EVALUATING THE THIACLOPRID INDUCED TOXICITY TO NERVOUS SYSTEM

INTRODUCTION
Neonicotinoids, the most important new class of synthetic insecticides of the past three decades are used in various agricultural fields worldwide (Tomizawa and Casida, 2003). Global annual trade in neonicotinoids is to the tune of one billion dollars that accounts for 11%–15% of the total insecticide market. They are readily absorbed by plants and act quickly by targeting the receptors of neurotransmitters, at low doses, on piercing-sucking insect pests (aphids, leafhoppers, and whiteflies) of major crops and companion animals (Tomizawa and Casida, 2005).

Ligand-gated ion channels (LGICs) are receptors with integral ion channel mediating the fast action of neurotransmitters at synapses in vertebrate and insect nervous systems. Prolonged activation, modulation, or inhibition of LGICs, irrespective of the receptor type involved, can result in toxicity. Neonicotinoids are a commercially important class of insecticides that target LGICs like nicotinic acetylcholine receptors (nAChRs) of insects in a selective manner (Matsuda et al., 2001). Neonicotinoids are synthetic, nicotine-derived pesticides which have similar effects like nicotine as an agonist to nAChRs of insects (Li et al., 2011).

Nicotine acts as an insecticide but is also toxic to mammals (Ujvary, 1999). In fact, nicotine has been reported to have a lower lethal dose for rats than flies (Yamamoto, 1999). This spurred a scientific search for compounds that retain the insecticidal properties of nicotine but have selectively less effect on mammals, though initial investigation of nicotine-related compounds (nicotinoids) as insecticides was unsuccessful (Ujvary, 1999). Nevertheless once succeeded the synthetic nicotinoids gain credence as successful broad spectrum insecticide.

Neonicotinoids, like nicotine, are nicotinic acetylcholine receptor agonists. This receptor is normally activated by the neurotransmitter acetylcholine. These receptors are located in both - the central and peripheral nervous systems of mammals but are limited to the CNS in insects; peripheral nervous system of insect is GABAergic not cholinergic. While low to moderate
activation of these receptors causes nervous stimulation, high levels over-stimulate and hence block the receptors (Yamamoto, 1999). This receptor blockage causes paralysis and death. Normally, acetylcholine is broken down by acetylcholinesterase (AChE) to terminate signals to these receptors. However, AChE cannot break down neonicotinoids, and the binding is irreversible. Most neonicotinoids bind much more strongly to insect neuron receptors than to mammal neuron receptors, and hence, these insecticides are selectively more toxic to insects than mammals (Tomizawa, 2004).

Thiacloprid, a cyanamide, shows low affinity for mammalian nicotinic acetylcholine receptors while exhibiting high affinity for insect nAChRs (Tomizawa et al., 1999; Yamamoto, 1999). Thiacloprid mimics the action of nicotine in the nervous system, binding at or near the site on the nAChR where nicotine binds, producing an unregulated barrage of nerve impulses, resulting in something akin to a nervous breakdown, and ultimately, death (Tomizawa and Casida, 2003; Tomizawa and Casida, 2005). Molecular difference between thiacloprid and nicotine is presented in Figure A.

![Figure A: Structural difference between nicotine and thiacloprid](image)

Mammals and insects have structural differences in nAChRs, which affect how strongly particular molecules bind, both in the composition of the receptor subunits and the structures of the receptors themselves (Tomizawa et al., 1999; Tomizawa, 2004). Nicotine, like the natural ligand acetylcholine, has a positively charged nitrogen (N) atom at physiological pH because it is protonated by water (Yamamoto, 1999; Tomizawa, 2004). Due to positive charge, these compounds have strong affinity towards mammalian nAChRs. At the same time, the charge on nicotine lowers its effectiveness as an insecticide, because the blood–brain barrier prevents free access of ions to the central nervous system, and insect nAChRs are only present in the central nervous system (Yamamoto, 1999; Tomizawa, 2004). The blood-brain barrier does not prevent nicotine poisoning in mammals, because mammalian nAChRs are located in the peripheral nervous system and are necessary for vital functions such as breathing. The low
mammalian toxicity of thiacloprid can be explained in large part by its lack of a charged nitrogen atom at physiological pH. The molecule shows weak affinity to mammalian nAChRs but strong affinity for insect \( \alpha_4\beta_2 \) nAChRs. Furthermore, the uncharged molecule can penetrate the insect blood-brain barrier, while the human blood-brain barrier filters it (Yamamoto, 1999).

All nAChR molecules contain five subunits, each of which contains a dicysteine loop (Cys-loop) with 13 intervening residues in the N terminal, extracellular domain (Corringer et al., 2000; Karlin, 2002). The subunits are classified into \( \alpha \) and non-\( \alpha \) types according to the presence (\( \alpha \) subunits) or absence (non-\( \alpha \) subunits) of vicinal cysteine residues in loop C, one of six separate loops (A-F) that make up the acetylcholine (ACh) binding site. Two \( \alpha \) and three non-\( \alpha \) subunits are recruited to form most heteromeric nAChRs, in which the ligand-binding sites are located at the interfaces of the \( \alpha \) and the adjacent non-\( \alpha \) subunits (Figure B). The \( \alpha_7 \), \( \alpha_8 \), and \( \alpha_9 \) subunits of vertebrates can form homo-oligomers (Couturier et al., 1990), whereas the 10 subunit forms a heteromer with the 9 subunit (Elgoyhen et al., 2001). In such cases, the ligand-binding sites are located between two adjacent \( \alpha \) subunits (Matsuda et al., 2005).

