



ORIGINAL ARTICLE

IMPACT OF PROPYLTHIOURACIL-INDUCED HYPOTHYROIDISM DURING PREGNANCY ON POST-NATAL LOCOMOTOR DEVELOPMENT IN RAT PUPS

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Background: The foetus depends on maternal thyroid hormones for neurogenesis during early days of pregnancy. The objective of this study was to determine the impact of maternal hypothyroidism on cortical lamination, resulting in impaired locomotion in rat pups. **Methods:** This study was conducted in the Anatomy Department of Islamic International Medical College over 9 weeks. Twenty-four female and 12 male Sprague-Dawley rats were divided into two equal groups. Control group received a standard diet and drinking water, while the hypothyroid group was given 4.5 mg propylthiouracil/rat/day via drinking water. Treatment began one week before mating and continued for three weeks after delivery. Pups were born after three weeks and nursed freely for 21 days. On day 22, locomotor testing was performed before dissection. Brains were extracted, and motor cortex was examined. Data from inclined plane test and microglial counts were analysed with *t*-test, while Chi-square test was applied for forelimb placing and cortical lamination assessment. **Results:** The control group exhibited typical locomotion, as evidenced by their performance in locomotor tests, which reflected a healthy pattern of neurogenesis. In contrast, the hypothyroid groups demonstrated a significant decline in their ability to stay on an inclined plane and a delayed response in forelimb placement, indicating atypical neurogenesis evidenced by an elevated number of microglia and disrupted cortical lamination. **Conclusion:** Experimentally induced maternal hypothyroidism in Sprague Dawley rats had a detrimental impact on the locomotion of pups associated with abnormal neurogenesis.

Keywords: Antenatal care, Impaired locomotion, Maternal hypothyroidism, Motor cortex, Neurogenesis

Pak J Physiol 2026;22(2):77–82, DOI: <https://doi.org/10.69656/pjp.v22i2.1895>
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INTRODUCTION

Maternal hypothyroidism is characterized by elevated levels of Thyroid Stimulating Hormone (TSH) in the bloodstream during pregnancy, affecting the development of the foetus.¹ During human development, the activation of foetal thyroid hormones typically takes place between the 10th and 16th week of gestation, which closely coincides with the second trimester of pregnancy. However, it is vital to recognize that the primary phase of neurogenesis occurs predominantly in the first trimester. Similarly, in rats, the activation of thyroid hormone typically begins after the 18th day of embryonic development, aligning with the critical period for normal brain development, which spans from the 12th to 18th embryonic days. Consequently, during this early developmental period, the foetus depends on maternal thyroid hormones for neurogenesis. Their impact extends beyond influencing neurons' proliferation, migration, and differentiation, along with myelination and is of vital importance.^{2,3}

The neocortex, a prominent cortical region, orchestrates the planning, initiation, and execution of voluntary movements in rodents. It also serves as the hub for receiving input from diverse brain regions and seamlessly integrating sensory data with motor

directives. Rats can acquire and enhance motor skills through repetitive training, with the neocortex assuming a pivotal role in storing and adjusting motor representations.⁴

Propylthiouracil (PTU) is classified as an anti-thyroid agent, hindering the transformation of inactive thyroid hormones into their active forms, thereby influencing the retention of thyroid hormones in the thyroid gland.⁵ It is used for the induction of maternal hypothyroidism in animal models, including rats.⁶

Maternal thyroid hormone deficiency affects the expression of approximately 500–1000 genes directly or indirectly and impairs the antioxidant defence system.^{7–9} This leads to oxidative stress and potential gene mutations, which significantly disrupt the laminar architecture of the cerebral cortex and cerebellum, ultimately provoking a microglial response.¹⁰ Additionally, thyroid hormone deficiency alters neuronal progenitor cell division dynamics, causing delayed symmetrical divisions and a reduced progenitor pool, factors that may contribute to behavioural deficits in the offspring.²

Numerous rat studies have demonstrated the impact of maternal hypothyroidism on cognitive functions and coordinated motor skills. However, most

of these studies have primarily focused on biochemical parameters and gene expressions, with limited research on histological parameters.^{3,11}

This study aims to evaluate how maternal hypothyroidism impacts the locomotor activity associated with abnormal neurodevelopment within the motor cortex of rat pups. This research proposes the implementation of thorough and structured screening initiatives aimed at ensuring optimal foetal brain development. Furthermore, it underscores the significance of incorporating serum TSH biochemical testing as a valuable indicator for detecting maternal hypothyroidism during prenatal checkups.

