	12.15 ± 0.73	40	0.216	0.8302
Axonal Length (Mean ± SEM)	MP	70.22 ± 2.89	59.48 ± 3.48	68	2.374	0.0204*
	PY	60.72 ± 4.13	61.23 ± 3.86	63	0.089	0.929
	ST	43.49 ± 2.88	35.63 ± 2.39	59	2.104	0.0396*

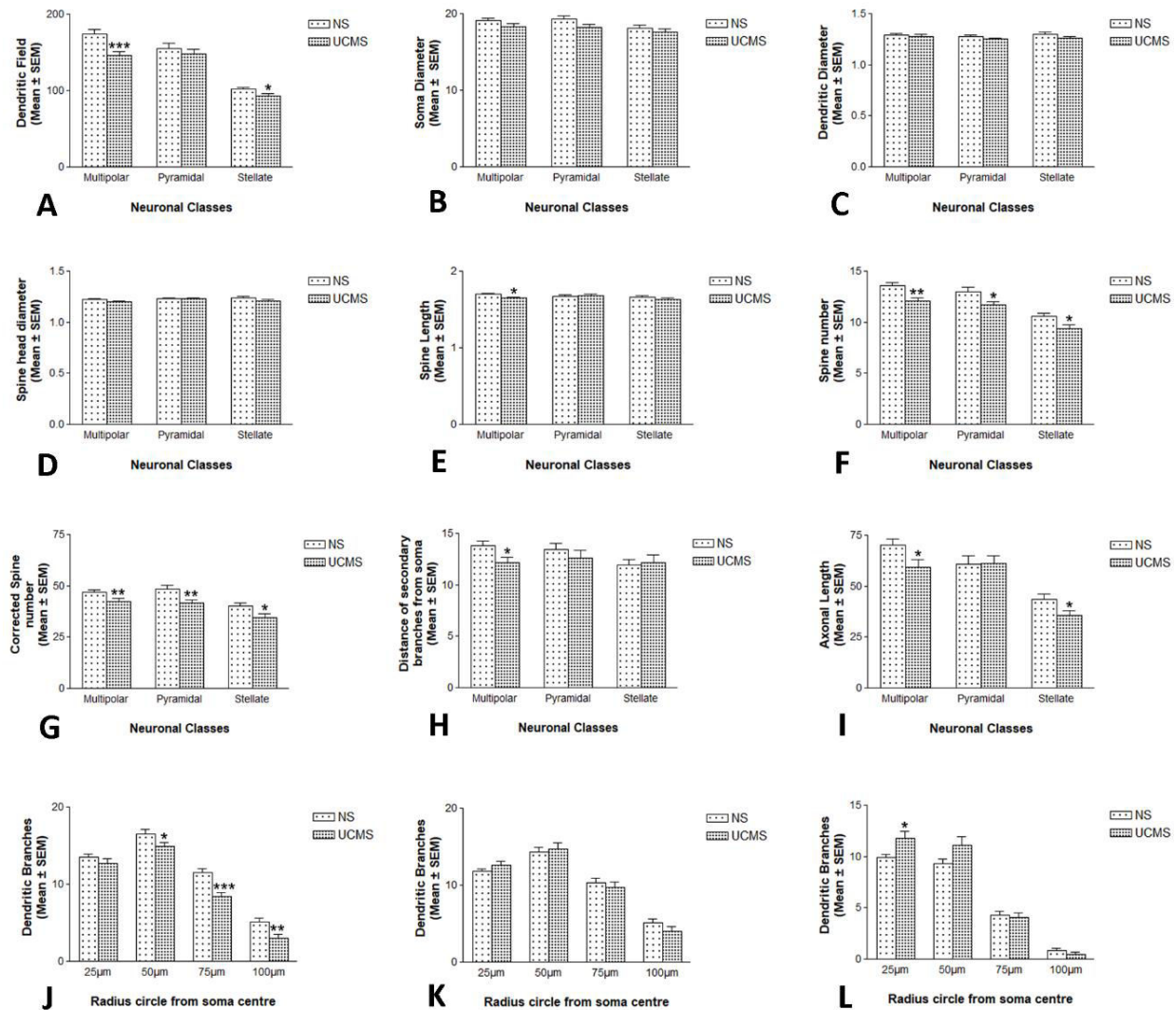


Fig. 4: Represent different neuronal characteristics: Dendritic field (A), soma diameter (B), dendritic diameter (C), spine head diameter (D), spine length (E), spine number (F), corrected spine number (G), distance of secondary branches from soma (H), axonal length (I) of all the neuronal classes. The dendritic branches at 25, 50, 75, and 100µm radius circles from soma centre of multipolar (J), pyramidal (K) and stellate (L) neurons in the HF of 3 weeks non-stressed (NS) and unpredictable chronic mild stressed (UCMS) chicks, *Gallus gallus domesticus*. Significant decrease was observed in the dendritic field and axonal length of multipolar and stellate neurons due to UCMS in 3 weeks old chicks. Similarly, spine length and distance of secondary branches from soma shows significant decrease only in multipolar neurons. The spine number and corrected spine number shows significant decrease in all the neuronal classes of 3 weeks old chicks. The mean dendritic branches at 50, 75, and 100µm radius circles from soma centre show significant decrease in multipolar neurons, but significant increase was observed only at 25µm radius circles from soma centre in stellate neurons in the HF of 3 weeks old chicks. Data is significantly different at level: *P < 0.05; ** P < 0.01; *** P < 0.001. Here, HF- hippocampal formation; MP- multipolar; PY- pyramidal; ST- stellate neurons.

Table 2: Depicts the mean dendritic branches at 25, 50, 75 and 100µm radius circles from soma centre of multipolar, pyramidal and stellate neurons present in the hippocampal formation of 3 weeks non- stressed (NS) and unpredictable chronic mild stressed (UCMS) chicks, *Gallus gallus domesticus*. The Unpaired t-test with Welch's correction revealed that the mean dendritic branches at 50, 75, and 100µm radius circles from soma centre show significant decrease in multipolar neurons whereas, no significant difference was observed in dendritic branches at 25µm. In pyramidal neurons, the dendritic branches at 25, 50, 75, and 100µm radius circles from soma centre show no significant difference, the same was observed in stellate neurons at 50, 75, and 100µm but significant increase was observed at 25 µm radius circles from soma centre. Data is significantly different at level: *P < 0.05; ** P < 0.01; *** P < 0.001. Here, MP- multipolar; PY- pyramidal; ST- stellate neurons.