**Figure B:** Schematic representations of a Nicotinic Acetylcholine Receptor (nAChR) and its ligand binding site (Matsuda et al., 2005)

Thiacloprid is specific for \( \alpha_4\beta_2 \) nAChR of insect CNS. Mammalian central and peripheral nervous system has \( \alpha_4\beta_2 \) nAChR. Human neuronal nicotinic receptors are pentamers formed by a single alpha subunit (\( \alpha_7 \), \( \alpha_8 \) or \( \alpha_9 \)) or a combination of \( \alpha \) and \( \beta \) subunits (Ortells and Lunt, 1995; Lindstrom et al., 1996). \( \alpha_4\beta_2 \) nicotinic receptors are abundant in the brain and they account for more than 90% of the high-affinity nicotine binding sites in the brain (Flores et al., 1992; Picciotto et al., 1995; Marubio et al., 1999). In rat, high affinity nAChR sites
revealed by $[^{3}\text{H}]^{\circ}-\text{nicotine}$, are abundant in selective areas of the cerebral cortex (predominantly layers III and IV), thalamus, interpeduncular nucleus and the superior colliculus, but are of low to moderate abundance in the hippocampus and hypothalamus (Clarke et al., 1985). The C=N-CN moiety of thiacloprid is hydrolyzed to the amide [C=NC(O)NH2] and also undergoes N–CN cleavage. Descyanothiacloprid is a particularly potent mammalian nAChR agonist (Tomizawa et al., 2000; Klein, 2003).

However, the endogenous agonist acetylcholine is an excitatory neurotransmitter of the cholinergic system. ACh released from the presynaptic membrane interacts with the binding site located at the extracellular domain of the nAChR/ion-channel complex. A conformational change of the receptor molecule then leads to channel opening, influx of extracellular Na$^+$ and efflux of intracellular K$^+$. The nAChR is responsible for rapid neurotransmission in the central nervous system (Matsuda et al., 2005). Thiacloprid is not protonated, but the electronegative (δ–) cyano- tip may bind to a lysine or arginine residue in a subsite of the insect nAChR. Nicotine is protonated at physiological pH and undergoes cation–π interaction with Trp at nAChR subsite in mammal (Figure C). Subsequent to excitation by the endogenous ligand ACh, enzyme acetylcholinesterase hydrolyses acetylcholine and turn off the stimulation. However AChE cannot hydrolyse bound thiacloprid from nAChRs, which results in excessive stimulation of cholinergic receptors (Matsuda et al., 2005).

![Figure C: Nicotinic receptor-ionophore complex in which subsite specificity confers selective toxicity of neonicotinoids (thiacloprid) for insects and nicotinoids (nicotine) for mammals (adapted from Matsuda et al., 2005).](image)

Thiacloprid poisoning causes increased stimulation at all three locations of nAChR: (1) the neuromuscular junction, (2) the autonomic ganglia and (3) the CNS. At the neuromuscular junction, overstimulation of nAChRs results first in muscle fasciculation that ultimately results
in weakness or even paralysis (Barthold and Schier, 2005). The weakness is probably due to desensitisation of the receptor. At the ganglia, excessive stimulation causes activation of the autonomic nervous system (Sheridan et al., 2005).

The main symptoms of thiacloprid toxicity are related to the excessive stimulation of the cholinergic receptors at neuronal, ganglionic and muscular interfaces. These symptoms include muscle fasciculation and weakness, papillary dilatation, ptosis and irregular respiration (Sheridan et al., 2005; Hassel, 2006). CNS symptoms are also observed, notable among which are giddiness, ataxia and seizures (Barthold and Schier, 2005). The cholinergic input to various brain nuclei gives rise to increased glutamatergic and GABAergic activity and causes brain seizures. The seizures progress rapidly to ‘status epilepticus’, which may lead to profound structural brain damage. The seizures and resulting brain damage are initiated by cholinergic over-stimulation which triggers seizures in susceptible brain regions. The seizures cause the release of excessive amounts of glutamate from affected neurons and the released glutamate gives rise to excitotoxicity and cell death (Solberg and Belkin, 1997).

Conventionally the extent of neurotoxicity is measured through the estimation of relevant biochemical markers. AChE activity is often estimated to measure the acute neurotoxicity however, delayed neuropathy is assessed by estimating the neuropathy target esterase (NTE, also called as neurotoxic esterase) (Ehrich et al., 1997). Neuropathy target esterase plays critical roles in embryonic development and maintenance of peripheral axons. NTE in vivo is poorly defined. It is known to hydrolyze lysophosphatidylcholine (LPC) in vitro and may protect cell membranes from cytotoxic accumulation (Vose et al., 2008). NTE mutations are associated with progressive upper and lower-motor neuron disease indicating the importance of NTE in maintaining the integrity of corticospinal tract and peripheral motor axons (Shirley et al., 2008). NTE-LysoPLA inhibition is known to lead to localized accumulation of lysolecithin, a known demyelinating agent and receptor-mediated signal transducer (Quistad et al., 2003).

NTE is anchored to the cytoplasmic face of the endoplasmic reticulum membrane and is particularly abundant in neurons, the placenta, and the kidney (Glynn, et al., 1998; Li et al., 2003; Moser et al., 2004; Zaccheo et al., 2004). Loss of NTE activity results in abnormally elevated levels of phosphatidylcholine in brain and impairment of the constitutive secretory pathway in neurons. Mouse or human neuroblastoma cell-lines can be considered useful in
vitro models to distinguish esterase-inhibiting neurotoxicants. NTE esterase domain accelerates the elongation of neurite processes in human neuroblastoma (SK-N-SH) cell line (Ping-An et al., 2005).