METHODOLOGY

This controlled interventional study, conducted in collaboration with the National Institute of Health and the Anatomy Department of the Islamic International Medical College, was executed for 9 weeks after obtaining approval for the study synopsis from the Ethics Review Committee, Reference No. Riphah/IRC/22/2087. A total of twenty-four female and twelve male Sprague Dawley rats were included in the study, with an age range of 12 to 16 weeks and weighing between 250 to 300 grams, selected by simple convenience sampling. The sample size was adequate according to the Resource Equation Method.¹² Animals were housed under carefully regulated environmental conditions. These conditions included maintaining a constant temperature of 22 ± 0.5 °C, a 12-hour light and dark cycle, and a humidity level of 50%.¹³

Twenty-four female and twelve male Sprague Dawley rats, aged 12 to 16 weeks and weighing between 250 and 300 grams, along with 12 of their offspring who were full-term rat pups (3 weeks) of both sexes without congenital abnormalities, were included in this study. Rats less than 12 weeks of age, >250 grams or <300 grams, along with pups born prematurely and pups born with visible congenital defects, were excluded.

The rats were divided into two distinct groups, with their dietary and water regimens beginning one week before mating and continuing consistently throughout gestation, weaning, and up to the 22nd day postpartum.¹⁴

Control group rats were provided with a standard chow diet and given plain drinking water through the oral route, and Experimental group rats were subjected to a daily oral administration of 4.5 mg of propylthiouracil/rat¹³, administered through drinking water. This treatment protocol began one week before mating and was maintained throughout gestation and the weaning period, continuing until the 22nd day postpartum.¹⁴

The study involved assessing the serum TSH levels in female rats before mating to ensure they didn't have natural hypothyroidism. Mating was conducted in

12 cages, with 3 cages serving as controls and 9 as experimental. Each cage contained 2 females and 1 male rat, and pregnancy was confirmed on the first day of gestation by observing a vaginal plug.¹⁴ On the 10th day of gestation, maternal serum TSH levels were reassessed to evaluate thyroid function, with values ranging from 25 ng/mL to 41 ng/mL confirming a sustained hypothyroid state (Table-1).

The pups were born at the end of pregnancy, and they were left with their mothers in the same cages to have unrestricted access to mother's milk for the next three weeks.¹⁵ At the end of three weeks after birth, the locomotor activity of 12 pups from each group was assessed.

Table-1: Serum TSH levels in the females of the hypothyroid group at the 10th day of gestation

Rat #	Serum TSH ng/ml	Rat #	Serum TSH ng/ml
1	25	7	32
2	35	8	51
3	36	9	38
4	65	10	39
5	45	11	56
6	35	12	41

Each pup's movement was assessed individually by placing it on an inclined plane. A unique inclined plane model was designed for this purpose, and the angle of descent of each rat pup was measured (Figure-1A). Between each measurement session, there was a one-hour gap. To ensure accuracy, the average of two readings were computed for each rat, providing a representative measure of their locomotion.¹⁶

The primary goal of the forelimb placing reaction test is to assess the motor function of the animal, particularly its ability to respond to sensory stimuli by accurately placing its forelimbs on a flat surface. Each pup's forelimb placing response was observed and evaluated for one minute¹⁷ (Figure-1B).

