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International Journal on Veterinary Medicine

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Research Article

Volume 2 Issue 1- 2023

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Article History

Received: : December 18, 2023 Accepted: December 21, 2023 Published: December 21, 2023

Abstract

Dramatic increase in incidences of learning and developmental disorders in children has attracted serious concern worldwide. Various reports have indicated that excitation and/or desensitisation of nicotinic acetylcholine receptors (nAChRs) might affect developing mammalian nervous systems. This study was undertaken to evaluate the effect of Thiamethoxam, a second generation neonicotinoid insecticide developmental neurotoxicity. The study design was based on Organization for Economic Cooperation and Development guideline 426, Developmental Neurotoxicity Study. Thiamethoxam was administered orally to groups of Wistar rats at doses of 15, 50 and 150mg/kg. The test item was administered to animals during gestation and lactation. Dams were tested to assess effects in pregnant and lactating females and also to provide comparative information (dams versus offspring). Offspring were randomly selected from within litters for neurotoxicity evaluation. The evaluation consists of observations to detect gross neurologic and behavioural abnormalities, including the assessment of physical development, behavioural ontogeny, motor activity, motor and sensory function and the evaluation of brain weights and neuropathology during postnatal development and adulthood.

Keywords: Thiamethoxam, Developmental Neurotoxicity, Rat

Abbreviations: GD: Gestation Day; LD: Lactation Day; nAChRs: Nicotinic Acetylcholine Receptor; OECD: Organization for Economic Cooperation and Development; PND: Post-Natal Day

Introduction

The developing human brain is inherently more susceptible to damage caused by toxic agents than is the brain of an adult [1,2]. One in every six children has a developmental disability and in most cases these disabilities affect the nervous system. The most common neurodevelopmental disorders include learning disabilities, sensory deficits, developmental delays and cerebral palsy [3]. Evidence has been accumulating over several decades that industrial chemicals can cause neurodevelopmental damage and that subclinical stages of these disorders might be common. Of the almost 200 chemicals known to be neurotoxic, many are developmental neurotoxicants. Exposure to these compounds in utero or during childhood can contribute to a variety

of neurodevelopmental and neurological disorders. Developmental neurotoxicants may also cause silent damage, which would manifest itself only as the individual ages and may contribute to neurodegenerative diseases such as Parkinson's or Alzheimer's diseases [4].

The neonicotinoids are the fastest growing chemical class of insecticides, now exceeding 15% of the total insecticide market. This tremendous success is based on their unique chemical and biological properties, such as broad-spectrum insecticidal activity, low application rates, excellent systemic characteristics, favourable safety profile and a new mode of action [5]. Thiamethoxam is a second generation neonicotinoid insecticide. It has a broad spectrum of activity against many types of insects. It acts by binding to the nicotinic receptors of the postsynaptic membranes from both nerve and muscle cells and thus disrupts the transmission of the nervous influx into the central and peripheral nervous system [6]. This study was aimed to evaluate developmental toxicity potential of Thiamethoxam as per the OECD 426, Developmental Neurotoxicity Study [7].

Materials and Methods

Chemicals

All chemical reagents, solvents and other chemicals used in the studies were of analytical or pharmaceutical grade.

Test Article

Technical-grade Thiomethoxam was used in the study. Thiomethoxam Technical was procured from Krish Rasayan Limited and the identity of the test item was provided by the supplier in form of Certificate of Analysis.

Test Animals

Animal usage was reviewed and approved by the Institutional Animal Ethics Committee. Male and female Wistar IGS Rat Crl:WI Outbred rats, approximately 9 weeks of age (Hylasco Bio-Technology (India) Pvt. Ltd., were acclimated to the laboratory (AAALAC International accredited) for 7 days before study initiation. The animal room was maintained at 22°C ± 2°C, 60% ± 5% relative humidity, and 12/12- hour light/dark cycle. Food (Certified Lab Diet no. 5L79, PMI Nutrition International, USA) and drinking water were available ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee of Vimta Labs Limited.

