CHAPTER I: STUDY OF PINEAL–THYROID INTERRELATIONSHIP
Study of Pineal – Thyroid Interrelationship

Introductory Note

Information concerning the influence of the pineal and its hormone melatonin on thyroid activity is inconclusive in mammals and birds. Contradictory results also preclude any conclusive inference regarding the role of pineal and melatonin in mammalian and avian thyroid functioning. Conversely, with respect to the thyroid gland, the relationship of thyroid hormone namely thyroxin, with the pineal gland as published in literature also appears to be inconclusive. In view of such, in the present chapter of the investigation, experiments were formulated involving either direct manipulations of the pineal and the thyroid gland through administration of melatonin on one hand and thyroxin and methimazole (a potent thyroid blocker) on the other. These were used as an important approach to establish a possible interrelationship between these two endocrine components in chicks.

Experimental Protocol

In the present investigation healthy neonatal chicks weighing about 40 – 45 gms. are used. They were first acclimatized to the laboratory conditions for ten days prior to start of experiments as mentioned in General Methodology. The experiments performed in this part have been categorized into two broad aspects.

**Group A: Manipulation of the Pineal Gland activity**

**Group B: Manipulation of Thyroid Gland activity**

EXPERIMENTAL PROTOCOLS

Group – A: Manipulation of pineal gland activity

Phase – I: Administration of Melatonin

Experiment I:

**Control - Ethanol – saline vehicle treatment:** This included the control group of birds. The chicks (N =6) were injected with vehicular ethanol-saline solution. A daily dose of
0.1 ml vehicle was injected for fifteen days. (Ethanol: Normal saline 1: 9 v / v) (Cardinali et al., 1979; Chakraborty, 1994).

Experiment II:

**Melatonin treatment:** Comparable to the control group, the chicks (N =11) were injected with melatonin (N - acetyl 1 - 5 methoxytryptamine, Sigma Chemicals, M.O., USA) dissolved in ethanol: injectable normal saline vehicle (1: 9 v / v) (Cardinali et al., 1979; Chakraborty, 1994). The dose of administration being 100 μg / 100 gm. body weight in 0.1 ml ethanol saline vehicle (Gromova et al., 1967) daily during the entire experimental schedule.

All the injections were given daily subcutaneously in the nape of the neck of the chicks approximately between 16.00 hrs. and 16.30 hrs. because more melatonin binding sites are available near the end of the photophase (Lang et al., 1983; Zisapel et al., 1988). The duration of the experiment was for fifteen consecutive days. The animals were housed in photoperiodic chambers fitted with lights and exhaust fan. The photoperiodic 12L: 12D lights (on at 06.00 hrs and off at 18.00 hrs) were controlled by timer switches (Surrey, UK). The chicks were killed by etherisation on the day sixteen (approximately twenty-four hours after the last injection of the drug). For the histological study the pineal and the thyroid glands were processed followed by evaluation and statistical analysis as mentioned in the General Methodology.

**OBSERVATIONS**

A. Histological studies

**Pineal Gland**

*Control- Ethanol-saline vehicle treatment:* Light microscopic study of chicks pineal gland indicated that the pineal parenchyma was seen to be compact with occasional lobules. The lobules were of irregular size separated from each other by narrow connective tissue strands. These lobules comprised of pinealocytes of principal cells,
arranged either of clusters of rosette without lumen or follicles and tubules with lumen interspersed with packed cells. The follicles or tubules contain lumen with periluminal columnar epithelial cells having basal nuclei (Fig. 1).

The staining procedures used in the present investigation failed to differentiate the parenchymal cell types of pinealocytes. The pinealocytes showed finely granular acidophilic cytoplasm with a spherical, rather vesicular nucleus which often showed presences of a nucleolus. The pinealocytes with distinct round nucleus characterized the pineal gland sections of these vehicular control birds.

*Melatonin treatment:* Melatonin administration evoked a significant alteration in the pineal indicative of stimulation. The pinealocytes were seen mostly with large round, well defined nuclei (Fig. 2). The values of nuclear diameter of the pinealocytes were increased compared to the control values \( p < 0.001, \) Table 1A, Fig. 1A. The nucleolus appeared to be dense and conspicuous.

The results from one-way analysis of variance for nuclear diameter \( [F (1, 10) = 24.614, p < 0.001] \) of pinealocytes from control and melatonin treated groups revealed that the experimental mean values were significantly different, indicating significant changes between the melatonin treated groups compared with control mean values.
Figure 1A - Histogram showing pinealocyte nuclear diameter (µm) following melatonin treatment as compared to control values. Melatonin caused a significant increase in nuclear diameter value compared to control group. The vertical lines signify the SEM.

**Thyroid Gland**

**Control- Ethanol-saline vehicle treatment:** Histological aspect of the control thyroid gland revealed follicles consisting of spheres formed by simple epithelium whose follicular radius was seen to be normal. Follicles were regular in size and shape and the follicle lumina were filled with homogenous colloid (Fig. 8). Plasma thyroxine level in these chicks was of moderate value (2.45 µg/dl). The value found in these chicks was within the range as reported earlier. It was seen that in chicks of 1 day-6 weeks old were in the range 1.45 – 3.0 µg/dl (Norris 2007)

**Melatonin treatment:** Melatonin treatment induced significant alteration in the overall histology of the thyroid gland (Fig.9). The epithelial cells were flattened leading to a significantly lower nuclear diameter value (p < 0.001, Table 1B, Fig.1B a). The thyrofollicular diameter of melatonin treated chicks was somewhat smaller compared to the control group (p<0.001, Table 1B, Fig.1B b). The epithelium height of thyroid
follicles in the melatonin chicks was reduced significantly compared to thyroid of control birds (p < 0.001, Table 1B, Fig. 1Bc). The D / N value was also less compared to those of control chicks (p < 0.01, Table 1B, Fig. 1Bd).