Thiacloprid being a neonicotinoid insecticide mainly targets the nervous system by mimicking the neurotransmitter acetylcholine and binds with nAChRs. Few studies have been performed in mammals regarding the toxic effects of neonicotinoids. Further, these studies have also mostly focussed on the mechanism of neurotoxicity of imidacloprid, a nitroimine derivative. Nevertheless, thiacloprid, a cyanamide though a frequently used agrochemical and a potent neurotoxicant like imidacloprid, there has been very little focus on its neurotoxic effects on a non-target organism like mammals.

Hence, it was thought pertinent to assess the possible esterase-dependent neuropathy induced by thiacloprid in the non-target mammalian system. This was done through estimating blood and plasma activities of esterases (AChE and NTE) and also by carrying out AChE localization in different regions of the brain. The study was also extended to explore any possible relation between NTE and neuronal damage, a likely toxic effect of thiacloprid. Neuronal damage was observed by Nissl staining on fresh frozen brain sections using cresyl violet stain. Further, inhibition of esterase may lead to development of paralysis or ataxia which is a known neurotoxic mechanism of pesticides. Hence, a rotarod test was also conducted to assess the neuromuscular co-ordination in thiacloprid intoxicated rats. Further, delayed neuropathy could reflect as possible development of demyelination which was explored through transmission electron microscopy of nerve tissues. Further, an in vitro study using IMR-32 cell line was also conducted to analyze the possible toxic effect of thiacloprid on the growth and elongation of neurite.

MATERIAL AND METHODS
Male Sprague-Dawley rats procured from a CPCSEA approved animal breeder (SPARC Ltd., Baroda) were used in all experiments. All experiments were carried out in accordance with protocols approved by IAEC according to CPCSEA guidelines, and all efforts were made to minimize the number of animals used and to take care that they were subjected to minimal suffering. Rats were kept in the departmental animal house (827/ac/04/CPCSEA) at controlled temperature (24 ± 2°C) and light-dark schedule (12:12) and were provided laboratory rat food (Pranav Agrochemicals) and water ad libitum. Thiacloprid (Allanto, 21.7%SC) was procured
from local market and diluted in distilled water to make the desired dose concentration. Experimental animals were grouped into three as control, low dose and high dose group, with five animals in each group for subacute as well as subchronic oral administration.

At the end of experiment, rats were sacrificed after overnight fasting. Blood was collected in EDTA vacutainer for cholinesterase activity. Target organ was removed and fixed in 10% buffered formalin. For transmission electron microscopy (TEM) 4% paraformaldehyde was used for tissue fixation.

**Protocol I: Assessment of Esterase**

**Acetylcholinesterase (AChE):** Acetylcholinesterase activity was performed by the method of Ellman *et al.* (1961). Activity of AChE was measured in whole blood, plasma, various regions of brain and neuroblastoma IMR cell line. The assay uses acetylthiocholine as a substrate. AChE hydrolysates the acetylthiocholine to produce thiocholine which in turn reduces DTNB liberating nitrobenzoate, which absorbs at 412nm.

**Neurotoxic esterase (NTE):** The procedure is a modification for rat brain of a standard method for hen brain (Johnson, 1977) and for IMR cell line (Correll and Ehrich, 1991). Phenyl valerate was used as a substrate and phenol liberated by NTE was measured at 490nm.

**Localization of AChE:** Direct colouring method described by Karnovsky and Roots (1964) was adapted for the localization of acetylcholinesterase in fresh frozen sections of brain.

**Protocol II: Functional observation battery (FOB)**

Forced swimming test (FST) was performed according to the protocol of Cryan *et al.* (2002). The rats were forced to swim for 10 minutes in the glass cylinders containing water. This activity was repeated every month. Parameters like active swimming, floating and dipping were recorded with time.

Rotarod test was used to assess motor coordination and balance in treated rats. Time (latency) taken by the rats to fall off the rotating rod at different speeds or under continuous acceleration (e.g. from 4 to 40rpm) was recorded. Each animal was observed for 5 minutes and each fall was noted.
Protocol III: Assessment of neuronal damage
Neuronal damage was assessed by cresyl violet staining. 15µm thick cryosections were stained with cresyl violet and observed under microscope (Leica DM2500). Demyelination was observed using Transmission electron microscopy (TEM) (Glauert, 1974). Tissues were fixed in 2.5% glutaraldehyde for 24 hrs at 4°C. The tissues were rinsed with buffer and osmicated in 2% osmium tetroxide. Tissues were rinsed again and dehydrated using increasing alcohol concentrations, followed by rinsing in propylene oxide. The specimen was put in a mixture of 50% propylene oxide and 50% resin for at least 2 hours and then in 100% resin as embedding medium. Resin blocks were prepared. Samples were cut on an ultra microtome and sections were stained with uranyl acetate for 20 minutes and then rinsed. Sections were observed under TEM for myelin pattern analysis.

Protocol IV: Histopathological evaluation
Brain tissue was fixed in 10% buffered formalin for 24hrs and then dehydrated in alcohol. After clearing in xylene, paraffin block was prepared. Sectioning was done using microtome and sections were stained with haematoxylin and eosin and observed under the microscope (Leica DM2500).

Protocol V: In vitro study on neuroblastoma cell line
IMR 32 human neuroblastoma cell line was used to study the effect of thiacloprid on neurite growth and process elongation. The cell line was procured from National Chemical Laboratory, Cell culture facility, Pune. Culture was maintained as an adherent cell line in T25 flask by providing Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100µl of antimycotic solution at 37°C in a 5% CO₂: 95% air-humidified atmosphere. IC50 value was calculated through MTT assay. Cell morphology was observed and esterase activity was also estimated.