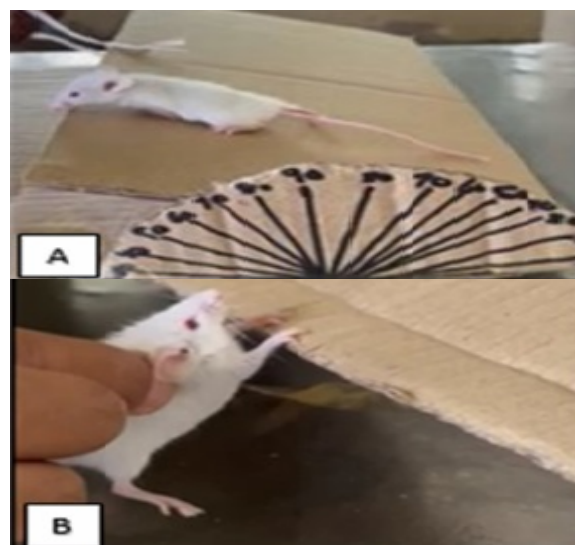


Figure-1: (A) The inclined plane test, (B) The forelimb placing reaction

This assessment employed a four-point scoring system as: 0= No observable reaction, 1= Weak and uncoordinated lifting of limbs without contacting the horizontal surface, 2= Delayed placement of the forelimb on the horizontal surface, and 3= Fast and accurate placement of both forelimbs on the horizontal surface.¹⁷

On the 22nd postpartum day, the same pups from each group were anesthetized and sacrificed. Subsequently, the skulls of each animal were split open, allowing access to the cerebrum, which was then carefully dissected. A portion of the specimens was preserved in neutral buffered formalin (10%) for 24 hours, followed by a process of dehydration using increasing concentrations of alcohol (50%, 70%, 80%, 90%, and 100%). These specimens were then subjected to clearing and embedding in paraffin wax. Afterward, 5-micron-thick tissue sections were obtained, and these sections were stained with H&E.¹⁸

High-resolution images of the slides were obtained using an Olympus BX53 microscope at magnifications of 4×, 10×, and 40×. Images captured were then transferred to a laptop for further analysis, utilizing Image J software. Parameters (Microglial count and differentiation of cortical layers) at the microscopic level were methodically evaluated in sections derived from both cerebral hemispheres. This assessment covered four distinct regions in each hemisphere, beginning at the midline and extending towards the right and left sides.

Microglial count was performed at 40× magnification to evaluate the cortical inflammatory response, which is associated with disrupted neurogenesis, impaired myelination, and altered locomotor activity. Neuronal migration is essential for forming the layered structure of the cerebral cortex during normal brain development. However, some neurons may fail to migrate correctly, forming ectopic clusters within the cortex.¹⁹ Arrangement of cortical neurons was observed at 10× magnification and graded using the following scale.²⁰

Grade 0, No Differentiation: None of the six cortical layers are preserved in any of the 4 HPF (high-power fields) per rat.

Grade 1, Minimal Differentiation: All six layers are preserved in 1 out of 4 HPF/rat.

Grade 2, Mild Differentiation: All six layers are preserved in 2 out of 4 HPF/rat.

Grade 3, Moderate Differentiation: All six layers are preserved in 3 out of 4 HPF/rat.

Grade 4, Well Differentiated: All six cortical layers are preserved in all 4 HPF/rat.

The data obtained from the calibrated images were recorded and analysed using SPSS-26. The Kolmogorov-Smirnov test was applied to assess the normality of the data. A significant p-value showed

normal distribution of data. Independent samples *t*-test was applied to assess potential differences in means for inclined plane test and microglial count (quantitative variables), with the results expressed as Mean±SD. For comparing group differences in forelimb placing reaction and lamination of cortical layers (qualitative variable), the Chi-square test was used. A significance level of $p<0.05$ was set by standard statistical practice.

RESULTS

The locomotive capabilities of the rat pups were evaluated based on their tendency to fall from an inclined plane. The outcomes demonstrated a notably strong statistical significance, ($p<0.001$). (Table-2).

In the forelimb placement reaction, the control group exhibited a quicker response than the hypothyroid group. In the control group, 83.3% of the rats showed a prompt forelimb placing reaction, while 16.7% exhibited a delayed response. In contrast, among the hypothyroid group, 66.7% showed no response, 25% displayed a weak reaction, and 8.3% had a delayed response. The Chi-square analysis revealed a significant disparity between the two groups (Table-3).