Analysis of variance (one-way) for thyroid nuclear diameter [F (1, 10) = 15.861, p < 0.001], thyrofolicular diameter [F (1, 10) = 14.935, p < 0.001], D / N [F (1, 10) = 7.715, p < 0.01] epithelial height [F (1, 10) = 35.315, p < 0.0001] of the thyroid gland from control and melatonin treated animals reveal significant changes of the experimental values compared to the control groups.

The plasma thyroxine level in chicks following melatonin treatment was found to be significantly reduced (1.33 μg/dl) when compared to control group of chicks injected with ethanol—normal saline control vehicle (p < 0.001 Table 1C, Fig. 1C).

One-way analysis of variance of thyroxin content in control and melatonin treated animals [F (1, 10) = 132.857, p < 0.001] reveal significant decrease of the mean thyroxin values in the melatonin treated groups compared with the control groups.

<table>
<thead>
<tr>
<th>Table 1 B. Thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Control [CON]</td>
</tr>
<tr>
<td>(N = 6)</td>
</tr>
<tr>
<td>Melatonin [MEL]</td>
</tr>
<tr>
<td>(N = 6)</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Figure 1B (a) - Histogram showing comparison between control and melatonin treated chicks as regard to thyroid nuclear diameter (µm) value. Melatonin caused a significant decrease in nuclear diameter (µm) value compared to control group. The vertical lines signify the SEM.

Figure 1B (b) - Histogram showing comparison between control and melatonin treated chicks as regard to thyroid follicular diameter (µm) value. Melatonin failed to produce any significant change in the same. The vertical lines signify the SEM.

Figure 1B (c) - Histogram showing comparison between control and melatonin treated chicks as regard to thyroid epithelium height (µm) value. Melatonin caused a significant decrease in epithelium height (µm) value compared to control group. The vertical lines signify the SEM.

Figure 1B (d) - Histogram showing comparison between control and melatonin treated chicks as regard to thyroid D/N (µm) value. Melatonin caused a significant decrease in D/N (µm) value compared to control group. The vertical lines signify the SEM.
**EXPERIMENTAL PROTOCOLS**

Group-B: - Manipulation of thyroid gland activity

**Phase - I: Administration of Thyroxin**

**Experiment I:**

*Control- Saline-vehicle treatment:* This included the control group of chicks. The chicks weighing between 40-45 gms (N = 6) were injected with saline vehicle (0.9 % NaCl) at a daily dose of 0.1 ml. vehicle per chick for seven consecutive days.

**Experiment II:**

*Administration of Thyroxin:* For this experiment the chicks (N = 6) were injected with thyroxin (Sigma – Aldrich) at a daily dose of 50 μg / 100 gm. body weight given in 0.1 ml vehicle per chick for seven consecutive days daily in the nape of the neck.
Phase - II: Administration of Methimazole

Experiment I:
Control: The chicks (N = 6) constituting the control group were supplied with chick mesh and normal tap water *ad libitum* for drinking daily for fifteen consecutive days. They were not subjected to any other treatment.

Experiment II:
*Methimazole treatment*: Comparable to the control group, the chicks (N = 6) were supplied with chick mesh and tap water in which methimazole powder was dissolved. For every 1 litre of tap water 1 gm. of methimazole powder was dissolved. This methimazole dissolved in water was supplied to the chicks instead of normal tap water. This treatment was given for fifteen consecutive days.

Phase - III: Administration of Methimazole + T4

Experiment I:
Control: The chicks (N = 6) constituting the control group were supplied with chick mesh and normal tap water *ad libitum* for drinking daily for fifteen consecutive days. They were not subjected to any other treatment.

Experiment II:
*Methimazole + T4 treatment*: The chicks (N = 6) were supplied with chick mesh and tap water in which methimazole powder was dissolved (For every 1 litre of tap water 1 gm. of methimazole powder was dissolved) and were injected with thyroxin (Sigma - Aldrich) at a daily dose of 50 µg / 100 gm. body weight given in 0.1 ml vehicle per chick for seven consecutive days daily in the nape of the neck.

After expiry of the experimental period, each set of control and treated animals were killed by etherisation, approximately 24 hrs. after the last injection or treatment of the drug.
In both the groups of control (N = 6) and treated (N = 6) chicks considered for histological studies. The excised pineal and thyroid glands were fixed in Bouin’s fluid and later processed for light microscopic studies as described in the General Methodology. From the same two groups serum was collected and stored at -20°C until thyroxin assay as described in general methodology.

OBSERVATIONS

A. Histological studies

Pineal Gland

*Control - Saline vehicle treatment:* The microscopic anatomy of the chicks pineal gland treated with saline vehicle was very much similar to that observed in chicks treated with ethanol - saline control vehicle. Thus nuclei of the pinealocytes were distinct and round with conspicuous nucleolus (Fig.3).