Statistical analysis
Data are expressed as a Mean ± SE. Statistical analysis was performed using One-way ANOVA followed by post hoc Bonferroni test for multiple comparisons using SPSS ver.12.0. The p values of ≤ 0.05 were considered statistically significant.
RESULTS

The effect of repeated exposure to thiacloprid during the subacute and subchronic studies on acetylcholinesterase (AChE) activity in blood, plasma and brain is shown in Table 3.1. The results indicate that activity of AChE was higher in blood and plasma in the thiacloprid treatment groups in the subacute study whereas the same was observed to be low in the subchronic study as compared to control values. However, these differences were not statistically significant. Brain AChE activity in animals treated at the dose of 100mg/kg body weight in subchronic study was found to be significantly decreased compared to control rats (p ≤ 0.05). Similar trend was observed for animals given a lower dose during subchronic study and higher dose during subacute study, but the differences were not statistically significant (Figures 3.1 and 3.2). Moreover, results of AChE localization also indicated much less intensely localized AChE in thiacloprid treated rat brain as compared to control rat brain (Figure 3.9).

Table 3.2 shows values for neuropathy target esterase (NTE) activity in brain of both the thiacloprid dosage groups for both the study periods. Activity of NTE was observed to be lower in both the treatment groups during both the subacute and subchronic studies as compared to control values. Statistically significant decrease (p ≤ 0.05) in brain NTE activity was observed for rats dosed at 100mg/kg body weight in the subchronic study (Figure 3.3). Neuromuscular coordination of animals was observed using rotarod test after 90 days, results of which are presented in Table 3.3. When rats were kept on the rotarod moving at 15rpm, the time taken to fall off the rod was not significantly different between the control and treatment groups. However, with increasing speeds of rotation, treated rats were observed to fall in less time as compared to control rats. At 20 rpm, time recorded for rats falling off the rotarod was significantly smaller (p ≤ 0.05) for the high dose treatment group as compared to control rats. Similarly at 25 rpm, both the treatment groups experienced much difficulty in staying on the rotarod and fell off the rod significantly (p ≤ 0.05) earlier (Figure 3.4).

Data compiled in Table 3.4 shows the performance of experimental animals in forced swimming test. Values represent the time duration in seconds, taken by the animals to become inactive (floating) or immobile (immobility time). Swimming test was done before intoxication of thiacloprid, values for which are given as on day ‘0’ and then thiacloprid was administered to rats for 28 and 90 days. On day 0, there was no significant difference in the values of the immobility time (Figure 3.5). After 28 days of intoxication of test compound, animals of low
dose group were observed to attain immobility earlier as compared to control and those of high
dose group also showed the same trend as the low dose group, with the difference being
statistically significant as compared to control ($p \leq 0.01$). 90 days of repeated thiacloprid doses
of 50mg/kg body weight and 100mg/kg body weight caused the animals to become inactive
significantly earlier as compared to reference group of rats ($p \leq 0.05$ for low dose group and $p
\leq 0.01$ for high dose group).

Figure 3.11 shows results of cresyl violet staining of brain. Control brain sections showed
nerves with prominent nuclei. Hippocampal region of brain also showed more intense staining
of cresyl violet, indicating greater neuronal cell density (Figure 3.11A). In the treatment
groups, at the end of 90 days of study period, the area of the cerebral cortex was observed to be
decreased compared to control as revealed by the cresyl violet staining of treated rat brain of
high dose group. Neuronal cell density was observed to be decreased as compared to the
control brain and clear pyknosis was also observed in brain sections of the treatment group in
the subchronic study (Figure 3.11B). Further investigation of neuronal damage was done by
transmission electron microscopy to assess demyelination and axonal loss in treated animals.
The demyelinated nerve in thiacloprid intoxicated CNS and sciatic nerve showed extensive
loss of myelin around axon, hypomyelination and vacuolar degeneration (Figure 3.12C-3.12F),
whereas the neurons in the control group were found to have intact myelin sheath (Figure

Histopathological evaluation of the brain of the control animals revealed intensely stained
neurons in cerebrocortex area and more granules in granular area of hippocampus and
thalamus in rat brain (Figure 3.13 I&II), whereas repeated exposure of thiacloprid for 90 days
developed axonal swelling, vacuolar degeneration, spongiform changes and pyknotic nuclei in
neuron in treated rat brain (Figure 3.14 V-VIII). Minimal haemorrhages, vacuolar degeneration
and mild gliosis with spongiform of changes were profoundly observed in the brain of treated
rat (Figure 3.13 III&IV).

Figure 3.6 shows percent inhibition of IMR 32 neuroblastoma cell line exposed with
thiacloprid. MTT assay was performed while assessing thiacloprid cytotoxicity for IC50 value.
The calculated IC50 value of thiacloprid for IMR 32 cell line is 7.02±1.3µg/ml (27µM).
Esterase activity of the cells was also assessed by exposing them to 0.35µg/ml and 0.7µg/ml
thiacloprid for different time intervals. AChE activity was found to gradually decrease with
increasing time of thiacloprid exposure and concentration. Statistically significant decrease in activity was observed at 60 hrs in cells given 0.35µg/ml and 0.7µg/ml of thiacloprid exposure (p ≤ 0.05) Table 3.5, Figure 3.7). Similar trend of decreased activity with increased time of exposure and concentration of test compound was also observed for NTE activity, however, decrease in activity was only observed to be significant with 0.7µg/ml of exposure for 60 hrs (p ≤ 0.05) (Table 3.5, Figure 3.8).

