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Unpredictable chronic mild stress shows neuronal remodeling in multipolar projection neurons of hippocampal complex in postnatal chicks

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Abstract

The hippocampal complex of birds is a narrow-curved strip of tissue that plays a crucial role in learning, memory, spatial navigation, and emotional and sexual behavior. This study was conducted to evaluate the effect of unpredictable chronic mild stress in multipolar neurons of 3-, 5-, 7-, and 9-week-old chick's hippocampal complex. This study revealed that chronic stress results in neuronal remodeling by causing alterations in dendritic field, axonal length, secondary branching, corrected spine number, and dendritic branching at 25, 50, 75, and 100 μm. Due to stress, the overall dendritic length was significantly retracted in 3-week-old chick, whereas no significant difference was observed in 5- and 7-week-old chick, but again it was significantly retracted in 9-week-old chick along with the axonal length. So, this study indicates that during initial days of stress exposure, the dendritic field shows retraction, but when the stress continues up to a certain level, the neurons undergo structural modifications so that chicks adapt and survive in stressful conditions. The repeated exposure to chronic stress for longer duration leads to the neuronal structural disruption by retraction in the dendritic length as well as axonal length. Another characteristic which leads to structural alterations is the dendritic spines which significantly decreased in all age groups of stressed chicks and eventually leads to less synaptic connections, disturbance in physiology, and neurology, which affects the learning, memory, and coping ability of an individual.

Keywords Hippocampal complex · Stress · Multipolar neurons · Dendritic spines · Golgi-Cox study

Introduction

The avian limbic system includes the hippocampal complex, a relatively small, curve strip of tissue which is located on the dorsomedial surface of the avian telencephalic hemisphere (Goodson et al. 2004). In birds, the hippocampal complex (HCC) is separated from the rest of the hemisphere by a lateral ventricle and is subdivided into two major regions that are the hippocampus proper (Hp) and the area parahippocampalis (Aph). The Hp appears widest at the dorsal side and tapers ventrally toward the septum (Atoji et al. 2001; Montagnese et al. 1996; Srivastava et al. 2007; Tömböl et al. 2000a), whereas the Aph act as a transitional zone between the hippocampus and the other adjacent telencephalic regions. In birds, the HCC plays a vital role in learning, memory, and spatial navigation (Bingman et al. 2005; Tömböl, et al. 2000b). As HCC is a prominent component of the avian limbic system, it also regulates sexual, emotional, and food-related behavior (Tracy et al. 2001).

Comparative anatomists defined the avian HCC as the phylogenetic homologue (Campbell and Hodos 1970) of the mammalian hippocampus because both structures are derived from a common ancestral reptile's dorsomedial cortex. Moreover, the mammalian Hp and the medial portion of the avian dorsal cortex (HCC) are similar in terms of development, topography, and functional perspectives (Craigie 1930; Kallen 1962) and involved in memory, learning, spatial navigation (Bingman and Yates 1992), and food storage behavior (Krebs et al. 1989). They also share some morphological similarities regarding the position of the lateral ventricle (Craigie 1934), laminar organization (Craigie

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1934; Tömböl et al. 2000a, b), neuronal types (Molla et al. 1986; Montagnese et al. 1996; Srivastava et al. 2007, 2009), and afferent and efferent connections (Bingman et al. 1989; Székely and Krebs 1996). However, the morphological arrangement of the avian Hp differs significantly from that of mammals, and parts that are similar to the subdivisions of the mammalian structure are not easily identifiable (Atoji and Wild 2006). The dorsomedial and medial walls of the caudal telencephalon are occupied by the avian HCC (or hippocampal formation), which includes the hippocampus, which is made up of layers of closely packed neurons, but a clear boundary is absent that separates Hp from the adjacent Aph (Atoji et al. 2002; Casini et al. 1997; Montagnese et al. 1996; Tömböl et al. 2000a). Contrarily, the mammalian hippocampal formation consists of the dentate gyrus and hippocampus (also known as the "Ammon's horn") which are two prominent three-layered structures which are covered by the neocortex and are easily recognized macroscopically and microscopically in transverse sections (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 behavioral reactions to stressors that may be harmful or protective (McEwen 1998; McEwen et al. 2015). Stress is a term used to describe situations in daily life that are physically and emotionally challenging. It can be considered as an alarm that alerts when the homeostasis disrupts and helps to rebuild it (McEwen 2007). The hypothalamic–pituitary–adrenal (HPA) axis and the autonomic nervous system are just two of the physiological reactions that lead to adaptation through "allostasis," but they also interact non-linearly with the metabolic system and the pro- and anti-inflammatory elements of the immune defense system. Multiple stressor exposure and dysregulation of non-linear interactions (ineffective response activation or suppression) result in allostasis (McEwen 1998; McEwen and Wingfield 2003), which is the active process of responding to stresses through the autonomic, metabolic, and immunological systems that work together to preserve homeostasis (McEwen 2006; McEwen et al. 2015). In response to environmental changes or altered internal states like stress or aging, the hippocampal complex exhibits dramatic structural and morphological alterations (Kerr et al. 1991; McEwen 1999; McKittrick et al. 2000) in the form of neuronal replacement, dendritic remodeling (dendritic spine shapes), and synapse turnover (dendritic spine density) (Mcewen and Gianaros 2010; Kumar et al. 2021). 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). Chronic stress reduces the complexity of the arbors and the overall length of the neuronal dendrites in the hippocampus (Watanabe et al. 1992; Magariños and McEwen 1995; Sunanda et al. 1995; McKittrick et al. 2000).

The foundation of modern neuroscience was meant because of the Golgi impregnation method (Golgi 1873) which was based on the principle of impregnation of neurons with metal, leads to the thorough visualization of the neuronal architecture i.e., cell soma, axons, dendrites, and spines. Golgi method is also used to understand the effect of induced gene alterations, neuropathology, and regenerative therapies on neuronal and brain morphology (Das et al. 2013). Many cytoarchitectural studies used the Golgi method to classify different types of neurons in the chicken (Molla et al. 1986; Tömböl et al. 2000a), pigeon (Atoji et al. 2002; Tömböl et al. 2000a), the zebra finch (Montagnese et al. 1996), and strawberry finch (Srivastava et al. 2009). The main feature of neuronal dendrites is the dendritic spines, specialized protrusions that shows variations in terms of size, shape, and number in response to activity (Leuner and Shors 2004; Mahmmoud et al. 2015). 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).