*Administration of T₄:* T₄ administration evoked significant changes of the pineal gland of chicks were observed (Fig.4). The pinealocytes were with large, well formed nuclei. In comparison with vehicular controls, the nuclear diameter was considerably larger (p < 0.01, Table 2A, Fig.2A). This indicated a significant hypertrophy of the pineal in the experimental groups.

The results from one - way analysis of variance for nuclear diameter [F (1,10) = 140, p < 0.01] of pinealocytes from control and T₄ treated groups revealed that the experimental mean values were significantly different, indicating a significant hypertrophy of the pineal gland in the experimental groups.
Control: The microscopic anatomy of the chick pineal gland was very much similar to that observed in chicks treated with ethanol – saline control vehicle. The pineal parenchyma composed of pinealocytes containing nucleus with conspicuous nucleolus (Fig.5).

Methimazole treatment: The pineal gland sections of chicks treated with methimazole indicate general inhibition (Fig.6). The pinealocyte nuclear diameters were much reduced (p < 0.001, Table 3A, Fig.3A) Compared to the control group of chicks. Consequently methimazole treatment caused a significant hypotrophy of the pineal gland.

Methimazole + T4 treatment. The pineal gland sections of chicks treated with methimazole + T4 indicate general stimulation (Fig.7). The pinealocyte nuclear diameters were significantly increased (p < 0.001, Table 3A, FigA.) Compared to the control group of chicks.
Analysis of variance (one-way) for mean values of pinealocyte nuclear diameter \([F(1, 10) = 18.171, p < 0.001]\) from control and methimazole treated animals reveal significant hypotrophy in mean values of the experimental groups compared to controls.

**Table 3 A. Pineal**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nuclear Diameter (μm)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CON)</td>
<td>4.33 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Methimazole (MET)</td>
<td>3.78 ± 0.07</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Methimazole+T4 (MET-T4)</td>
<td>4.65 ± 0.08</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

**Figure 3A** - Histogram showing pinealocyte nuclear diameter (μm) following methimazole, methimazole+T4 treatment as compared to control values. Methimazole caused a significant decrease and methimazole + T4 caused a significant increase in nuclear diameter value compared to control group. The vertical lines signify the SEM.
Thyroid Gland

Control Saline vehicle treatment: The thyroid histology of the chicks injected with saline vehicle was more or less identical to that described for animal injected with alcoholic saline control vehicle (Fig.10). The control thyroid gland reveals follicles consisting of spheres formed by simple epithelium whose follicular radius was seen to be normal. Follicles being regular in size and shape and lumina filled with homogenous colloid. Plasma T4 level was (2.32 µg/dl)

Administration of T4: T4 treatment induced significant alteration in the overall histology of the thyroid gland (Fig.11). The diameter of epithelial cells was significantly lower than the epithelial cells of the control (p < 0.001, Table 2B, Fig.2B (a)). Thyrofollicular diameter of treated chicks were significantly change (p<0.01, Table 2B, Fig.2B (b)). Significant decrease was seen in epithelium height of thyroid follicles in the treated chicks (p < 0.01, Table 2B, Fig.2B(c)). The D / N value was also significantly lower when compared to those of control chicks (p < 0.01, Table 2B, Fig.2B (d)).

Analysis of variance (one - way) for thyroid nuclear diameter [F (1,10) = 3.704, p < 0.01], thyroid follicular diameter[F (1, 10) = 5.753, p < 0.01],D / N [F (1, 10) = 7.531, p < 0.01] and epithelial height [F (1, 10) = 5.757, p < 0.01] of the thyroid gland from control and thyroxin treated animals reveal significant hypertrophy of the experimental mean values of thyroxin treated groups compared to the controls.

PlasmaT4 level (3.58 µg/dl) was significantly higher than the control group of chicks (p<0.001 Table2C, Fig.2C). One – way analysis of variance of thyroxin content in control and T4 treated animals [F (1, 10) = 32.743, p < 0.001] reveal significant increase of the mean values of the thyroxin treated groups compared with the control animals.
Table 2 B. Thyroid

<table>
<thead>
<tr>
<th></th>
<th>D/N (µm)</th>
<th>Nuclear Diameter (µm)</th>
<th>Epi Height (µm)</th>
<th>Follicle Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CON) (N = 6)</td>
<td>3.56 ± 0.06</td>
<td>4.24 ± 0.06</td>
<td>7.05 ± 0.37</td>
<td>28.97 ± 0.23</td>
</tr>
<tr>
<td>Thyroxin (T4) (N = 6)</td>
<td>2.74 ± 0.05</td>
<td>3.73 ± 0.03</td>
<td>5.86 ± 0.33</td>
<td>26.26 ± 1.10</td>
</tr>
</tbody>
</table>

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

Figure 2B (a) - Histogram showing comparison between control and thyroxin treated chicks as regard to thyroid nuclear diameter (µm) value. Thyroxin caused a significant decrease in nuclear diameter (µm) value compared to control group. The vertical lines signify the SEM.

Figure 2B (b) - Histogram showing comparison between control and thyroxin treated chicks as regard to thyroid follicular diameter (µm) value. Thyroxin caused a significant decrease in follicle diameter value compared to control group. The vertical lines signify the SEM.
Figure 2B (c) - Histogram showing comparison between control and thyroxin treated chicks as regard to epithelium height (μm) value. Thyroxin caused a significant decrease in epithelium height (μm) value compared to control group. The vertical lines signify the SEM.