Figure 3.15 show acridine orange-ethidium bromide staining of IMR 32 cells. Figure 3.15B shows that thiacloprid-exposed cells experienced greater degree of cell-death as compared to control cells. Cells emitting green fluorescence are viable cells and those emitting red fluorescence are necrotic cells. Moreover, the test compound treated cells were observed to have less neurite processes, due to which proper growth of these cells could not have been achieved. Due to decreased elongation of processes, the communication between cells was also diminished as compared to that in control cells (Figure 3.16 A&B).

**DISCUSSION**

The current study was aimed at finding the possible neurotoxic effects of the neonicotinoid thiacloprid on the mammalian system. One of the many analyses carried out in this regard was assessing the acetylcholinesterase activity in thiacloprid intoxicated rats. It was observed that rats given subacute exposure to thiacloprid showed increased AChE activity in blood and plasma and decreased brain AChE activity, even though the changes were not statistically significant. Thiacloprid works as an agonist to nAChR and binds with it, stimulating the nerve. This nerve activation, it is proposed, induces a signal to release the substrate from the receptor and hence, AChE levels tend to elevate to cleave the substrate from the receptors of stimulated nerve (Kimura-Kuroda *et al.*, 2012). This probably may be the reason for the observed increase in AChE activity in blood and plasma of treated rats. However, activity of AChE in brain of treated rats was found to be decreased in the 28 days study (not significant) and also in 90 days study (significant decrease). In similar studies done by Rodrigues *et al.* (2010), decreased activity of acetylcholinesterase in rat brain and decreased choline uptake in synaptosomes from hippocampus area of rat brain treated with cyanogroup of neonicotinoid thiamethoxam was observed. Bhardwaj *et al.* (2010) also reported significant decreased activity of AChE in the brain of female rats given 90 days repeated exposure of imidacloripid at a dose of 20mg/kg body weight. Chao and Casida (1997) reported the accumulation of imidacloripid in mouse brain following direct intra-peritoneal administration. However, Brunet *et al.* (2004) have also
reported that neonicotinoid is highly absorbed in human intestinal cells, suggesting its potential anticholinergic effects. The cause of this inhibition is unknown because neonicotinoid is not ChE inhibitor, since plasma AChE is synthesized in the liver, the decrease in plasma AChE activity may be related to observed changes in liver function (EPA, 2006a). Results of the current study were also supported with localization of AChE in frozen sections of brain, which was observed to be much less intense in thiacloprid treated rat brain as compared to control. This pattern was more prominent in high dose treatment group of experimental animals in the subchronic study. Similar localization studies related to the toxic effects of neonicotinoids have not been reported.

Neurotoxic target esterase (NTE) activity in rat brain was also analyzed in present study. Activity of NTE was observed to be decreased in treatment group of rats in both 28 and 90 days studies. However, decrease was significant only for animals of high dose treatment group in the subchronic study. Similar studies about thiacloprid or any other neonicotinoid pesticide have not been reported till date. Nevertheless, the above results could emerge as a pertinent reason for observed neuronal damage in thiacloprid intoxicated rats.

In this study, rats exposed to thiacloprid for 28 days and 90 days were observed to attain immobility earlier as compared to control rats in the forced swimming test (FST), an indication of lesser coordination in movement of legs. The attainment of early immobility caused by thiacloprid in the FST can be attributed to a locomotors effect caused by this test compound, since there was a change in total locomotion after thiacloprid administration. During the FST of rats given 90 days of exposure to the test compound, it was observed that the rats started dipping in water in much less time as compared to control and eventually became rather inactive (floating) and were unable to make an effort to swim. In addition to the inactivity observed during the FST, it was also observed that thiacloprid treated rats developed a dragging locomotion and even poking the animal with a blunt object did not cause the animal to respond and move further. This observation has been video-graphed. Such a significant decrease in locomotor activity in the rats treated for 90 days with thiacloprid could be an indication of the accumulation of thiacloprid or its metabolites in the brain. This discussed notion does not find any support from any report published with regard to thiacloprid. However, it has been reported that oral exposure of neonicotinoid imidacloprid for 90 days at different doses in female rats resulted in significant alteration in various aspects of spontaneous locomotor activity. A significant decrease in distance travelled, ambulatory time
and stereotypic time was noted after 90 days in animals exposed with 20 mg/kg/d imidacloprid (Bhardwaj et al., 2010).

Hamm et al. (1994) had suggested that compared to other behavioural tests, the rotarod test is a more sensitive and efficient index for assessing motor impairment produced by brain injury. The present study on rats exposed to thiacloprid for 90 days showed decreased neuromuscular coordination which was reflected as increasing number of falls from the rotating rod as compared to the control animals. It was found that thiacloprid treated rats could not keep balance on the rod at 25 rpm. This observation could be co-related to the development of ataxia in thiacloprid exposed rats in the subchronic study as mentioned earlier. Significant sensorimotor impairments similar to the current results have been reported in female rats after exposure to a single dose of imidacloprid (Abou-Donia et al., 2008). The motor and behavioural alterations reported here could also be related to alterations in the brain chemistry of animals treated with thiacloprid. Development of ataxia in rats could be a cumulative result of thiacloprid intoxication effects like neuronal damage, thyrotoxicosis and lack of neuromuscular coordination which lead to dysdiadochokinesia observed in the rotarod test. Barnard et al. (1971) have even suggested a preclinical picture of cerebellar syndrome with dominant ataxia of gait and less ataxia of the limbs occurring in patients with hypothyroidism. A sensorimotor disorder associated with depression and characterized by a strong urge to move the legs associated with paresthesias and motor restlessness has also been described in patients with chronic kidney disease caused by the medication used which proved to be neuropathic (Szentkiralyi et al., 2009; Cavallini et al., 2010). It is possible that thiacloprid could possibly act as a depressant also. Tomizawa (2004) suggested that stimulation of the nervous system by acute or sustained exposure to neonicotinoid chemicals may lead to synaptic plasticity or attenuated neuronal functions.