Many previous studies highlighted that chronic stress causes alteration in dendritic length and spine density in the HCC. The neuronal morphology was studied in the HCC, because this region is highly prone to stress, plays important role in spatial learning and memory (Bliss and Lomo 1973; Mahmmoud et al. 2015). Additionally, various behavioral studies have reported that hippocampus is also involved in spatial cognition and spatial orientation (Casini et al. 1997; Tömböl et al. 2000a). The main purpose of this study is to evaluate the effect of unpredictable chronic mild stress (UCMS- 4 types of stresses; one stress/day were given to chicks for 4 h) in the neurons of the HCC in different age groups (3, 5, 7, and 9 weeks old) of chicks (*Gallus gallus domesticus*), by concentrating on various neuronal characteristics, such as dendritic field, secondary branching, number of branching, and the dendritic spines.

Methods

Animals

The 5-day-old male chicks (*Gallus gallus domesticus*) identified by feather sexing method (Kaleta and Redmann 2008) were purchased from the Pahari Poultry farm (a government department), Hawalbagh, Almora. The chicks were

reared in two animal pens, i.e., non-stressed chicks in one, whereas stressed chicks in other, each having the dimension of $0.90 \times 0.60 \times 0.60$ m³, were kept in an animal house, with ad libitum food and water, 12/12 light/dark cycle, and in a moderate temperature of 24–27 °C. The chicks were kept for a day to overcome the consequences of stress caused by transport.

Grouping and stress protocol

In the study, total 48 male chicks of 5 days old were divided into five groups: Group A (24 chicks) was the control or non-stressed (NS) group which was left undisturbed, whereas Group B (6 chicks), Group C (6 chicks), Group D (6 chicks), and Group E (6 chicks) were the experimental or stressed groups which were exposed to different unpredictable chronic mild stress daily for 4 h (10:00 am to 2:00 pm) up to 2, 4, 6, and 8 weeks. As per the experimental design, the four types of UCMS conducted in this study were food deprivation, darkness, isolation, and cold temperature (12–16 °C). All the experimental procedures were conducted according to the guidelines of Institutional Animal Ethical Committee (IAEC) of Kumaun University, Nainital (Protocol no. KUDOPS/157).

Golgi-Cox staining method

After 2 weeks, Group A (6 chicks) and Group B were sacrificed; similarly, after 4 (Group A—6 chicks and Group C), 6 (Group A—6 chicks and Group D), and 8 weeks (Group A—6 chicks and Group E), the chicks were anaesthetized with ketamine and sacrificed for Golgi-Cox technique.

The brain was removed from the skull immediately followed by the immersion in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) at room temperature (RT) for 30 min. The brain was then stored in the Golgi-Cox solution (Levine et al. 2013) in dark for 24 h at room temperature. The brain was immersed in fresh Golgi-Cox solution the next day and again kept for 14 days in the dark at RT. Following impregnation, the brain was immersed in 1% potassium dichromate solution ($K_2Cr_2O_7$) for 24 h, rinsed 2–3 times with distilled water (15 min), then dehydrated in ascending grades of alcohol (30%, 50%, 70%, 90%, and 100%) for 30 min each. The brain was then cleaned in xylene for 15 min before being imbedded in paraffin wax. The 120 μm brain slices were cut with a rotary microtome, deparaffinized in xylene for 10 min, followed by rehydration in descending alcohol grades (100%, 90%, 70%, 50%, and 30%—5 min in each). Sections were then placed for 5 min in each—1% $K_2Cr_2O_7$ solution, 28% ammonia solution, and 1% sodium thiosulfate ($Na_2S_2O_3.5H_2O$) solution. Sections were then dehydrated in 100% alcohol, cleaned in xylene (5 min each), and mounted in D.P.X. (Kumar et al. 2021).

Microscopic analysis

Under the light microscope, the neurons in the Golgi-stained sections were examined. Selected neurons and dendritic segments with spines were photographed using a computer-aided microscope (Leica-DMIL) at 400X (40X × 10X) magnification. Camera Lucida coupled to a light microscope at 400X (40X × 10X) primary magnification was used to generate drawings of the selected neurons from the prepared slides. The neuron's drawings were scanned, and all microphotographs as well as drawings were annotated and photo plates were created using Adobe Photoshop 7.0.
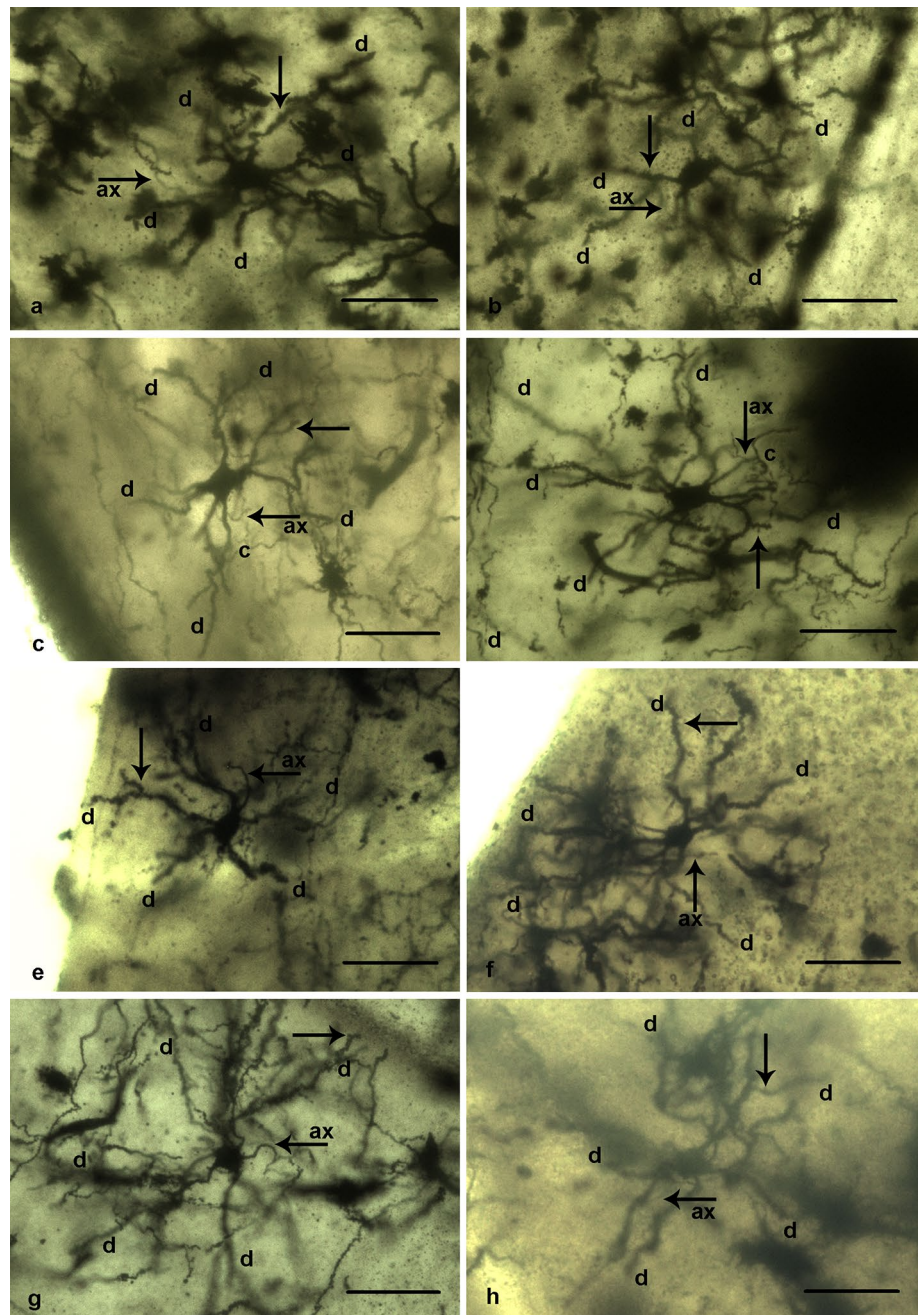
Neuronal data analysis