Figure 2B (d) - Histogram showing comparison between control and thyroxin treated chicks as regard to D/N (μm) value. Thyroxin caused a significant decrease in D/N (μm) value compared to control group. The vertical lines signify the SEM.

**Table 2C.**

<table>
<thead>
<tr>
<th></th>
<th>T4 (μg / dl)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control [CON] (N = 5)</td>
<td>2.32 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Thyroxin [T4] (N = 5)</td>
<td>3.58 ± 0.19</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Figure 2C - Histogram comparing the effect of thyroxine treatment on serum thyroxin content (μg / dl) with respect to control value. Thyroxine caused significant increase in thyroxin content value as compared to the control group. The vertical lines signify the SEM.
Control: The thyroid histology of the chicks taken as control was more or less identical to that described for animal injected with alcoholic saline control vehicle (Fig. 12)

Methimazole treatment: Methimazole treatment induced a drastic alteration in the overall histological aspect of the thyroid gland. Majority of follicles were irregularly shaped and narrowed due to largely resorbed colloid. Epithelium often formed infoldings towards lumen. In fewer follicles luminal content remained (Fig. 13). The size of epithelial cells was significantly increased (p < 0.01, Table 3B, Fig. 3B (a)). The thyrofollicular diameter of methimazole treated chicks was significantly larger than the follicular diameter of the controls (p < 0.001, Table 3B, Fig. 3B (b)). The most important aspect was the greatly increased epithelium height so much that the lumina which was seen filled with homogenous colloid in the control was almost absolutely minimized or in most of the follicle not visible at all (p < 0.001, Table 3B, Fig. 3B (c)). The D / N value was also significantly increased compared to those of control chicks (p < 0.001, Table 3B, Fig. 3B (d)).

Analysis of variance (one - way) for thyrofollicular diameter [F (1,10) = 34.343, p < 0.001], thyroid nuclear diameter [F (1,10) = 6.634, p < 0.01], D / N [F (1, 10) = 32.954, p < 0.001] and epithelial height [F (1, 10) = 291.524, p < 0.001] of the thyroid gland from control and methimazole treated animals reveal significant blockage of the methimazole treated groups compared with control mean values.

PlasmaT4 level (0.75 pg/dl) was significantly lower when compared to control group of chicks injected with ethanol – normal saline control vehicle (p<0.001 Table 3C, Fig. 3C).

One - way analysis of variance of thyroxin content in control and methimazole treated animals [F (1, 10) = 75.379, p < 0.001] reveal significant decrease of the mean values when compared with the control animals.

Methimazole + T4 treatment: Methimazole + T4 treatment induced significant alteration in the overall histology of the thyroid gland (Fig. 14). The diameter of epithelial cells was
significantly lower than the epithelial cells of the control \((p < 0.05, \text{Table 3B, Fig.3B (a)})\). Thyrofollicular diameter of treated chicks were significantly change \((p<0.01, \text{Table 3B, Fig.3B (b)})\). Significant decrease was seen in epithelium height of thyroid follicles in the treated chicks \((p < 0.01, \text{Table 3B, Fig.3B(c)})\). The D / N value was also significantly lower when compared to those of control chicks \((p < 0.01, \text{Table 3B, Fig.3B (d)})\).

Analysis of variance (one - way) for thyroid nuclear diameter \([F (1,10) = 6.104, p < 0.01]\), thyroid follicular diameter\([F (1, 10) = 8.927, p < 0.01]\),D / N \([F (1, 10) = 4.684, p < 0.05]\) and epithelial height \([F (1, 10) = 9.890, p < 0.01]\) of the thyroid gland from control and thyroxin treated animals reveal significant hypertrophy of the experimental mean values of thyroxin treated groups compared to the controls.

PlasmaT4 level \((3.37 \mu g/dl)\) was significantly higher when compared to control group of chicks \((p<0.001 \text{ Table 3C, Fig.3C})\).

One – way analysis of variance of thyroxin content in control and methimazole + T4 treated animals \([F (1, 10) = 16.457, p < 0.001]\) reveal significant increase of the mean values when compared with the control animals.

<table>
<thead>
<tr>
<th></th>
<th>D/N</th>
<th>Nuclear Diameter</th>
<th>Epithelial Height</th>
<th>Follicle Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu m)</td>
<td>(\mu m)</td>
<td>(\mu m)</td>
<td>(\mu m)</td>
</tr>
<tr>
<td><strong>Control [CON]</strong> ((N = 6))</td>
<td>2.75 ± 0.13</td>
<td>4.12 ± 0.08</td>
<td>7.42 ± 0.39</td>
<td>26.38 ± 0.81</td>
</tr>
<tr>
<td><strong>Methimazole [MET]</strong> ((N = 6))</td>
<td>3.83 ± 0.12</td>
<td>4.55 ± 0.06</td>
<td>33.33 ± 1.46</td>
<td>35.84 ± 1.39</td>
</tr>
<tr>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
</tr>
<tr>
<td><strong>Methimazole + T4 [MET+T4]</strong> ((N = 6))</td>
<td>2.40 ± 0.06</td>
<td>4.35 ± 0.05</td>
<td>6.15± 0.40</td>
<td>28.83 ± 0.72</td>
</tr>
<tr>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.05)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.01)</td>
<td>(p &lt; 0.01)</td>
</tr>
</tbody>
</table>

Table 3 B. Thyroid
Figure 3 B(a) - Histogram showing comparison between control and methimazole, methimazole+T4 treated chicks as regard to thyroid nuclear diameter (μm) value. Methimazole caused a significant increase and methimazole+T4 caused a significant decrease in nuclear diameter (μm) value compared to control group. The vertical lines signify the SEM.