It is reported that such neurobehavioral deficits may reflect dysfunction at multiple anatomical areas in the central nervous system. Brain injury or damage may result into abnormal sensorimotor coordination (Hamm et al., 1994). With this notion, neuronal damage was assessed in the current study by cresyl violet staining. Cresyl violet stains Nissl bodies of neuron. Nissl bodies represent aggregations of rough endoplasmic reticulum, containing numerous ribosomes involved in the synthesis of neurotransmitter such as acetylcholine (Bear et al., 2007). Hippocampus area of brain of thiacloprid treated rat exhibited pyknosis and less density of neurons as compared to control rats when stained by cresyl violet indicating
neuronal damage. In vivo studies in rat as well as in vitro studies have suggested links between decreased hippocampal neurogenesis and depression (Malberg et al., 2000; Manev et al., 2001), which support the behavioural observations noted in the present study. Trauth et al. (1999) also reported evidence supporting hippocampal cell damage in female rats exposed to nicotine. The inhibitory effect on AChE activity due to thiacloprid intoxication can be correlated with results of neuronal cresyl violet staining, which stains RNA (Nissl body involved in acetylcholine synthesis).

Further, neuropathy target esterase (NTE) activity was also estimated in the brain of the experimental animals. NTE is an integral membrane protein in vertebrate neurons (Glynn, 1999) and it plays an important role in neural development, possibly via involvement in a signalling pathway between neurons and glial cells. In the current study, NTE activity was found to be decreased in the thiacloprid treated rats indicating neuronal damage of the brain in these animals. This decrease was significant, as compared to control animals, for the animals given subchronic exposure to the test compound. Vacuolated myelin degeneration of neuron was observed in TEM images. NTE inhibition is also related to localized accumulation of lysolecithin, a known demyelinating agent and receptor-mediated signal transducer (Quistad et al., 2003). In the present study, the myelination status of the nervous tissue was also assessed in the experimental animals. As expected, the thickness of myelin was found less in the treated group of animals compared to control neurons. It is reported that on exposure to organophosphorous, negatively charged phosphate group attaches to the active site of serine, triggers demyelination by inhibiting catalytic activity of NTE and AChE (Lotti and Moretto, 2005). Researchers have suggested that myelin degradation is induced via Ca\(^{2+}\) influx into myelin and subsequent activation of cytosolic phospholipase A\(_2\) and calain, which break down the myelin lipids and protein and ultimately lead to excessive stimulation of Ca\(^{2+}\)-dependent degradative pathways (Fu et al., 2007; Trapp and Stys, 2009). Significant excitatory Ca\(^{2+}\) influxes have been reported to be evoked by acetaminophen, imidacloprid and nicotine at concentrations greater than 1µM in small neurons in cerebellar cultures that expressed the mRNA of the α3, α4, and α7 nAChR subunit (Zoli et al., 1995; Kimura-Kuroda et al., 2012). Hypothetically speaking, the reasons discussed above may also probably be true for the neurotoxic mechanism of thiacloprid in causing demyelination of neurons.

The pattern of myelin degeneration observed in the treatment group in the present study is comparable to Alexander disease, a foetal neurodegenerative disease in which dysplasia of
myelin occurs as a vacuolar pattern which leads to neuronal degeneration in humans (Roessmann et al., 1980). Neuronal degeneration in this disease is due to the lack of glial fibrillar acidic protein (GFAP). GFAP is proposed to play a role in cell-cell communication and is also known to modulate phosphorylation at various serine or threonine residues by PKC and PKA which are two kinases important for the cytoplasmic transduction of signals (Tuccari et al., 1986). This mechanism may also be one possibility in the present study, causing inhibition of AChE and axonal degeneration by inhibition of GFAP. It has also been reported that loss of GFAP in nerve impairs Schwann cell proliferation and delays nerve regeneration after damage. In many peripheral neuropathies, axonal loss causes disabling and permanent deficits and may result from inefficient nerve regeneration due to a defective relationship between Schwann cells, axons and the extracellular matrix. These interactions are mediated by surface receptors and transduced by cytoskeletal molecules (Triolo et al., 2006).

AChE inhibition and NTE inhibition due to thiacloprid exposure induced histopathological changes in brain as could be concluded from the histological observations made for rat brain. Axonal swelling, pyknotic nuclei brain cells with loss of granules in granular layer of hippocampus and thalamus region of brain were the several pathological changes observed in the brain of treatment group of animals. In the absence of published reports it could be hypothesized that demyelination may be the result of axonal swelling which in turn disturbs the integrity of myelin sheath. Mild focal vacuolar degeneration and gliosis with spongiform change was also observed in treated rat cerebrum. Gliosis is a nonspecific reactive change of glial cells in response to damage to the central nervous system (CNS) and the process of gliosis involves a series of cellular and molecular events that occur over several days (Fawcett and Asher, 1999). Proliferation of astrocytes is increased in gliosis which accompanies traumatic brain injury as well as many neuropathologies (Zhang et al., 2010; Rivera-Zengotita and Yachnis, 2012). Microglia is one of the glial cells which is increased during gliosis and induce the release of neurotoxic factors that promote increased degeneration of the neuron and more rapid phagocytosis by the microglia (Streit et al., 1999). Oligodendrocytes are another type of glial cell which generate and maintain the formation of myelin around the axons of large neurons in the CNS, allowing for rapid transmission of neural signals. Unlike astrocytes and microglia, oligodendrocytes undergo a much more limited reaction to injury (Rivera-Zengotita and Yachnis, 2012).
The degeneration of axons as a result of trauma or pathology invariably results in the degeneration of the myelin sheath (Fawcett and Asher, 1999). Earlier studies have shown that concentration of pesticides and metabolites in plasma and brain generally correlate with the severity of toxicity and symptoms of neurotoxicity which are found to increase with the pesticide concentration in brain (Nagata et al., 1996). The histopathological changes observed in the brain of treated rats in the current study also provide support to the neurobehavioural effects observed earlier indicating accumulation of thiacloprid and its metabolites in the brain (although kinetic studies of thiacloprid have not been done). Pathological alteration due to the exposure of imidacloprid for the period of 90 days has also been observed in rat by Bhardwaj et al. (2010). Nicotine exposure to brain tissue reportedly leads to nAChR induced apoptotic cell death in hippocampal area of brain (Berger et al., 1998). Carlson et al. (2000) have also observed nicotine induced axonal degeneration in brain caused due to several days of its administration.