Wistar rats obtained from Vimta Labs Limited, were housed in sterilized polycarbonate cages in which they had access to reverse osmosis filtered water and certified standard pelleted laboratory animal diet (PMI Nutrition International, USA) ad libitum. Throughout acclimatization and treatment periods, animal rooms were maintained at 20.7 - 24.8 °C and 35 -70% relative humidity with light/dark cycles of 12 hours. Animals were identified by unique accession number.

Study Design

Parental animals were assigned to four groups of 10 animals each based on a weight-balanced random allocation scheme. Dose levels of 0 (vehicle control, purified water), 15, 50, and 150mg/kg/day were administered once daily by oral gavage with a catheter. Males received Thiomethoxam Technical from 2 weeks prior to the mating, 2 weeks during mating and 6 weeks post mating period (to cover one complete spermatogenic cycle). Males were sacrificed after 10 weeks of treatment. Whereas, females received Thiomethoxam Technical 2 weeks prior to mating (which covers 3-4 complete oestrous cycles in order to detect any adverse effects on cyclicity), treatment was continued during mating, throughout pregnancy and up to the weaning of the F1 offspring, after which parental females were sacrificed. Males and females were cohabited in a 1: 1 ratio. The day of finding seminal plugs in the vagina was defined as gestational day (GD) 0.

Animals were observed at least once daily prior to dosing for mortality and adverse physical signs; an additional observation was made l-5 h after each dose. Body weights were recorded periodically during gestation and lactation. From GD 21 until completion of delivery, each presumed-pregnant female was observed frequently. At 21 day postpartum of F1 litter, randomly selected 10 male and 10 female pups per group were assigned to Cohort A and Cohort B as adults and at weaning (PND 21 or 22) respectively for neurobehavioural testing followed by neurohistopathology assessment. The dose formulation of test item was administered to F1 offspring of Cohort A from weaning (PND 21) to PND 90. The animals were sacrificed on PND 91 ± 5 days whereas dose formulation of test item was not administered to F1 offspring of cohort B and were sacrificed upon weaning (LD 21).

Animals were observed for morbidity and mortality twice daily. General clinical observations occurred daily and detailed clinical examinations occurred before randomization and weekly thereafter throughout the treatment period. Body weights for all groups were measured prior to gavage on day 1 and at weekly interval. Female body weight was recorded twice weekly during gestation and lactation periods.

Necropsy of P animals ↓

Thiamethoxam Dosing					
	Pre-mating	Mating	Post-mating		
P Males	2 weeks	2 weeks	6 weeks		
P Females	2 weeks	2 weeks	Pregnancy	Lactation	Post-weaning

Parental generation	Cohort	Designation	Animals/Cohort
10 litters per group	A	Neurotoxicity: Adult	10 M + 10 F
	B	Neurotoxicity: Young animals	10 M + 10 F

Behavioral Assessment

Functional Observation Battery (FOB) evaluation was performed between PND 63 to PND 75 for Cohort A offspring (10 males and 10 females). The FOB, which was conducted according to previously described procedures [8], included cage-side, handheld, and open-field observations with ranked and categorical observations, as well as sensory evaluations, which included approach response, touch response, click response, tail pinch response and pupil response measurements and forelimb and hind limb grip performance, landing foot splay, and motor activity. The FOB was conducted by an observer who was blind to the treatment status of the animal. The same observer was used for all rats.