The hypothyroid group displayed microglial cells with oval-shaped nuclei (Black arrows in Figure-2), indicative of an inflammatory response, whereas the control group showed no microglial cells. There was a high statistical significance ($p<0.001$), indicating a substantial differences between the two groups (Table-4).

Table-2: Mean degree of fall, *t*-test of pups by inclined plane test

Groups	Mean Degree of Fall±SD
Control	41.66±6.06*
Hypothyroid	31.66±3.58*

* $p<0.001$

Table-3: Chi-square analysis of pups in forelimb placing reaction [n (%)]

Forelimb Placing Reaction	Control	Hypothyroid
Prompt Response	10 (83.3)	0 (0.0)
Delayed Response	2 (16.7)	1 (8.3)
Weak Response	0 (0.0)	3 (25.0)
No Response	0 (0.0)	8 (66.7)

Table-4: Mean microglial count in both groups *t*-test

Groups	Mean Microglial Count (cells/hpf)±SD
Control	0±0.0
Hypothyroid	6.63±0.52

* $p<0.001$

In the control group, 100% of the rats demonstrated good differentiation across all 6 cortical layers, with no observed clusters of ectopic cells (Black circles in Figure 2). In contrast, within the hypothyroid group, 8.3% of the rats exhibited minimal differentiation (Grade 1) across all six cortical layers, 50% showed mild differentiation (Grade 2), and 41.7% demonstrated

moderate differentiation (Grade 3). The statistical analysis, conducted using the chi-square test, highlighted an extremely significant difference ($p < 0.001$) between the two groups concerning the severity of differentiation. (Table 5).

Table-5: Percentage of rats with different grades of differentiation of cortical layers [n (%)]

Severity of Cortical Layer Differentiation	Control	Hypothyroid
Grade 1 (Minimal Differentiation)	0 (0.0)	1 (8.3)
Grade 2 (Mild Differentiation)	0 (0.0)	6 (50.0)
Grade 3 (Moderate Differentiation)	0 (0.0)	5 (41.7)
Good Differentiation (All 6 Cortical Layers)	12 (100)	0 (0.0)

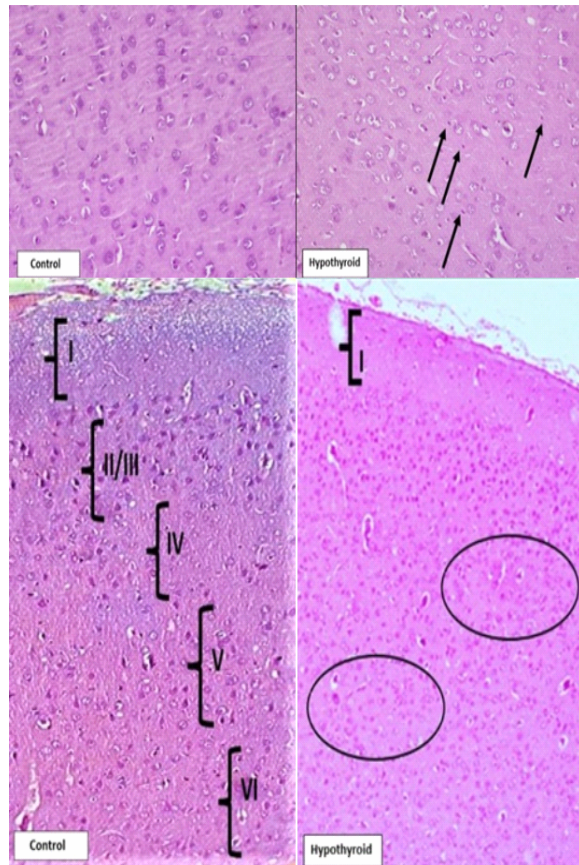


Figure-2: Microglia, represented by black arrows at 400× and ectopic cell clusters, represented by black circles at 400× in hypothyroid groups while Control groups showed no microglia with normal cortical lamination (I-VI)

DISCUSSION

Maternal thyroid hormones play a crucial role in foetal neurogenesis, and their significance is particularly pronounced during pregnancy. This heightened importance arises from the increased demands placed on the maternal thyroid gland due to physiological changes in the mother's body. Of particular note is the fact that, up to the 12th week of gestation, the foetus relies entirely on the maternal provision of thyroid hormones²¹. The

findings of this study align with those reported in previous research.