Under the light microscope at 400X (40X × 10X) magnification, the total number of neurons (x) was counted, and their percentage was calculated. A computer-aided microscope (Leica-DMIL) at 400X magnification was used to measuring various neuronal morphological features such as dendritic field, dendritic diameter, spine length, spine head diameter, visible spine number, distance of secondary dendrite from soma, and axonal length of all the well-stained neurons, and then the Mean ± SEM was calculated for each parameter. The axonal projection was also observed in computer-aided microscope (Leica-DMIL) at 400X magnification. The Camera Lucida's drawing of the neurons was used to count the number of dendritic branches at 25, 50, 75, and 100 μm radius circles from the soma center. The number of dendritic spines (n) per 25 μm of a dendritic segment was counted to calculate spine density for each kind of neuron. The average dendritic diameter was computed after measuring it three times from different places, while dendritic radius (Dr) was computed as half of the dendritic diameter. The spine length (Sl) was determined by measuring the perpendicular linear distance from the surface of the dendritic shaft to the tip of the dendritic spine, as well as the mean of three spine head diameters (Sd).

Spine density 1 represents the total number of visible spines (Horner and Arbuthnott 1991), and spine density 2 provides a more realistic spine density because it also includes spines on the other side of the dendritic circumference (Srivastava et al. 2014). Spine density 1 = n/Dl, and spine density 2 = N/Dl, where N represents the corrected spine number and Dl represents the dendritic length across which spines were counted. The following equation was used to calculate spine density (N) (Feldman and Peters 1979):

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

Fig. 1 Microphotographs depicts multipolar projection neurons in the hippocampal complex of 3- (**a** NS; **b** UCMS), 5- (**c** NS; **d** UCMS), 7- (**e** NS; **f** UCMS), and 9- (**g** NS; **h** UCMS) week-old chicks (*Gallus gallus domesticus*). Here, d- dendrites, ax- axon, c- axon collaterals, arrow- spines, *NS* non-stress, *UCMS* unpredictable chronic mild stress. Scale bar- 50 μm


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

$$Cos\theta = \frac{Dr + Sd}{Dr + Sl}.$$

Statistical data analysis

An unpaired t-test with Welch's correction was used to examine the mean value of several neuronal morphological features. As a significant difference, a minimum criterion of probability level is $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. The results are displayed as Mean ± Standard error of the mean. Microsoft Excel and Graph Pad Prism 9.0 were used for the statistical analysis of neurons.

Fig. 2 Camera Lucida drawings of multipolar projection neurons in the hippocampal complex of 3- (**a** NS; **b** UCMS), 5- (**c** NS; **d** UCMS), 7- (**e** NS; **f** UCMS) and 9- (**g** NS; **h** UCMS) week-old chicks (*Gallus gallus domesticus*). F is circle diagram in which circles of 25, 50, 75, and 100 μm were drawn to calculate the number of dendritic branches. Inset shows the respective position of the multipolar cells. Here, d- dendrites, ax- axon, c- axon collaterals, arrow- spines, *NS* non-stress, *UCMS* unpredictable chronic mild stress. Scale bar- 50 μm
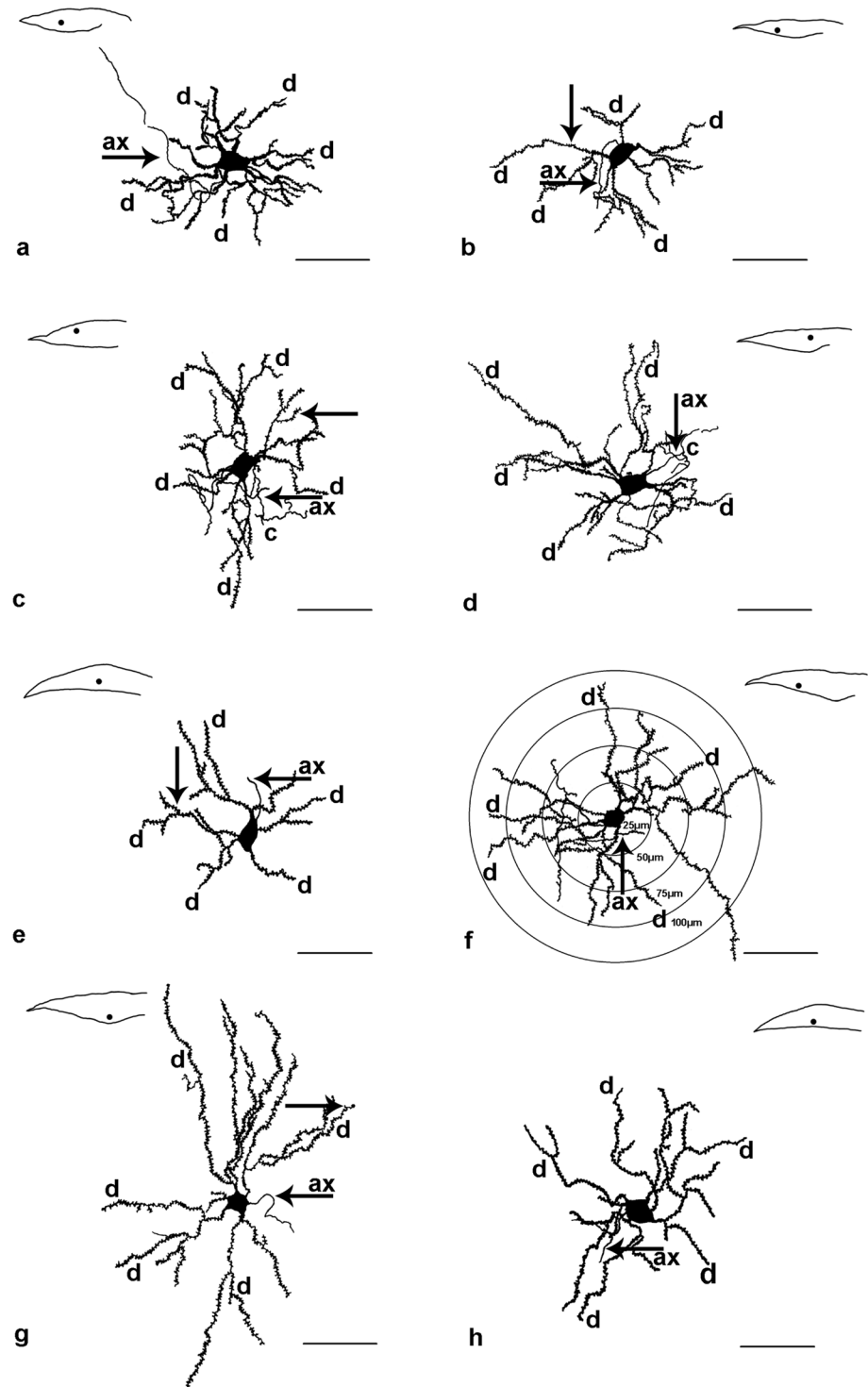
Results