Figure 3 B(b) - Histogram showing comparison between control methimazole methimazole+T4 treated chicks as regard to thyroid follicular diameter (μm) value. Methimazole caused a significant increase and methimazole+T4 caused a significant decrease in follicular diameter (μm) value compared to control group. The vertical lines signify the SEM.
Figure 3B(c) - Histogram showing comparison between control and methimazole and methimazole+T4 treated chicks as regard to thyroid epithelium height (µm) value. Methimazole caused a significant increase and methimazole+T4 caused a significant decrease in epithelium height (µm) value compared to control group. The vertical lines signify the SEM.

Figure 3B (d) - Histogram showing comparison between control methimazole and methimazole+T4 treated chicks as regard to thyroid D/N (µm) value. Methimazole caused a significant increase and methimazole+T4 caused a significant decrease in D/N (µm) value compared to control group. The vertical lines signify the SEM.
Table 3C.

<table>
<thead>
<tr>
<th>Condition</th>
<th>T4 (µg / dl)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CON) (N=5)</td>
<td>2.32 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Methimazole (MET) (N=5)</td>
<td>0.75 ± 0.15</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Methimazole+T4 (MET+T4) (N=5)</td>
<td>3.37 ± 0.23</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

Figure 3C - Histogram comparing the effect of methimazole and methimazole+T4 treatment on serum thyroxin content (µg / dl.) with respect to control value. Methimazole caused significant decrease and methimazole+T4 caused significant increase in thyroxin content value as compared to the control group. The vertical lines signify the SEM.
EXPLANATION OF PHOTOMICROGRAPHS –

Figure 1 - Microphotograph of pineal gland section from control chick showing normal nuclear diameter.

Figure 2 - Microphotograph of pineal gland section in melatonin treated neonatal chick showing hypertrophied nuclei.
EXPLANATION OF PHOTOMICROGRAPHS –

Figure 3 - Microphotograph of pineal gland section from control chick showing normal nuclear diameter.

Figure 4 - Microphotograph of pineal gland section in thyroxin treated neonatal chicks. Note the increase in nuclear diameter.
EXPLANATION OF PHOTOMICROGRAPHS -

Figure 5 - Microphotograph of pineal gland section from control chick showing normal nuclear diameter.

Figure 6 - Microphotograph of pineal gland section in methimazole treated neonatal chicks. Note the decrease in nuclear diameter.

Figure 7 - Microphotograph of pineal gland section in methimazole and thyroxine treated neonatal chicks. Note the increase in nuclear diameter.
EXPLANATION OF PHOTOMICROGRAPHS –

Figure 8 - Microphotograph of thyroid gland section from control neonatal chicks showing normal nuclear diameter, epithelium height and follicular diameter.

Figure 9 - Microphotograph of thyroid gland section from melatonin treated neonatal chick showing significantly decreased nuclear diameter and decreased epithelium height.
EXPLANATION OF PHOTOMICROGRAPHS –

Figure 10 - Microphotograph of thyroid gland section from control neonatal chicks showing normal nuclear diameter, epithelium height and follicular diameter.

Figure 11 - Microphotograph of thyroid gland section from thyroxin treated neonatal chick showing significantly decreased nuclear diameter and decreased epithelium height.
EXPLANATION OF PHOTOMICROGRAPHS –

Figure 12 - Microphotograph of thyroid gland section from control neonatal chicks showing normal nuclear diameter, epithelium height and follicular diameter.

Figure 13 - Microphotograph of thyroid gland section from methimazole treated neonatal chick showing blocked thyrofollicular activity.

Figure 14 - Microphotograph of thyroid gland section from methimazole and thyroxine treated neonatal chick showing increased nuclear diameter and reduced epithelium height.
Discussion

The present investigation offers an insight into the morphological and functional organization of the pineal and thyroid in response to differential modulation of both the glands through administration of pineal indoleamine: melatonin, thyroxin and thyroid blocker- methimazole. This study further gives emphasis on the cytophysiological behavior of the glands as reflected in concomitant alterations in the karyomorphological values in both the glands and their possible interrelationship. In addition, estimation of thyroxin content following experimental modulation of both the glands was also performed.

It may be noted that present studies related to the cytological features included measurement of the nuclear diameter, an indicator of the glandular activity (Oehler and Schultze, 1960; Citoler et al., 1965; Edwards and Gray, 1970; Reiter, 1977). The alteration in the nuclear size influences the synthetic and secretory activity of both pineal gland and thyroid in mammals and birds. The nuclear size responds to various physiologically induced changes and reflects glandular activity in several mammalian and avian species. An active phase is characterized by increased pinealocyte nuclear size indicating stimulation of synthesis activity, whereas an inhibitory phase is characterized by decreased nuclear size suggestive of inhibition of gland cells (Quay, 1965, 1976; Chakraborty and Maiti, 1981; Chakraborty et al., 1981; Chakraborty and Maitra, 1982; Diehl et al., 1984; Sahu and Chakraborty, 1983, 1986; Hira et al., 1989; Peschke et al., 1989; Martinez Soriano et al., 1990; Chakraborty et al., 1992,1994; Chakraborty, 1981, 1993,1994; Chakraborty and Sarkar, 1994; Ganguli et al., 1998; Sinha et al., 2009; Sinha and Chakraborty,2010).