Neuronal Classes	Radius Circles from soma centre	Mean dendritic branches at different radius circles (Mean ± SEM)		Unpaired t-test (with Welch's correction) at P < 0.05		
		NS	UCMS	Degree of Freedom	t _{calculated} value	P value
MP	25 µm	13.54 ± 0.34	12.74 ± 0.59	49	1.177	0.2447
	50 µm	16.54 ± 0.59	14.87 ± 0.54	81	2.093	0.0395*
	75 µm	11.54 ± 0.52	8.42 ± 0.50	80	4.310	0.0001***
	100 µm	5.14 ± 0.44	2.97 ± 0.59	61	2.959	0.0044**
PY	25 µm	11.79 ± 0.35	12.63 ± 0.47	51	1.425	0.1602
	50 µm	14.33 ± 0.63	14.70 ± 0.77	55	0.3716	0.7116
	75 µm	10.31 ± 0.65	9.67 ± 0.78	56	0.6340	0.5287
	100 µm	5.10 ± 0.56	4.00 ± 0.59	60	1.358	0.1795
ST	25 µm	9.93 ± 0.28	11.80 ± 0.63	26	2.691	0.0123*
	50 µm	9.34 ± 0.41	11.10 ± 0.85	28	1.870	0.072
	75 µm	4.27 ± 0.40	4.10 ± 0.43	48	0.2937	0.7702
	100 µm	0.86 ± 0.21	0.45 ± 0.22	49	1.358	0.1807

µm in multipolar neurons (Table 2, Fig. 4J). The significant increase was observed in the number of dendritic branches only at 25 µm radius circles from soma centre in stellate neurons whereas there was no significant difference in 50, 75, and 100 µm (Table 2, Fig. 4 K).

No significant difference was observed in several neuronal characteristics like soma diameter (Table 1, Fig. 4B), dendritic diameter (Table 1, Fig. 4C), spine head diameter (Table 1, Fig. 4D) in all the neuronal classes i.e., multipolar, pyramidal and stellate neurons, similarly, no significant difference was observed in dendritic

branches at different radius circles from soma centre (25, 50, 75, and 100µm) in pyramidal neurons (Table 2, Fig. 4K).

Discussion

The Golgi technique has been crucial for neuronal study in the past years and due to its highly effective role, the technique is being modified to get better results or to stain the neurons more efficiently. In the present study, the UCMS affects the normal growth like length and weight of chicks, though there was no significant difference observed in the length of chicks but the weight of

the chicks decreased.