In the present study, the Golgi-Cox method revealed multipolar (MP) projection neurons in different age groups in the HCC of chicks (*Gallus gallus domesticus*). The projection neurons are the neurons that acquire spinous dendrites (highly or moderately), their axons extend from the soma that bifurcate and form axon collaterals which projects within the same or adjacent regions (Tömböl et al. 2000a; Chand and Srivastava 2010; Chand et al. 2013). Various characteristics like soma diameter, dendritic field, corrected spine number, axonal length, axonal projection, secondary branching, and branching at different radius circles (25, 50, 75, and 100 μm) from the soma center show variations in
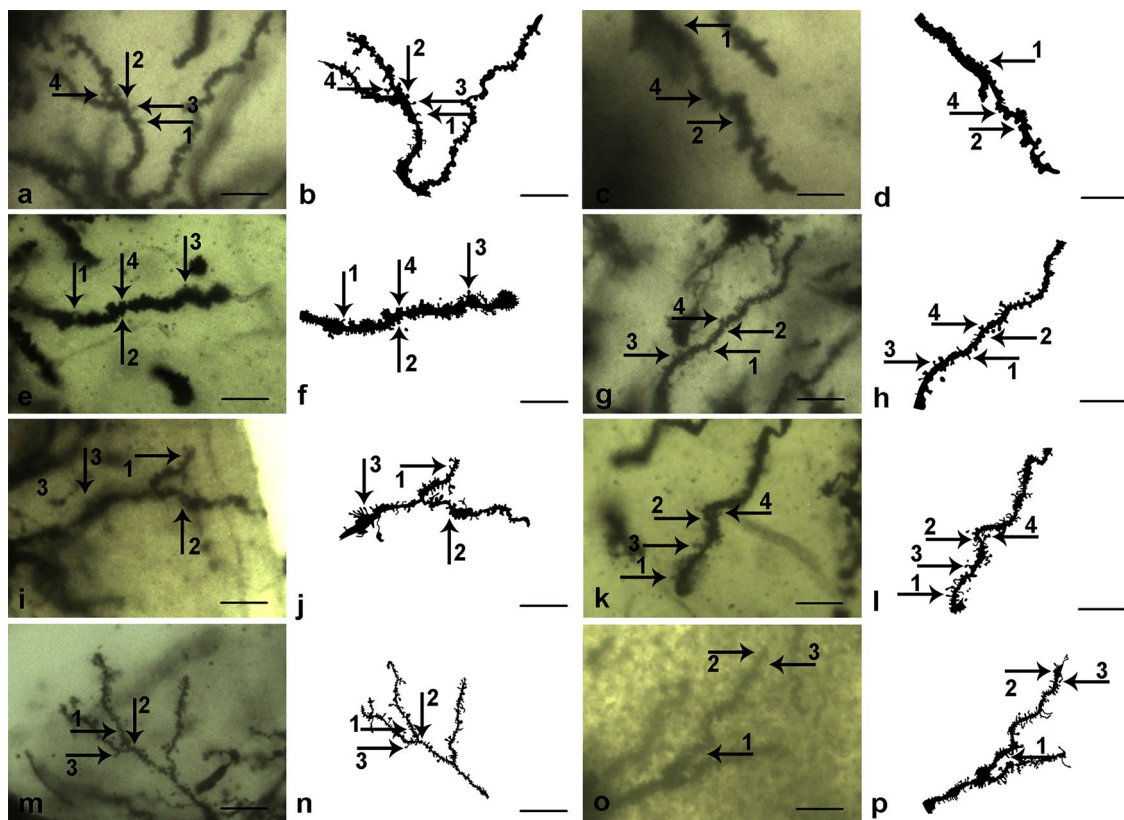
Fig. 3 Microphotographs showing four types of dendritic spines present in 25 μm dendritic segment of multipolar projection neurons in the hippocampal complex of 3-, 5-, 7-, and 9-week-old chicks (*Gallus gallus domesticus*). **a**, **b** (3-weeks), **e**, **f** (5-weeks), **i**, **j** (7-weeks), and **m**, **n** (9-weeks) depicts the microphotographs and camera lucida drawings of multipolar projection neurons of NS chicks. **c**, **d** (3-weeks), **g**, **h** (5-weeks), **k**, **l** (7-weeks), and **o**, **p** (9-weeks) depicts the microphotographs and camera lucida drawings of multipolar projection neurons of UCMS chicks. Here, 1- filopodia, 2-stubby, 3-thin, 4-mushroom-shaped spines, *NS* Non-stress, *UCMS* unpredictable chronic mild stress. Scale bars = 20 μm

different age groups. The technique also stained the small protrusions, i.e., dendritic spines (four types) present on the dendritic segment of multipolar neurons in the avian HCC.