Motor activity was performed using OPTO VARIMEX and automated animal activity measuring system (Columbus Instruments, USA) equipped with a computer analyser. Each clear square acrylic activity monitor has a floor area of approximately 1640 cm^2 and each monitor was used exclusively by one sex. Different dosage groups were tested sequentially in each monitor. The floor of each activity cage was cleaned with water and dried between animals. The test was performed in a room with red light illumination. An auditory startle test (responsiveness to sharp noise) was performed on PND 24 (± 1 day) using Responder-X instrument. All surviving Parental, Cohort A and Cohort B animals were euthanized by CO_2 asphyxiation followed by exsanguination. The animals were subjected to detailed gross pathological observation during necropsy. Whole body perfusion fixation was performed for all animals belonging to Cohort A. Brain was collected from Cohort A and Cohort B animals and processed for histopathological evaluation. Sections (3-5µm) were cut and stained with Haematoxylin and Eosin.

Figure 1 Seven transverse (coronal) haematoxylin and eosin-stained sections corresponding to levels based on anatomic target landmarks. The brain sections of control and high dose group animals were examined under a microscope with a camera and imaging software (DP2-BSW). Histology images were captured at the desired section. Microscopic linear measurements of Cohort A animals were taken at representative locations to estimate the thickness of major layers, which included cerebral cortex and corpus callosum thickness and cerebellum thickness. Brain measurements for bilaterally symmetrical structures were taken, wherever feasible. A 5-step grading system of minimum, mild, moderate, severe and marked was used to rank microscopic findings for comparison among groups. Data was compiled based on incidences and severity of changes. The data was analysed using Graphpad Prism, version 4.2. All statistical tests were performed at 5% and 1% level of significance.

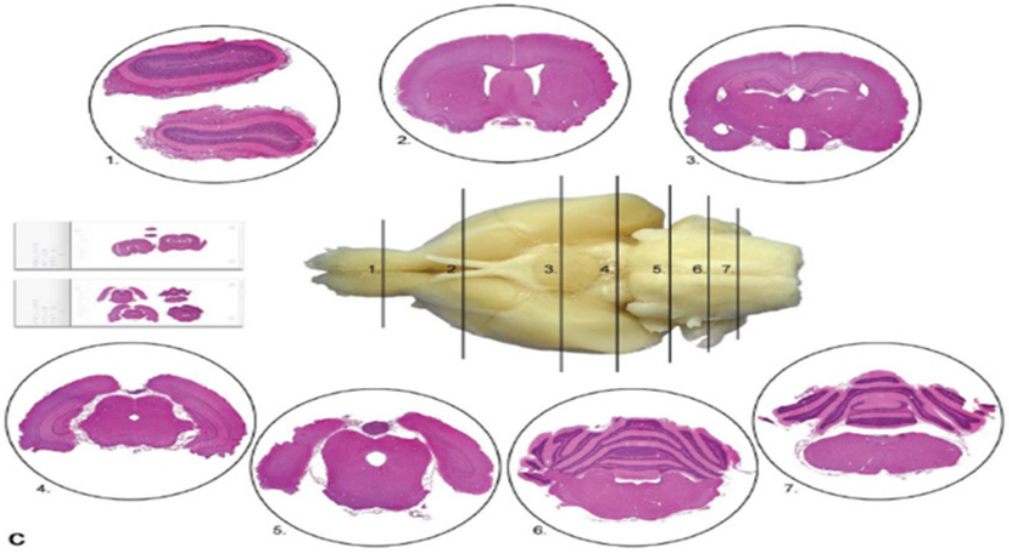

Figure 1: Brain Sectioning.

Results

Parental Animals

There was no mortality and morbidity throughout the study period. All animals from vehicle control and test item treated groups were found to be normal throughout the study period. Further, there were no significant changes in the mean weekly body weights and food consumption in any of the treated groups including females during gestation and lactation periods as compared to the vehicle control group. There were no treatment related changes in oestrous cycle length between control and treatment groups. Oestrous cyclicity was evaluated for its length and normality by examining the vaginal smears daily for 14 days prior to mating. The calculated mean oestrous cycle length was 4.1, 4.0, 4.3 and 4.1 days in vehicle control, low, mid and high doses, respectively.