Moreover, it is well documented that transient but significant expression of nAChRs during the perinatal stage is important for brain development (Role and Berg, 1996; Dwyer et al., 2009). In the developing brain, α4β2 and α7 subtypes of the nAChR have been implicated in neuronal proliferation, apoptosis, migration, differentiation, synapse formation, and neural-circuit formation. Due to nicotine-like mechanism of neonicotinoids, they are likely to affect these important processes when they activate nAChRs (Role and Berg, 1996; Dwyer et al., 2009). *In vitro* effects related to thiacloprid exposure on a human cell line may be useful in predicting *in vivo* alteration in the animal model. Thus, an *in vitro* study was also performed to observe the neuronal growth-related damage due to thiacloprid exposure, which can be useful in understanding observations made in the *in vivo* study and can also be correlated with the brain development process of neonates. For this study, neuroblastoma IMR 32 cell line was selected because neuroblastoma is a childhood solid tumor composed of primitive cells derived from precursors of the autonomic nervous system. This neoplasm has the highest rate of spontaneous regression of all cancer types and has been noted to undergo spontaneous and chemically induced differentiation into elements resembling mature nervous tissue. As such, neuroblastoma has been a prime model system for the study of neuronal differentiation (Abemayor and Sidell, 1989).

In the present study, thiacloprid-induced percent cell-inhibition concentration (IC50) was measured to be 7.02±1.3µg/ml (27µM) in human neuroblastoma cell line using MTT assay.
Tomizawa and Casida (2000) have also reported a nearby IC50 value in mouse fibroblast M1 cell line. IC50 value reported for imidacloprid was 70µM and that for thiacloprid was 19µM in fibroblast M1 cells. Results given by Tomizawa and Casida (2000) suggested that thiacloprid is a more potent toxicant than imidacloprid. It is also mentioned in this study that the descyano metabolite was more potent than the parental thiacloprid for M1 cells. In metabolically active cells, MTT is reduced by mitochondrial succinate dehydrogenase to give a dark purple-coloured product (Mosmann, 1983). According to the calculated IC50 value of thiacloprid obtained in the present study, it can be concluded that thiacloprid can lead to mitochondrial dysfunction at very low dosage and develop neurotoxicity by disturbing the developing neurons.

Subsequently, we estimated the esterase activity in IMR 32 cells exposed to 1/10th (0.35µg/ml) and 1/20th (0.7µg/ml) of IC50 value of thiacloprid for different time intervals. Thiacloprid exposure for 60 hours significantly inhibited the activity of acetylcholinesterase in both the studies of different concentrations of exposure, indicating that within 60hrs thiacloprid could bind with nAChR and inhibit the choline uptake from the synaptosome of developing cells. NTE activity was also observed to be significantly low in these cells on 60hrs of exposure at a concentration of 0.7µg of thiacloprid. The neonicotinoid imidacloprid has also been reported to act as an agonist or an antagonist of nAChRs at 10µM in rat pheochromocytoma (PC12) cells (Nagata et al., 1998) and to change the membrane properties of neurons at ≥10µM in the mouse cochlear nucleus (Bal et al., 2010).

IMR cells exposed to these different thiacloprid concentrations were also observed for the neurite growth. At higher concentration we observed that neurite growth was inhibited and elongation of cell processes was very less and not properly attained among the cultured cells which may also probably disturb communication between these cells. Moreover, acridine orange and ethidium bromide staining of the cells evidently showed more cell death in thiacloprid exposed cells as compared to control cells. The dead cells emitted red fluorescence by taking ethidium bromide while live cells were stained with acridine orange and gave green fluorescence. These observations being the first of its kind could not be supported with reports on any studies related to such pesticides. However, recent studies have reported that gestational nicotine exposure modulates the cell-adhesion and cell-death/survival systems in the brains of adolescent rats and may lead to numerous behavioural and physiological deficits (Cao et al., 2011; Wei et al., 2011).
CONCLUSION