Morphological analysis of multipolar neurons

The multipolar projection neurons are the dominant subtype of neurons observed in the HCC of chicks (Figs. 1 and 2). The neurons observed in 3-week-old chicks were 278 (43.78%) in NS, whereas 121 (42.76%) in UCMS chicks. In 5-week-old chicks, the neurons were 187 (47.70%) in NS, whereas 324 (50.31%) in UCMS. Similarly, in 7-week-old chicks, the neurons were 250 (37.31%) in NS, whereas 230 (37.10%) in UCMS chicks. In 9-week-old chicks, 260 (36.56%) is the neurons observed in NS, whereas 227 (38.67%) is the neurons in UCMS chick's HCC. The multipolar neurons of the avian HCC have medium-sized soma having a diameter between 15 and 19 μm in different age groups of chicks. There was no difference in the shape of cell bodies and the soma diameter was approximate of same range in each group. The soma shape of multipolar neurons whether in NS or UCMS chicks of all the age groups was multiangular, oval, rectangular, or spherical from where various thick spinous primary dendrites of about 6 to 9 originate and extended toward all possible directions. Many side branches arise from the main primary dendrite, i.e., secondary, tertiary, and quaternary dendritic branches. Along with the dendrites, the soma also gave rise to a single axon that extends in all directions like Aph, Hp, local, dorsal, and ventral sides; sometimes, the axon bifurcates into two side branches and forms axon collateral (c) that observed in MP projection neurons. The present study analyzes the length of dendrites and axons, but it is possible that the tracing is inaccurate because of the overlap with glial cells and dendrites and axons may be truncated at horizontal plane of sectioning (Figs. 1 and 2).

The Golgi method also revealed single and tiny structures that are present in the neurons. The dendritic surface of the MP neurons possesses small protrusions called dendritic spines. There are four types of dendritic spines—filopodia,

Table 1 Mean ± SEM of various neuronal characteristics like dendritic field, secondary branches, corrected spine number, and axonal length of multipolar neurons in the hippocampal complex of 3-, 5-, 7-, and 9-week non-stressed (NS) and unpredictable chronic mild stressed (UCMS) chicks, *Gallus gallus domesticus*

Neuronal Characters	Chick's age	NS (Mean ± SEM)	UCMS (Mean ± SEM)	Unpaired t-test (with Welch's correction) at $P < 0.05$		
				Df	$t_{calculated}$ value	P value
Dendritic field	3-weeks	196.60 ± 5.35	154.20 ± 7.45	34	4.6150	$P < 0.0001$***
	5-weeks	189.00 ± 7.59	188.50 ± 6.00	36	0.0605	0.9521
	7-weeks	162.40 ± 5.12	174.00 ± 7.39	33	1.2880	0.2068
	9-weeks	213.00 ± 7.27	175.40 ± 4.89	33	4.2880	0.0001***
Secondary branches	3-weeks	12.90 ± 0.76	13.03 ± 0.61	36	0.1323	0.9000
	5-weeks	13.28 ± 1.13	13.20 ± 1.12	37	0.0505	0.9600
	7-weeks	12.95 ± 1.09	14.25 ± 0.94	37	0.9045	0.3716
	9-weeks	12.62 ± 0.87	12.52 ± 0.60	33	0.0932	0.9263
Corrected spine no	3-weeks	47.07 ± 1.54	41.96 ± 1.27	36	2.5590	0.0148*
	5-weeks	48.22 ± 2.06	41.23 ± 1.60	35	2.6770	0.0112*
	7-weeks	42.49 ± 1.78	37.70 ± 1.32	34	2.1620	0.0378*
	9-weeks	58.27 ± 2.82	43.89 ± 1.52	29	4.4910	0.0001***
Axonal length	3-weeks	73.38 ± 4.79	66.74 ± 3.89	36	1.0740	0.2900
	5-weeks	59.39 ± 4.06	69.86 ± 5.60	34	1.5130	0.1396
	7-weeks	62.92 ± 5.14	70.41 ± 5.84	37	0.9626	0.3420
	9-weeks	93.34 ± 4.98	67.58 ± 4.11	36	3.9890	0.0003***

The unpaired *t*-test with Welch's correction revealed significant decrease in the dendritic field of 3- and 9-week-old UCMS chicks. The t-test also revealed significant decrease in the corrected spine number of all age groups (3, 5, 7, and 9 weeks old), whereas significant decrease in the axonal length was only observed in 9-week-old UCMS chicks. Data are significantly different at level: *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$. *Df* degree of freedom

stubby, thin, and mushroom-shaped spines observed in the MP neurons present in the HCC of different age groups of chicks (Fig. 3). First, the filopodia spines are thin hair-like protrusions that lack a bulbous head, the second is the stubby spines which are shorter and devoid of neck, the third is the thin spines which acquire distinct head and neck but with much slender diameters, and the last is the mushroom spines that possess a wider head than the neck.

Statistical analysis

The statistical data analysis was conducted by Unpaired t-test and Welch's correction to compare various neuronal morphology of 3-, 5-, 7-, and 9-week-old NS with UCMS chicks by analyzing several neuronal parameters.

Dendritic field

Significant decrease (***) in the dendritic field was observed in 3-week UCMS chicks, whereas no significant difference was observed in 5- and 7-week-old chicks, but 9-week-old UCMS chicks show significant decrease (***) than NS chicks (Table 1, Fig. 4).

Secondary branches

The secondary branches of 3-, 5-, 7-, and 9-week UCMS chicks show no significant difference than in NS chicks (Table 1, Fig. 4).

Corrected spine number

Significant decrease in the corrected spine number was observed in 3- (*), 5- (*), and 7- (*) week-old UCMS chicks as compared to NS chicks. 9-week-old UCMS chicks show significant decrease (***) in the corrected spine number when compared to NS chicks (Table 1, Fig. 4).

Axonal Length

3-, 5-, and 7-week UCMS chicks show no significant difference, but significant decrease (***) was observed in 9-week UCMS chicks as compared to NS chicks (Table 1, Fig. 4).