Cohort A Animals

There was no mortality and morbidity throughout the study period. All animals from vehicle control and test item treated groups were found to be normal throughout study period. Further, there were no statistically significant changes in the mean body weights and food consumption in any of the treated groups as compared to the vehicle control group. However, significant increase in the mean body weight of females was observed during week 6 and 7 with the increase of 8.7% and 8.4% respectively at 50mg/kg b.w. and were considered to be of no toxicological significance since they were transient changes and non-dose dependent.

Vaginal patency: Vaginal patency was evaluated for a visible break in the membranous sheath covering the vaginal orifice. The acquisition of vaginal patency began on post-partum day 30 in all the groups and completed in the all the pups on post-partum day 34, 35, 36 and 35 in control, low, mid and high doses respectively. The mean age at acquisition of vaginal patency were not affected by the treatment when compared to control.

Balano-preputial separation: Acquisition of balano-preputial separation started on post-partum day 44 in G1 and G2, day 45 in G3 and day 46 in G4 and completed on postpartum day 47 in G1, day 48 in G2, day 49 in G3 and day 50 in G4. Delay was observed by one day in G2, two days in G3 and three days in G4 group when compared to control.

Neurobehavioural observation: Home cage, handling and open field observations of treated animals were comparable to control group. All animals displayed normal gait throughout the treatment period. No

clonic or tonic movements, stereotypic movements and bizarre behaviour were observed during handling and open field observations throughout the study period.

Sensory activity and neuromuscular measurements: All animals of treated groups as well as the control groups displayed normal approach, tail pinch, touch, click and pupil responses.

Air righting reflex: Air righting reflex of all the animals of both the control and treated groups were found to be normal.

Grip strength: No alterations in the fore and hind limb grip strength were recorded in any of the treated group animals as compared to concurrent control groups (Figure 2,3).

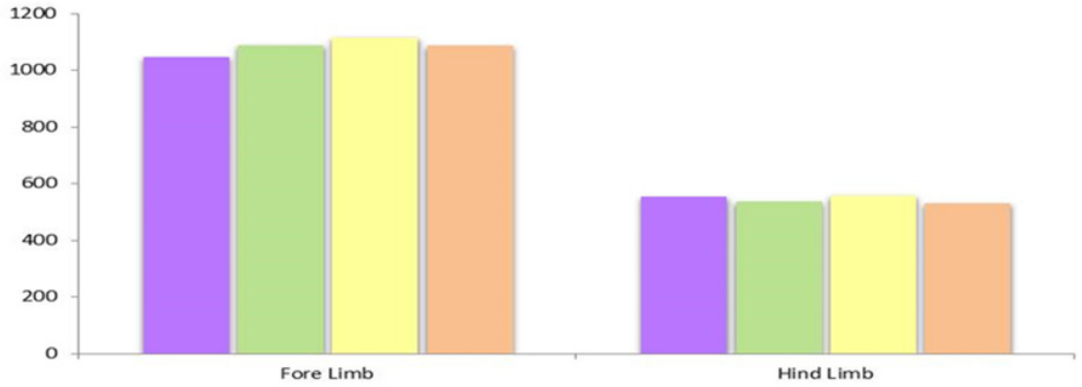

Figure 2: Effect on Grip Strength in Male Animals.

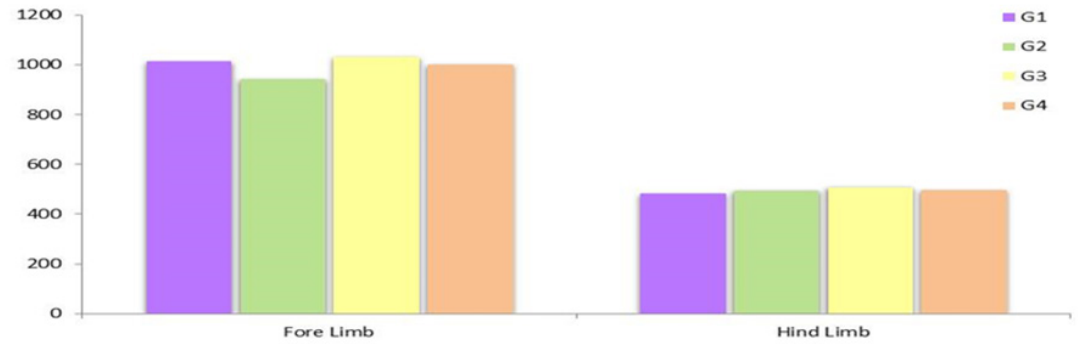

Figure 3: Effect on Grip Strength in Female Animals.