The current experimental observations reveal that the pineal gland reacts to the indoleamine input by becoming significantly hyperactive, in so far as the synthetic phase of the pineal parenchymal cells are concerned. Melatonin administration in neonatal
chicks induce a significant increase in the pinealocyte nuclear diameter with further augmentation of overall picture of the pineal parenchyma as compared to control group of chicks (Table 1A, Fig.1A, Fig.2). Also in support is a previous finding showing that melatonin increases the ability of pineal homogenates to bind to colchicines, used to arrest cell division at metaphase stage (Freire and Cardinali, 1975; Reiter, 1978). Thus this quantitative nuclear morphology indicates that in this species, melatonin has a hormonal action on the pineal gland, influencing its metabolism. In fact, the present cytological observations corroborate with earlier biochemical findings in rats where it was observed that melatonin administration caused alteration in the lipid content of the pineal gland (Ebels and Prop, 1965) and also elevated HIOMT and S-NAT activity, required for melatonin synthesis by the pineal gland (Freire and Cardinali, 1975; Benson, 1977).

Our current studies lend support to the contention that in addition to alterations in pineal biochemistry (Wright and Preslock, 1975; Preslock, 1976) and pineal induced physiological activities (Turek et al., 1976a, b), cellular morphology of pineal (Freire and Cardinali, 1975; Chakraborty, 1994; Bandyapadhyay et al., 2011) also seems to serve as a target organ for exogenously administered indoleamine. In addition, the result from a later study indicated an acute regulatory action of exogenous melatonin on the pineal melatonin synthesis pathway (Miguez and Aldegunde, 1996).

Contrary to the current experimental results, reports are present indicating that exogenous melatonin either prevents normal diurnal serotonin rhythm in rats (Fiske and Huppert, 1968; Minneman and Wurtman, 1975). It also fails to influence melatonin rhythm in the rat pineal gland (Lang et al., 1983). Additionally melatonin administration exerts no acute effect on the endogenous, circadian melatonin profile, independent of dosage and sex (Niklowitz, et al., 1996).

However, these publications are in conflict with reports about the stimulatory action of the indoleamine on pinealocyte morphology (Chakraborty, 1994; Bandyapadhyay et al., 2011), pineal ultrastructural components (Przybylska et al., 1994) and serotonin secretion.
(Miguez and Aldegunde, 1996) that confirm our foregoing observations, revealing melatonin to have a stimulatory action on the pineal gland activity. Additionally, in view of the present findings, it is indicated that the pineal gland in mammals may itself be a target organ for exogenously administered melatonin (Ralph, 1978) and the changes brought about in this species may be a direct action of exogenously administered hormonal impact on the pineal gland.

In fact cytological observation indicating the ability of exogenous melatonin to stimulate pinealocyte morphology and function corroborates with findings by several group of researchers. The fact that pineal gland possesses melatonin receptors (Angeloni and Fraschini, 2006) confirms the ability of the pinealocytes to respond to melatonin stimulation as evidenced from the current study. Additionally a number of reports substantiate the present finding, in as much as melatonin’s ability to stimulate pineal morphological and ultrastructural features (Chakraborty, 1994; Bandyapadhyay et al., 2011; Vollrath et al. 1985; Benson and Krasovich, 1977; Przybylska et al. 1994; Redins et al. 2001) and enhance the biochemical and hormonal activity of the pineal gland (Freire and Cardinali, 1975; Benson 1977; Halder et al. 1983 a, b).

An in-depth study of published literatures indicated that melatonin besides influencing the pineal activity, may also regulate thyroid function (Singh and Prasad, 1981; Lewinski et al., 1986; cf Chakraborty et al., 1992). Contradiction exists regarding the influence of the pineal gland and melatonin on thyroid activity, ranging from no effect (Rowe et al., 1970) to inhibition (Narang et al., 1967; De Prosopo et al., 1969; De Fronzo and Roth, 1972; Relkin, 1972) or even significant stimulation of thyroxine production by thyroid in mammals (Orosz et al., 1964; Panda and Turner, 1968; De Fronzo and Roth, 1972; Nir et al., 1978).

The present experimental results show that exogenous melatonin treatment appears to have an inhibitory effect on the thyoidal activity in neonatal chicks. This is characterized by a significant decrease in the epithelial nuclear size in melatonin treated chicks supported by the significant decrease in epithelial height and follicular diameter. With the
decrease in size of epithelial nuclei there was an increase in their number all around the epithelium lining the follicular lumen (Table 1B, Fig.1c-f, Fig.8). Hence a decrease in the ratio of follicular diameter to the number of nuclei present in these follicles corroborated suppression of thyrofollicular activity induced by treatment of melatonin in birds (Chakraborty et al., 1992).

The biochemical data of the present experimentation further substantiate the histological observations indicating that melatonin administration significantly depresses the plasma thyroxin content in the neonatal chicks (Table 1C, Fig.1C).