Dendritic branches at 25 μm

Only 3-week-old UCMS chicks show significant increase (*) in the dendritic branches at 25 μm radius circle from
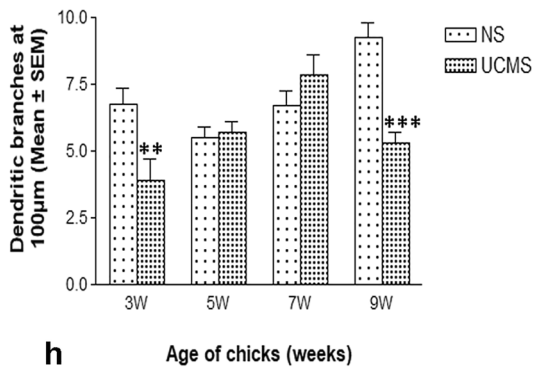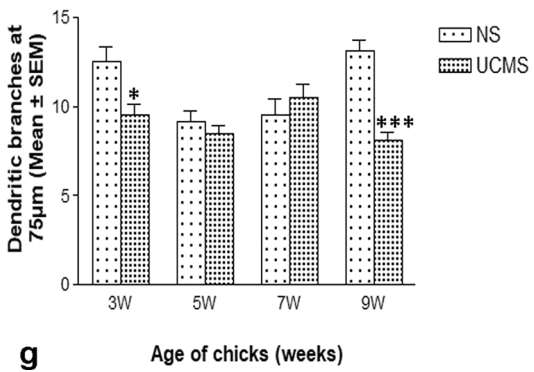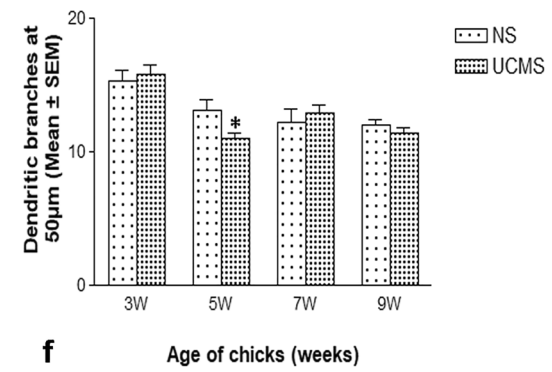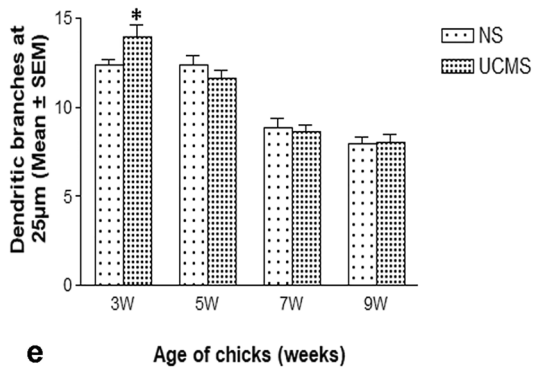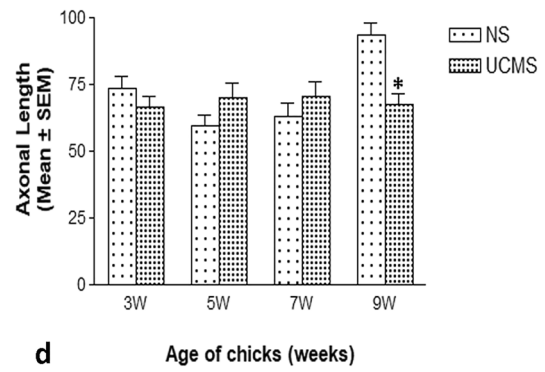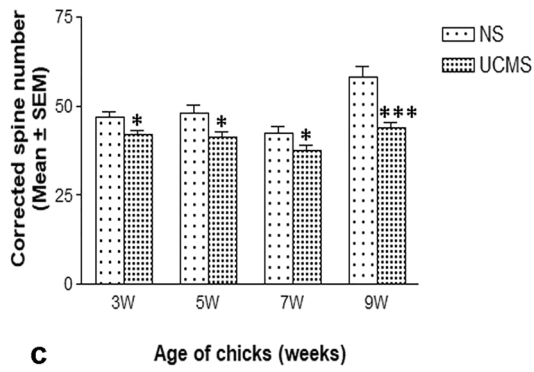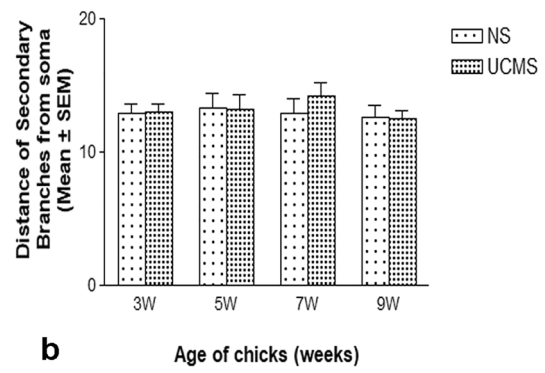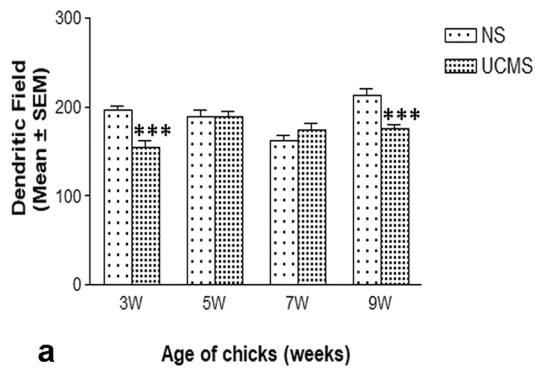
◄**Fig. 4** Mean ± SEM of different neuronal characteristics: Dendritic field (**a**), Distance of secondary branches from soma (**b**), corrected spine number (**c**), Axonal length (**d**), the dendritic branches at 25 (**e**), 50 (**f**), 75 (**g**), and 100 μm, (**h**) radius circles from soma center of multipolar projection neurons in 3-, 5-, 7-, and 9-week-old chicks (*Gallus gallus domesticus*). The dendritic field (**a**) decreases significantly in 3- and 9-week-old UCMS chicks, whereas no significant difference was observed in distance of secondary branches soma, **b** in all age groups. Significant decrease in the corrected spine number (**c**) of all age groups (3, 5, 7, and 9 weeks old) was observed, whereas significant decrease in the axonal length (**d**) was observed only in 9-week-old UCMS chicks. The dendritic branching at 25 μm, **e** radius circle from soma center in 3-week-old chicks shows significant increase, whereas significant decrease was observed in branching at 50 μm, **f** in 5-week-old UCMS chicks. The figure also revealed that the dendritic branching at 75 (**g**) and 100 μm (**h**) was significantly decreased in 3- and 9-week-old UCMS chicks. Data are significantly different at level: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

soma center than NS group of chicks, whereas no significant difference was observed in 5-, 7-, and 9-week-old UCMS chicks (Table 2, Fig. 4).