In this study, the offspring of hypothyroid rat mothers showed impaired motor coordination and balance, struggling to maintain posture and adjust body position on an inclined plane. Their task performance lacked the precision and stability observed in pups. These motor deficits are likely linked to reduced expression of key proteins involved in neuronal maintenance, migration, and plasticity. Similar alterations in motor skills were reported by Barez-Lopez S *et al*²² and Khairinisa MA *et al*²³.

Forelimb placing reactions were assessed using a four-point scoring system to evaluate motor activity in rats. The control group showed quick and strong responses, whereas the hypothyroid group exhibited delayed and weakened reactions. These differences are likely due to maternal hypothyroidism and disrupted cortical lamination, potentially driven by oxidative stress and down regulation of key genes related to motor function. Similar motor deficits were reported by Manoj P. Dandekar, supporting the findings of this study.²⁴

Microglia serve as a supportive element in neuronal survival and engage in phagocytic activities²⁵, primarily in inflammatory response to the demise of neuronal precursors and the surplus of neurons during perinatal development. When faced with stressful conditions, there is an observable rise in microglia²⁶. Recent research has furnished substantiating evidence of this phenomenon, with the hypothyroid group exhibiting a more pronounced prevalence of microglial cells when contrasted with the control group. In 2022, Chamas L conducted research indicating that hypothyroidism, specifically within the hippocampus of mice leads to the activation of glial cells.²⁷ This finding was further supported by Yizhen Wang *et al*, who also found a connection between hippocampal hypothyroidism in rats and an uptick in inflammatory immune responses.²⁸

In this study, a significant disruption in the organization of cortical layers within the hypothyroid group was observed. This irregularity was characterized by blurred boundaries between layers II, III, and IV, primarily resulting from the infiltration of layer III pyramidal cells into layer IV, as well as the migration of large pyramidal cells from layer V into layer IV. The root cause of these irregularities in cortical layer differentiation associated with hypothyroidism has been associated to reduced expression of the reelin gene. In 2020, O'Shaughnessy KL's research demonstrated that chemicals disrupting thyroid function during developmental neurotoxicity could lead to abnormalities in cortical lamination.²⁹ Furthermore, in 2021, Montero *et al*, indicated that rats experiencing dysfunction in thyroid hormones exhibited downregulated genes and delayed neuronal radial migration.³⁰

Although the study gave invaluable insight into the histological parameters of the developing motor cortex. The current research did not provide insights into ultrastructural alterations, molecular mechanisms, or the dynamics of gene expression due to limited resources.

Future studies should incorporate molecular and gene expression analyses to further elucidate the mechanisms underlying the observed effects. Given the critical role of thyroid hormones in neural development, effective prenatal monitoring and timely management of maternal thyroid function are essential to prevent neurodevelopmental deficits and promote healthy brain development in offspring.

CONCLUSION

This study highlights the detrimental effects of maternal hypothyroidism on locomotor activity. The observed disruptions in neuronal layer organization underscore a critical connection between maternal thyroid dysfunction, especially under stress, as evidenced by increased microglial cell counts, and altered motor cortex development.

ACKNOWLEDGEMENT

The authors would like to express sincere gratitude to Dr. Hussain Ali, Scientific Officer at NIH, for his invaluable technical support, practical guidance, and insightful recommendations.

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Received: 2 Sep 2025

Reviewed: 12 Jun 2026

Accepted: 18 Jun 2026

Contribution of Authors:

Authors approved the draft and are accountable in ensuring that questions related to accuracy or integrity of the work are duly investigated and resolved.

TF: Concept, methodology and prepared the original draft

SA: Supervision, review, and editing

TQ: Methodology and review

AH: Data analysis and reviewed the manuscript

SF: Methodology and review

MY: Discussion and review

Conflict of Interest: No conflict of interest is declared

Funding: No funds have been received from any agency