Hind limb foot splay: The hind limb foot splay of all treated groups was comparable to concurrent control groups (Figure 4,5).

Motor activity: Administration of test item did not cause significant alterations in the total, ambulatory and stereotypic activities of treated rats as compared to control group animals. However, male rats displayed significant decrease in the total and ambulatory counts in mid and high dose as compared to control group animals. The observed change might be correlated with reduction in hippocampus of brain (Figure 6,7).

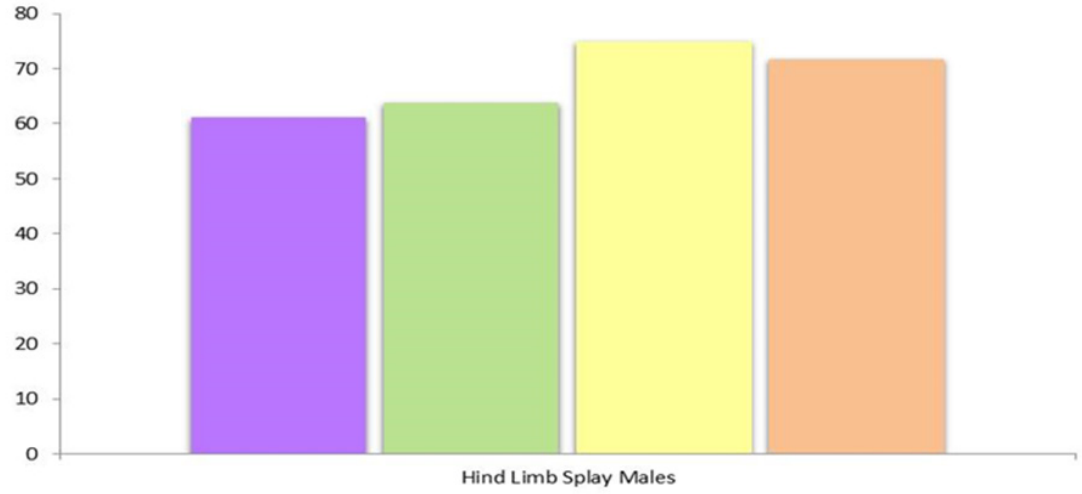

Figure 4: Effect on Hind Foot Splay in Male Animals.

Figure 5: Effect on Hind Foot Splay in Female Animals.

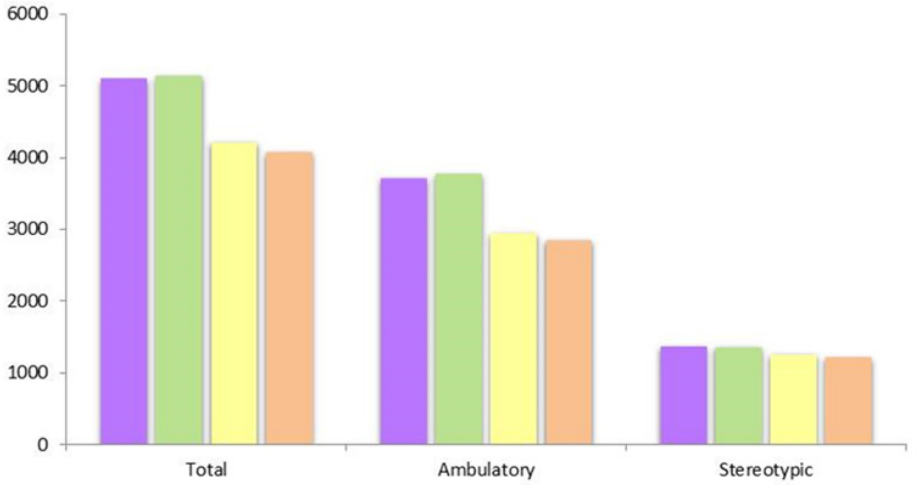

Figure 6: Effect on Motor Activity in Male Animals.