Dendritic branches at 50 μm

Similarly, the dendritic branches at 50 μm radius circle from soma center show significant difference (*) only in 5-week-old UCMS chicks, whereas other age groups, i.e., 3-, 7-, and 9-week-old UCMS chicks show no significant difference when compared to NS chicks (Table 2, Fig. 4).

Dendritic branches at 75 μm

Significant decrease (*) was observed in dendritic branches at 75 μm radius circle from soma center in 3-week-old UCMS chicks, but no significant difference was observed in 5- and 7-week-old UCMS chicks. Later on, the 9-week-old UCMS chicks shows significant decrease (***) than NS chicks (Table 2, Fig. 4).

Dendritic branches at 100 μm

Similarly, significant decrease (*) in the dendritic branches at 100 μm radius circle from soma center was observed in 3-week-old UCMS chicks, whereas no significant difference was observed in 5- and 7-week-old UCMS chicks. But dendritic branches in 9-week-old UCMS chicks shows significant decrease (***) than those in NS chicks (Table 2, Fig. 4).

Discussion

The present study was conducted to evaluate the effect of UCMS in the hippocampal neurons of different age groups of chicks, i.e., 3-, 5-, 7-, and 9-week-old chicks. The present study includes four types of chronic stressors, namely, food

deprivation, isolation, dark, and cold temperature which were reported in birds and in other animal models. Various studies show similar types of chronic stressors and their adverse effects in the organism's body. Food deprivation decreases neurogenesis in an avian brain (Robertson et al. 2017) as well as spine density in the hippocampal neurons in *Gallus gallus domesticus* (Kumar et al. 2023). Neonatal chicks (*Gallus gallus*) on exposure to low environmental temperature show hypothermia and decreased behavioral activity (Mujahid and Furuse 2009). Darkness causes neuroplastic variations, i.e., dendritic arborization in chick (Fosser et al. 2005). Short photoperiod (dark stress) induces a depression-like response in a diurnal rodent (Einat et al. 2006). Social isolation alters the calling dynamics (Ma et al. 2017) and decreases the new neurons in adult songbirds, i.e., in zebra finches (Lipkind et al. 2002) as well as impaired spatial learning in rats (Frisone et al. 2002). All these previous researches include four stressors and their consequences separately in different studies which encouraged us to select all the four types of stressors for the present study and included them for unpredictable chronic stress method to analyze their overall effects on the neuronal morphology of the HCC of different age groups of chicks. However, the limitation of this study is that four different chronic stress stimuli which were gathered together as one parameter may reduce the accuracy of the analysis on the alteration of neuronal ultrastructure.

In the present study, multipolar projection neurons are the dominant type of neurons in the chick's HCC or dorsomedial forebrain. The percentage of multipolar neurons decreases in the HCC of 3-week-old chicks after UCMS stress exposure which clearly represents the stress in postnatal chicks, and then their percentage increases in 5-week-old chicks which represents the increased adaptation in harsh conditions. The 7-week-old chicks adapted to the given condition showing little fluctuation, while during age of 9-week, the better adaptability has been observed in chick's HCC neurons showing increased neuronal percentage. UCMS not only leads to alterations in the neuronal percentage but also causes variations in many other neuronal parameters. On comparing neuronal morphological characteristics between NS and UCMS chicks of different ages revealed the significant changes in the dendritic field, axonal length, corrected spine number, branching at 25, 50, 75, and 100 μm of 3-, 5-, 7-, and 9-week-old chicks. But no significant difference was observed in secondary branching in all age groups of UCMS chicks. All these significant decreases in the neuronal parameters, namely, the changes in dendrites alteration in spines shape and density, are the major results of unpredictable chronic stress exposure which cause neuronal remodeling; moreover, repeated stress exposure for longer

Table 2 Mean ± SEM of dendritic branches at 25, 50, 75 and 100 μm radius circles from soma center of multipolar neurons in the hippocampal complex of 3-, 5-, 7-, and 9-week-old non-stressed (NS) and unpredictable chronic mild stressed (UCMS) chicks, *Gallus gallus domesticus*

Radius Circle	Chick's age	NS	UCMS	Df	$t_{calculated}$ value	P value
25 μm	3-weeks	12.35 ± 0.36	13.95 ± 0.67	29	2.105	0.0400*
	5-weeks	12.40 ± 0.51	11.60 ± 0.48	37	1.145	0.2597
	7-weeks	8.85 ± 0.51	8.65 ± 0.37	34	0.3151	0.7546
	9-weeks	7.95 ± 0.39	8.05 ± 0.41	37	0.1724	0.8641
50 μm	3-weeks	15.35 ± 0.77	15.85 ± 0.62	36	0.5073	0.6200
	5-weeks	13.15 ± 0.73	11.00 ± 0.40	29	2.587	0.0150*
	7-weeks	12.25 ± 0.93	12.95 ± 0.55	30	0.6500	0.5206
	9-weeks	12.00 ± 0.46	11.40 ± 0.46	37	0.9002	0.3738
75 μm	3-weeks	12.50 ± 0.89	9.55 ± 0.61	33	2.7290	0.0100*
	5-weeks	9.15 ± 0.64	8.50 ± 0.45	34	0.8291	0.4128
	7-weeks	9.55 ± 0.90	10.50 ± 0.77	37	0.8029	0.4271
	9-weeks	13.10 ± 0.61	8.10 ± 0.47	34	6.271	<0.0001***
100 μm	3-weeks	6.75 ± 0.62	3.90 ± 0.82	35	2.766	0.0100**
	5-weeks	5.50 ± 0.43	5.70 ± 0.42	37	0.3328	0.7411
	7-weeks	6.70 ± 0.58	7.85 ± 0.77	35	1.1970	0.2394
	9-weeks	9.25 ± 0.55	5.30 ± 0.40	33	5.617	$P < 0.0001$***

Table 2 spanning headers: "Mean dendritic branches at different radius circles from soma center (Mean ± SEM) of Multipolar neurons" over NS/UCMS columns; "Unpaired t-test (with Welch's correction) at $P < 0.05$" over Df / $t_{calculated}$ / P value columns.