The Golgi Cox technique revealed three types of neurons (Multipolar, Pyramidal and Stellate neurons) in the HF and four types of spines (stubby, thin, filopodia and mushroom shaped spines) in the dendritic segment of multipolar and pyramidal neurons, few dendritic spines was observed in the dendrites of stellate neurons of 3 weeks old NS and UCMS chicks. In the dorsomedial cortex (DMC) of newborn chick brain, the Golgi-stained sections revealed six types of neurons-long axon multipolar neurons, short axon multipolar neurons, pyramidal neurons, bipyramidal neurons, horizontal neurons, and periventricular neurons (Molla *et al.*, 1986b). Two main classes of neurons: projection neurons and local circuit neurons was reported in chick's (*Gallus domesticus*) and homing pigeon's (*Columba livia*) hippocampus. Moreover, both chick's and pigeon's HF possess three types of projection neurons, pyramidal, pyramidal-like, and multipolar projection neurons.

The neuronal types in the HF of aves and mammals are morphologically similar, the mammalian hippocampus possesses the dominant projection neuron i.e., pyramidal neurons (Tömböl *et al.*, 2000). It was noted that birds of different species share some common divisions of the brain but it was observed that reptiles also have some common brain structures with those of birds, one highlighted structure is the anterior dorsal ventricular ridge (DVR), a telencephalic structure that protrudes medially into the lateral ventricle in all birds and reptiles. The very spiny and moderately spiny neurons of alligators were reported similar to the four types of neurons recognized in starling, *Sturnus vulgaris*, (Saini and Leppelsack, 1981). The Projection neurons with spiny dendrites and local circuit neurons with aspiny dendrites were reported in zebra finches (*Taeniopygia guttata*) (Montagnese *et al.*, 1996; Atoji and Wild, 2006). Two types of neurons such as projection neurons (multipolar, pyramidal and pyramidal-like) and local circuit neurons were reported in the dorsomedial forebrain of

strawberry finches (*Estrilda amandava*) (Srivastava *et al.*, 2007). Golgi sections are also helpful for studying neuronal plasticity in terms of variations in spine density and dendritic thickness in APH. An increased dendritic thickness and spine density in the female Indian Ringneck Parrot (*Psittacula krameri*) was observed during the breeding period (Srivastava and Singh, 2012).

The mammalian HF is known as homologous to the avian HF so it can be stated that the changes that are caused by stress in the neuronal morphology of the avian brain will be similar to the mammalian brain. Stress affects the neuronal morphology and neural circuits of an individual's brain, and this change in its neuronal morphology leads to various mood disorders like aggression, anxiety, depression etc. So, it is necessary to obtain anatomical as well as morphological knowledge about the HF, its neurons and neural circuits to study the neurobiological effects of stress (Keuker *et al.*, 2003). Stress usually affects the functioning of the brain or more specifically the HF (Woolley *et al.*, 1990). The present study revealed that UCMS exposure for 2 weeks showed high fluctuations in various neuronal parameters of the HF of 3 weeks old chicks. Due to chronic stress, the dendritic field or dendritic length was retracted in multipolar, pyramidal and stellate neurons of 3 weeks old chicks. Similarly, many studies have reported that chronic stress disrupts neuronal architecture by reducing the length of apical dendritic branches of pyramidal neurons present in CA3 (Woolley *et al.*, 1990).

Additionally, the density of spines also decreased which represents the negative effect of UCMS over the neuronal morphology of chick's HF. Hence, the changes in spine shape, spine density, and dendritic remodeling were the major cause of chronic stress. In several animal models, various stressors decrease the spine density in the CA1 and CA3 pyramidal neurons which leads to depression-like behaviour (Patel *et al.*, 2018). Similarly, in the present study, various other neuronal characteristics such as axonal length, distance of secondary branches from soma in all

neuronal classes decreased. The dendritic branching pattern at 25, 50, 75, and 100 μm radius circle from soma centre in multipolar neurons decreased but many fluctuations were observed in pyramidal and stellate neurons which clearly depicted that chronic stress was the major cause for alteration in neuronal morphology. Therefore, it can be stated that all the neuronal parameters are somehow connected to each other. But it was more appropriate for dendritic field and spine density because when dendritic field contracts, the number of spines that was localized on the surface of dendrites also reduced, which may lead to decrease in formation of synaptic connections, by this way signal transmission, neural circuits, and neural pathways are affected, moreover, the changes in dendrites or dendritic remodeling, alteration in spines shape and density influence the neuronal plasticity and cognitive performance.

Conclusion

The present study showed that the neuronal morphology of chicks was affected after 2 weeks of chronic stress exposure as it was observed that various neuronal parameters such as, dendritic field, axonal length, spine number and corrected spine number decreased as compared to non-stressed group. Among these, the majorly affected neuronal characteristic were the dendritic field, spine number and corrected spine number that showed decrease in all the neuronal classes (multipolar, pyramidal and stellate neurons). Therefore, all these alteration in the dendrites i.e., dendritic remodeling and structural plasticity of dendritic spines eventually contributed to neuronal plasticity which is essential for the survival of an animal.

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