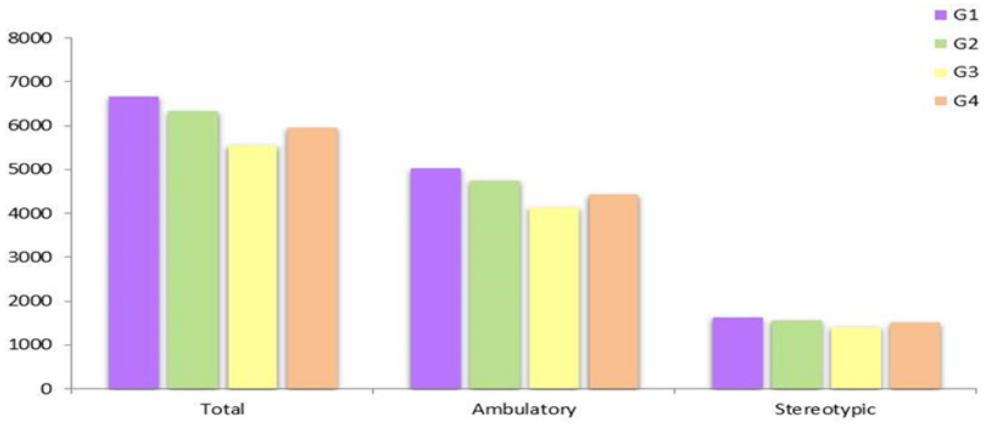

Figure 7: Effect on Motor Activity in Female Animals.

Startle response: Significant decrease in acoustic startle response at 65 to 120db on PND 24 (± 1 day) in males treated with 150mg/kg Thiamethoxam as compared to vehicle control was considered treatment related change. However, no such treatment related effect was observed in females (Table 1).

Pathological evaluation with histomorphometry evaluation: No treatment related changes were observed in absolute and relative organ weights in treated group animals when compared to control. Further, no gross pathological findings were observed in both sexes during terminal sacrifice. Histomorphometric analysis revealed a decrease in overall width of hippocampus of high dose treated adult animals (150mg/kg b.w.) as compared to concurrent control animals which were considered as treatment related. The overall width of all other regions (cerebral cortex motor, cerebral cortex sensory, corpus callosum and cerebellum) was comparable to control (Table 2).

Table 1: Effect on Startle Response.

Amplitude (Pre-pulse & Stimulus)	Group & Dose (mg/kg b.w.)							
	G1(0)		G2 (15)		G3 (50)		G4 (150)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Male								
65 & 100	89.88	18.27	100.77	18.17	101.01↓	13.53	97.68↓	18.94
70 & 110	132.03	37.56	133.02	25.30	135.31↓	31.13	122.91↓	28.09
75 & 115	147.12	31.24	143.00	25.45	153.10↓	31.39	132.41↓	22.67
80 & 120	151.29	42.17	145.61	39.25	146.91↓	33.79	134.66↓	27.57
Female								
65 & 100	91.74	16.95	95.17	11.45	92.18	8.78	101.18	27.41
70 & 110	126.73	31.16	133.90	18.34	139.17	24.26	140.46	44.22
75 & 115	139.03	33.82	157.48	39.03	145.37	28.81	150.27	39.45
80 & 120	131.53	35.73	146.14	32.89	142.40	24.86	137.55	29.88

Table 2: Histomorphometry of Brain Tissue.