These findings are supported by studies, which point to a modulatory depressant effect of the pineal on this gland. Enhancing pineal activity by exposing animals to continuous darkness (De Prospo et al., 1969; Relkin, 1972) or injecting melatonin (Narang et al., 1967; De Fronzo and Roth, 1972) brought about a generalized depression of thyroid function, whereas abolition of possible pineal influence by its removal (Csaba and Nagy, 1973; Csaba and Barath, 1974), or inhibition of pineal activity by exposing the animal to continuous light (Singh and Turner, 1969; Relkin, 1972) or blocking indoleamine synthesis by p-chlorophenylalanine (De Prospo and Melgar, 1975), was followed by increased thyroid activity.

It was unequivocally shown that the thyroid weight increased following pinealectomy (De Fronzo and Roth, 1972; Peschke et al, 1988) and decreased after administration of pineal substance (Naber et al, 1969). These in vivo results point to an inhibitory effect on the pituitary – thyroid axis by the pineal through increased pineal indoleamine.

The observations of Puschett and Goldberg (1968) on thyroid abnormalities and the earlier results of De Luca et al. (1961), Scepovic (1963) suggest that the pineal exerts an inhibitory effect over thyroid function and that the active principle may be melatonin. Results of experiments show a significant increase in thyroid weight following pinealectomy. These results substantiate those of Baschieri et al. (1963) and suggest the existence of an inhibitor over the pituitary-thyroid axis produced by the pineal gland. The
decrease in thyroid weight seen in pinealectomized-ovariectomized animals receiving melatonin suggests that the inhibitor may be melatonin.

Experiments with rats and hamsters have provided evidence for an inhibitory action of the pineal gland on the neuroendocrine-thyroid axis. While maintenance of these animals in short photoperiod results in reduced levels of circulating thyroxin (T₄), pinealectomy restores the level to normal. Studies suggest that an active pineal gland produces a substance, which inhibits thyrotrophin releasing hormone release from the hypothalamus. Several investigators have concluded that endogenous melatonin, produced in the pineal gland, could account for the inhibitory action of the pineal gland on blood T₄ levels. The effect of pinealectomy and exogenous melatonin on circadian rhythm of triiodothyronine (T₃), thyroxin (T₄), in sham operated and pinealectomized rats were investigated. The findings suggest that pinealectomy disturbs the circadian rhythm of T₃ and T₄. Exogenous melatonin has the suppressive effect of diurnal secretion of T₃, T₄ in pinealectomized rats (Zwirska-Korezala et al., 1991). In female Syrian hamsters, daily afternoon injections lead to a loss of vaginal cyclicity and reduced gonadotrophin and thyroid hormone levels (Stetson and Hamilton, 1981; Vriend et al., 1982).

The influence of a single dose of melatonin administration to pinealectomized and sham operated rats at different times of day on serum T₃, T₄ concentrations was investigated. It was found that such melatonin influence depends upon a time of day. Both pinealectomy and exogenous melatonin did not influence the thyroid activity in the morning. The most remarkable pineal effect on the thyroid appeared during the night (Kniazevski et al., 1990).

Our results also corroborate the reports of others where daily injections of melatonin in microgram amounts for several weeks are associated with a depression of plasma thyroxin levels (Vriend and Reiter, 1977). Additional reports in support of our results (Vriend et al., 1982; Vaughan et al., 1982, 1984), which indicated that serum T₄ levels are significantly reduced by melatonin administration. Vriend et al. (1982) have reported a depression in serum T₄, T₃ and TSH levels in female hamsters following melatonin
injections for 8 weeks. It was found that serum T₄ levels were lowered by melatonin injections while T₃ levels fluctuated with the estrous cycle unaffected by the administration of melatonin. The result of the study indicated that when melatonin is given late in the light phase of the photoperiod, it acts to reduce thyroxin levels. The study thus demonstrated that the day of the cycle and the time of the day needed to be taken into consideration in studies of the effects of melatonin on the thyroid axis in female hamsters (Petterborg and Rudeen, 1989).

Apparently, it may be noted that the antithyroid principle of the pineal gland is dependant on both the day-time (Vaughan et al., 1984) and the dose of melatonin application (Vriend et al., 1982). In addition a short-lived inhibitory influence of the pineal on TSH secretion was found (Relkin, 1972). In view of these results melatonin is believed to produce an antithyroid and a counter-antithyroid action (Vriend et al., 1982; Vriend and Gibbs, 1984).

However, in contrast to our findings a few earlier studies reported thyroid hyperactivity as evidenced by increased thyroid weight and histological changes, increased blood TSH levels produced by melatonin (Panda and Turner, 1968) and increased serum thyroxin (Orosz et al., 1964) levels in rats exposed to continuous darkness.

Along the same lines were the findings of increased thyroid glandular activity, as evidenced by an elevated ratio of RNA to DNA following melatonin application (De Fronzo and Roth, 1972). Continuous darkness, which stimulates pineal activity, also brought about an increase in thyroid cAMP, supporting the finding of a stimulatory effect of the indolic compounds on thyroid hormone release (Nir et al., 1978).

It could be that the pineal is exerting a dual action on the thyroid, one via the hypothalamic – pituitary axis (inhibitory) and occasionally under extreme environmental conditions directly (stimulatory).
Hence from a close consideration of earlier reports in mammals compared with the currently obtained experimental results regarding the influence of melatonin on pineal and thyroid, it is summarized that the effect of melatonin under similar experimental conditions are that of stimulation of the pineal and inhibition of thyroid as evident from both histological and biochemical evaluations.