The unpaired t-test with Welch's correction revealed significant increase in the dendritic branching at 25 μm radius circle from soma center in 3-week-old chicks, whereas significant decrease was observed in branching at 50 μm in 5-week-old UCMS chicks. The test also revealed that the dendritic branching at 75 and 100 μm was significantly decreased in 3- and 9-week-old UCMS chicks. Data are significantly different at level: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Df- degree of freedom

duration leads to less synaptic connections that affects learning, memory, and coping ability of chicks.

In brain, dendrites are known as the entry site of neural signals. In the dendritic branches of most neurons, the neural signals such as synaptic inputs for postsynaptic neurons and external stimuli for sensory neurons are processed and transformed into the electrical signals (Kanamori et al. 2015). In the present study, chronic stress significantly retracts the dendritic field during the early days, i.e., during 3-week-old chicks, but after sometime, no significant retraction was observed in 5- and 7-week-old chicks, which depicts some modifications, initiated in the neurons as well as in the neural circuits of the chick's HCC. The 9-week-old stressed chicks show a significant decrease or retraction in parameters, such as dendritic field and axonal length, revealed that repeated chronic stress exposure for a longer duration hinders the chicks to adapt the environment and ultimately shows adverse effects on the neuronal morphology of chicks, i.e., neuronal remodeling or plasticity. Stressors like food deprivation significantly reduced dendritic field in the hippocampal neurons of 30-day-old chicks (Kumar et al. 2023). Similarly, the dendritic length was reduced in house sparrow (*Passer domesticus*) due to reduced spatial availability, i.e., captivity (Roth et al. 2016). The 21 days of exposure to chronic stress (6 h/day) cause neuronal plasticity in terms of dendritic retractions in the CA3 area of a rat's hippocampus (Watanabe et al. 1992; Conrad et al. 1999). Based on the

present study similar findings were reported that revealed repeated stress exposure causes neuronal remodeling in terms of shortening of dendrites, loss of spine synapses, and suppression of the neurogenesis in the hippocampal complex of various animal models (Radley et al. 2004; Seeman et al. 2010; Liston and Gan 2011; Leuner et al. 2012; Gualtieri et al. 2019).

The spine density of the hippocampus was directly associated with the formation of excitatory synapses and plays a major role in learning and memory (Mahmmoud et al. 2015; Ojha and Singh 2021).

The dendritic spines undergo changes in size and shape throughout life, and slight changes in the spine cause significant changes to the somatic depolarization, or plasticity of the spine (Rall 1978). In the present study, filopodia, stubby, thin, and mushroom-shaped spines in the HCC of different age groups of chicks were observed. The same types of dendritic spines were observed in the hippocampus of chick (*Gallus gallus domesti*cus) (Kumar et al. 2023). In this study, out of all the parameters, dendritic spine number shows a decline in all the age groups (3, 5, 7, and 9 weeks old) of chicks due to UCMS exposure, which clearly indicates that spines are more vulnerable to stress and the disruption in the morphology of spines as well as in the spine density that cause neuronal remodeling. To support the study, similar findings were reported in 15- and 30-day-old chicks which demonstrate that food and water deprivation cause

structural plasticity in terms of a decrease in the number of dendritic spines in multipolar neurons (Kumar et al. 2023). A decrease in spine density in the CA1 and CA3 neurons due to chronic stress leads to depression-like behaviors (Patel et al. 2018). Moreover, the reduced dendritic spine number in the hippocampus was also proposed to cause schizophrenia (Law et al. 2004; von Bohlen und Halbach 2009).

In the present study, dendritic branches at 25, 50, 75, and 100 μm radius circle from soma center shows many fluctuations due to UCMS. Dendritic branching at 25 μm increases in 3-week-old chicks, but decreases at 50 μm in 5-week-old chicks. The decrease in number of branching at 75 and 100 μm was observed in both 3- and 9-week-old chicks, which clearly depicts that the number of dendritic branches or branch points was significantly reduced in UCMS chicks as compared to NS. Similar studies regarding unpredictable chronic stress for one month lead to reductions in the number of branch points in CA3 Hippocampal region of rats (Sousa et al. 2000); even a short duration of chronic stress also influences neuronal atrophy in the hippocampus by decreasing the number of branch points (Lambert et al. 1998). Restraint stress (21 days) in male rats and psychosocial stress (28 days) in tree shrews leads to a significant decrease in the number of branch points of hippocampal CA3 neurons (Watanabe et al. 1992; Magariños et al. 1996). The decrease in the number of branching points leads to less synaptic connection as it was reported that more branching leads to more synaptic connection between the neurons so that the information or signal can transmit more rapidly and increases the memory capacity (McEwen 1999; Roth et al. 2016).

During the initial days of stress exposure, the organism adapts to these difficulties and protects itself with the help of physiological reactions of the autonomic nervous system, cardiovascular, HPA axis, metabolic, and immunological systems which are referred to as allostasis, a crucial part for preserving homeostasis (Sterling and Eyer 1988). Overcoming certain stressful events can result in development, adaptability, and useful learning methods that foster future resiliency (McEwen 2007). Yet, further stressful events might result in behavioral, cognitive, physiological, and neurological alterations (McEwen and Gianaros 2010). These outcomes of the present study indicates that UCMS causes neuronal variations, such as dendritic retraction, alterations in the spine density, and reduction in the number of neurons that affects behavioral activity, learning ability, and causes depression-like behaviors.

Conclusions

Various studies have indicated the adverse effects of unpredictable chronic mild stress in the hippocampal neurons. In the present study, the initial stress exposure disrupts the multipolar projection neurons in the HCC by producing negative and significant alterations in neuronal parameter (decrease in spine density and dendritic length). Later, due to the coping ability of the HCC, the structural modification in neurons reverses the effects of stress by demonstrating no significant changes in the neuronal parameters (except spine density) as compared to NS chicks. But the chronic stress exposure for longer duration again shows significant changes in the neuronal characteristics which ultimately hinders the normal functioning of neurons, neural circuits, and synaptic connections and these changes which reflect the behavior, physiology, coping ability, learning, memory of an organism and sometimes leads to severe stress-related disorders. This study deals with the variation at the neuronal level due to unpredictable chronic stress, but it enlightened the broader perspective for research related to various alterations in neurotransmitter pathways and neurological disorders caused by chronic stress.

Declarations

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