Parameters	Group & Dose (mg/kg b.w.) Measurement (µM)			
	G1 (0)		G4 (150)	
	Mean	Standard Deviation(SD)	Mean	Standard Deviation(SD)
Male				
Cerebral Cortex Motor (CCM)	1831.97	174.65	1922.51	112.98
Cerebral Cortex Sensory (CCS)	1700.61	137.19	1802.68	125.91
Corpus Callosum (CST)	422.67	84.02	477.19	73
Hippocampus (HPC)	1403.62	153.42	1221.12↓	154.01
Cerebellum (CB)	4996.14	841.26	4845.51	861.61
Female				
Cerebral Cortex Motor (CCM)	1920.56	136.6	1874.35	84.76
Cerebral Cortex Sensory (CCS)	1783.74	179.82	1760.87	90.27
Corpus Callosum (CST)	446.31	79.03	401.79	64.58
Hippocampus (HPC)	1257.36	145.2	1094.22↓	69.34
Cerebellum (CB)	5035.49	1465.01	5300.41	353.5

On morphometric evaluation of Cohort A animals, there was decrease in overall width of the hippocampus of adult animals at 150mg/kg dose as compared to control. The overall width of all brain regions (cerebral cortex motor, cerebral cortex sensory, corpus callosum and cerebellum) was comparable to control. Figure 8 Arrows depict histomorphometry of brain in which width of hippocampus is decreased compared to control animals (Normal) and width of Cerebral cortex and Corpus callosum are comparable in high dose and control treated animals.

Cohort B Animals

There was no mortality and morbidity and all animals were found to be normal. Absolute and relative brain weights were comparable to vehicle control in both sexes and gross pathological observations did not reveal any lesions attributable to treatment in both sexes. No treatment related histopathological findings were observed in brain tissues in both sexes of Cohort B animals.

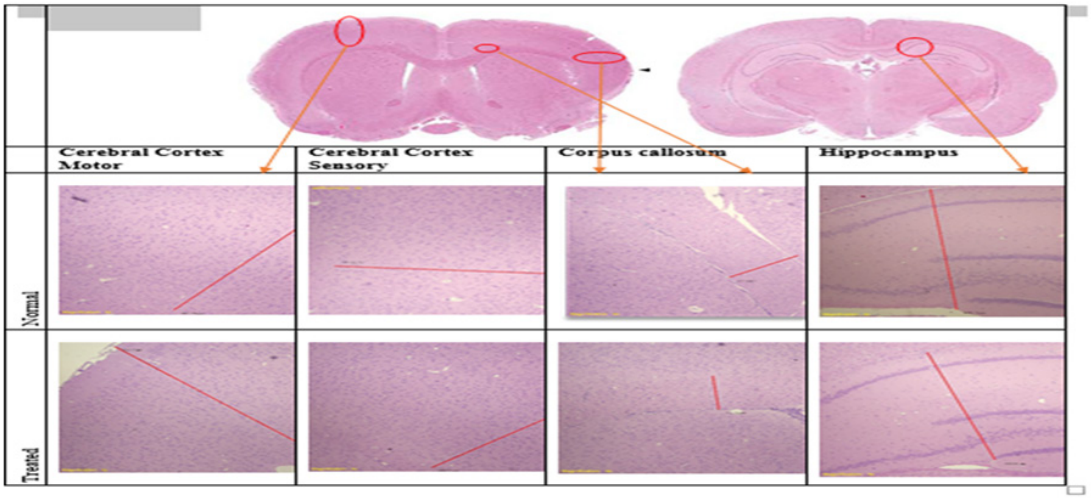

Figure 8: Brain Morphometry.