Also evidences elucidate that thyroid physiology has a regulating effect on the pineal synthetic and secretory activity. The present study support the idea that pineal cellular morphology can be influenced by thyroid modulations which can be performed either by administration of thyroxin, thyroidectomy or using thyroid blocker – methimazole.

The present experiments demonstrate that thyroid and its principle hormone thyroxin had a stimulatory effect on the pineal thus enhancing the pineal activity. Administration of thyroxin in neonatal chicks augmented the nuclear size of pinealocytes when compared with the data obtained from the control group of chicks (Table 2A, Fig.2A, and Fig.4). Furthermore it was observed that administration of thyroid blocker -- methimazole significantly depressed pineal activity (Table 3A, Fig.3A, and Fig.6). Whereas administration of thyroid blocker -- methimazole followed by administration of thyroxin increased the nuclear diameter of pinealocytes (Table3A, Fig.3A, Fig.7).

The biochemical data of the present set of experimentation show increased levels of thyroxine in the serum of chicks treated with thyroxin (Table 2A, Fig. 2A) and chicks that were administered with methimazole and thyroxin show increased levels of thyroxine in the serum and significantly low levels of thyroxin was obtained in serum of those chicks which were supplied with drinking water in which methimazole was dissolved (Table 3C, Fig.3C).

In support of the above experiments earlier studies demonstrate that $T_4$ affects the cells of the pineal, mainly by enhancing the pineal activity. Observations showed that $T_4$ augmented the nuclear size of pinealocytes while thyroidectomy or thiouracil – (a thyroid blocker) application diminished the nuclear size (Peschke, 1981). Recent study on rats
have shown that thyroxine administration evoked hyperactive changes in pineal gland cytomorphology along with enhanced serum $T_4$, as evidenced from increased pinealocyte nuclear diameter ($\mu$m) and decreased nuclear density and enhanced serum $T_4$ level ($\mu$g/dl). Contrarily, thyroidectomized ($T_x$) rats with undetectable $T_4$ levels showed pineal inhibition, as seen from significantly decreased pinealocyte nuclear diameter ($\mu$m) values and an increased nuclear density per microscopic field. However thyroidectomized animals, supplemented with thyroxine ($T_4 + T_x$), induced pineal activation as seen from increased pinealocyte nuclear diameter, associated with increased serum $T_4$ level. The present study argues for a direct pineal-thyroid relationship as interpreted from cytomorphological level and hormone profiles in male albino rats (Sinha and Chakraborty, 2010). Furthermore $T_4$ stimulated histochemically demonstrated enzymes of pinealocytes in vitro (Milcoul et al., 1968) and increased pineal activity (Champney et al., 1985). These results and the fact that $T_4$ showed an excitatory action of a part of pineal cells (Semm et al., 1981a).

Alterations in diurnal pineal indoleamine metabolism by thyroid hormones have only received a limited amount of study. Thyroxin increased hydroxyindole-O-methyl transferase (HIOMT) activity two fold in chick pineal cell culture (Mezei and Wainwright, 1979). $T_4$ also increased serotonin (5HT) metabolism (Csaba and Bernard, 1973) and melatonin production (Nir and Hirschman, 1978) in rat P cell cultures. In vitro application of $T_3$ increased 5HT, N-acetyl serotonin (NAS) and melatonin content while TSH had no effect on indole production (Nir and Hirschman, 1978). In an in vivo study, $T_4$ was found to increase maturation of the NAT activity rhythm in neonatal rats (Yuwiler and Brammer, 1981).

Dillman and Cady (1971) found that thyroxin was preferentially taken up by bovine pinealocytes. Mezei and Wainwright (1979) observed a two fold increase in HIOMT activity after addition of thyroxin to chick pineal culture preparation. Csaba and Bernard (1973) reported increased serotonin metabolism when thyroxin was added to cultured rat pineal glands and thyroxin increased cell excitability in guinea pig pineal cells (Semm et al., 1981a,b).
As compatible with our observation that thyroidectomy induced an overall depressant effect on pineal cytomorphology, thyroidectomy was found to lower nocturnal levels of rat pineal melatonin content when compared to control animals (Johnson, 1982; Reiter et al., 1982; Vriend, 1983). However some contradictory results were also observed where it was reported that thyroidectomy had no effect on pineal ultrastructure (Karasek, 1981a,b, Karasek and Stephen, 1981).

It appears that a direct relationship between the pineal gland and the thyroid may exist as evidenced from both morphological and hormonal estimates in chicks. Additionally in the present study exogenously administered melatonin caused an inhibition of thyroid gland activity as evidenced from both morphometric and hormonal analysis. However, conversely a more precise approach concerning pineal gland responsiveness in hypothyroid and hyperthyroidic animals indicates that hypothyroidic animals show inhibited pinealocyte activity and on the reverse hyperthyroidic condition causes stimulation of the pineal gland. Thus conclusively it appears that there exists a direct relationship between pineal and thyroid gland. Logistically melatonin administration in the present instance should have inadvertently been thyroid stimulatory but our results, on the contrary, indicate inhibition of the thyroid gland activity. Such a result could be because of the dosage, time of administration and duration of melatonin administration adversely affecting the thyroid gland activity. Although earlier reports do affirm our finding. It was previously shown that serum T4 levels are significantly reduced by melatonin administration (Stetson and Hamilton, 1981; Vriend et al., 1982; Vaughan et al., 1982, 1984; Zwirska Korezala et al., 1991).