Discussion

Potential concern that the neonicotinoid insecticides could affect the developing nervous system is associated with their nicotinic mode of insecticidal action and recognition of nicotine as a likely developmental neurotoxicant in humans and laboratory animals. In Parental, Cohort A and Cohort B animals, there was no mortality and morbidity throughout the study period. Further, there were no significant changes in the mean weekly body weights and food consumption in any of the treated groups including parental females during gestation and lactation periods as compared to the vehicle control group. No effect on body weight and food consumption at 50 and 150ppm but dams fed 4000ppm Thiamethoxam during gestation period had statistically significant lower body weight [9,10]. The acquisition of vaginal patency began on post-partum day 30 in all the groups and completed around day 36. No treatment-related effect on the day of vaginal opening was observed in dams treated with 4000ppm. However, preputial separation was delayed by an average of 1.5 days relative to the control at 4000ppm Thiamethoxam [9,11]. In present, study balano-preputial separation was delayed in dose dependent manner in Thiamethoxam treated groups as compared to control.

No treatment related changes were observed in FOB parameters up to 150 mg/kg b.w. [9] except significant decrease in the total and ambulatory counts at 50 and 150 mg/kg and acoustic startle response (65 to 120db) at 150mg/kg dose in males. Motor activity is measured in guideline-compliant [12] DNT studies during the period of exposure on PND 13, 17, and 21, as well as on PND 60 to test for latent or persistent effects [13]. The effects noted in the guideline studies with neonicotinoids consisted of decreased motor activity at the highest dietary level with imidacloprid on PND 17 and 21 [14] and with clothianidin on PND 21 [15], without an effect on habituation in either case. Treatment related significant decrease in acoustic startle response in males treated with 150mg/kg Thiamethoxam was recorded on PND 24 (± 1 day) whereas no such treatment related effect was observed in females. McGregor [9] did not report effect of maternal treatment with thiamethoxam on startle amplitude or the time to maximum amplitude of the F1 rats on day 23 or 61 up to 4000ppm however, some statistically significant differences (especially in the 400ppm group) from control provided no evidence for any treatment-related effects. Difference in findings may be attributed to dietary administration of test item vs. gavage dosing.

In present study, absolute and relative brain weights were comparable to vehicle control and gross pathological observations did not reveal any lesions attributable to treatment up to 150mg/kg b.w in animals from both the cohorts. In males and females of dams treated with Thiamethoxam at 50, 400 and 4000ppm, absolute brain weight was lower than that of controls at 400ppm thiamethoxam, but again, this effect was not apparent following adjustment for terminal body weight [9,16]. No treatment related histopathological findings were observed in brain tissues in both sexes of Cohort B animals. On morphometric evaluation of Cohort A animals, there was decrease in overall width of the hippocampus of adult animals at 150mg/kg dose. McGregor [9] reported revealed a number of statistically significant differences in thalamus and hippocampus width at day 63 in the 4000ppm group prior to adjustment for body weight. However, many of these were not apparent after adjustment for body weight, suggesting that they are a consequence of the reduced birth weight and lower body weights seen at this dose level. In present study, there was no effect on body weight and brain weight hence, observed changes were attributed to treatment effect. However, there are no reports of neuropathology with any of the neonicotinoids evaluated, which includes brain regions identified as rich in nicotinic acetylcholine receptor (nAChR) [17], regions reported to express structural abnormalities following developmental exposure to nicotine [18] and regions that showed an excitatory response to neonicotinoids in primary cell cultures [19,20]. The functional significance of observed decrease width of the hippocampus to be evaluated.

Conclusions

Treatment with Thiamethoxam technical did not reveal any systemic toxicity and abnormal changes in to dams and offsprings at 150mg/kg b.w. However, males exhibited delay in acquisition of balano-preputial separation, decreased total and ambulatory counts at 50 and 150mg/kg along with decrease overall width of hippocampus at 150mg/kg.

Declaration of Conflicting Interest

The authors declared no additional conflicts of interest in regard to the research, authorship, and/or publication of this article.

Funding

The authors disclose that financial support for the research described here in was provided by Vimta Labs Limited.

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