Administration of thiacloprid caused adverse motor and behavioural alterations. Based on the results of the behavioural tests, it can be believed that thiacloprid may act as a depressant. The current study is the first to report that thiacloprid has enough potential to cause neuronal damage and inhibit the acetylcholinesterase and neuropathy target esterase activities. Cresyl violet staining of the brain tissue gave clear indication about the hippocampus neuronal pyknosis and damage as well as less neuronal density in brain caused by thiacloprid administration. Thiacloprid was also found to be a potent toxicant for growing neuronal cells as could be established from results of the in vitro study. The neuroblastoma IMR 32 cell growth inhibition could be concluded from observations of disruptions in neurite elongation and cell communication. Based on the results of the in vitro study, it is possible that thiacloprid has the potential to damage the developing foetus. Thyroid hormone is known to play a role in normal brain development and it has already been observed that thiacloprid also results in development of thyrotoxicosis (Chapter 2). Further, the pathological changes in brain and specific AChE and NTE inhibition observed in the present study suggest that multiple brain region abnormalities may be involved in disruption of neuromuscular coordination, which is reflected as abnormal locomotor activity. Therefore, we can conclude that thiacloprid may potentially affect human health adversely, especially the developing brain. Moreover, such a toxicant that affects AChE levels or acts as agonist or antagonist at the nicotinic ACh receptors may negatively influence the latent processes of children’s neurological function.
### Table 3.1. Effect of thiacloprid administration on acetylcholinesterase activities in various tissues of SD rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Subacute (µM ATC/min/dl)</th>
<th>Subchronic (µM ATC/min/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Blood</td>
</tr>
<tr>
<td>Control</td>
<td>0.63±0.09↑</td>
<td>0.68±0.1</td>
</tr>
<tr>
<td>Low dose</td>
<td>0.58±0.1</td>
<td>0.84±0.12</td>
</tr>
<tr>
<td>High dose</td>
<td>0.67±0.1</td>
<td>1.1±0.29</td>
</tr>
</tbody>
</table>

### Table 3.2. Neurotoxic esterase activity in the brain of thiacloprid intoxicated SD rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subacute</td>
<td>2.6±0.13↑</td>
<td>2.1±0.16</td>
<td>2.0±0.25</td>
</tr>
<tr>
<td>Subchronic</td>
<td>2.4±0.11</td>
<td>1.9±0.14</td>
<td>1.8±0.16↓*</td>
</tr>
</tbody>
</table>

### Table 3.3. Effect of 90 days exposure of thiacloprid on neuromuscular coordination using rotarod test

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (Latency) in minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 rpm</td>
</tr>
<tr>
<td>Control</td>
<td>2.4±0.03↑</td>
</tr>
<tr>
<td>Low dose</td>
<td>2.3±0.07</td>
</tr>
<tr>
<td>High dose</td>
<td>2.1±0.15</td>
</tr>
</tbody>
</table>

Values are expressed as Mean±SE; n=5 for each group; *p≤0.05; ** p≤0.01

### Table 3.4. Time to float during the forced swimming test in control and thiacloprid treated rats

<table>
<thead>
<tr>
<th>Group N=5</th>
<th>Immobility time (In second)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0 day</td>
<td>64.8±0.9↑</td>
</tr>
<tr>
<td>28 day</td>
<td>65.2±0.8</td>
</tr>
<tr>
<td>90 day</td>
<td>66.4±0.9</td>
</tr>
</tbody>
</table>
Table 3.5. Effect of thiacloprid on esterase activity in proliferative neuroblastoma cells at different time interval

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Hours of exposure</th>
<th>24hrs</th>
<th>36hrs</th>
<th>48hrs</th>
<th>60hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.18±0.007@</td>
<td>0.18±0.008</td>
<td>0.19±0.01</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>AChE</td>
<td>0.35μg</td>
<td>0.18±0.008</td>
<td>0.15±0.013</td>
<td>0.14±0.012</td>
<td>0.13±0.013↓*</td>
</tr>
<tr>
<td></td>
<td>0.7 μg</td>
<td>0.15±0.008</td>
<td>0.14±0.01</td>
<td>0.11±0.014</td>
<td>0.1±0.015↓*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.2±0.12@</td>
<td>3.3±0.12</td>
<td>3.3±0.11</td>
<td>3.4±0.13</td>
</tr>
<tr>
<td>NTE</td>
<td>0.35μg</td>
<td>3.3±0.17</td>
<td>3.1±0.17</td>
<td>2.9±0.1</td>
<td>2.7±0.18</td>
</tr>
<tr>
<td></td>
<td>0.7 μg</td>
<td>3.2±0.12</td>
<td>2.9±0.14</td>
<td>2.9±0.12</td>
<td>2.5±0.13↓*</td>
</tr>
</tbody>
</table>

@Values are expressed as Mean ± SEM; n=5 for each group; *p≤0.05; ** p≤0.01
Figure 3.1. Effect of subacute thiacloprid exposure on AChE activity in rat

Figure 3.2. Effect of subchronic thiacloprid exposure on AChE activity in rat
Figure 3.3. Effect of thiacloprid exposure on NTE activity in rat brain

Figure 3.4. Effect of thiacloprid on neuromuscular coordination and grip strength
Figure 3.5. Immobility time recorded for animals of various groups during forced swimming test

Figure 3.6. Effects of thiacloprid on cell viability of IMR cells
Figure 3.7. Effect of thiacloprid on AChE activity in IMR 32 cell line at different time intervals

![Graph showing the effect of thiacloprid on AChE activity in IMR 32 cell line at different time intervals.](image)

Figure 3.8. Effect of thiacloprid on NTE activity in IMR 32 cell line at different time intervals

![Graph showing the effect of thiacloprid on NTE activity in IMR 32 cell line at different time intervals.](image)
Figure 3.9 Histochemical localization of acetylcholinesterase (AChE) activity in the rat brain (40X).

(A) AChE activities are localized in the brain of control rat (in brown color); (B) weak acetylcholinesterase activity was found in treated brain.
Figure 3.10 Histochemical localization of AChE activity in hippocampal area of rat brain (40X).

(A) Control hippocampal region AChE localized more due to its activity, (B) Treated section with less localization due to inhibition, deficiency of enzyme activity marked by blue arrow.
Figure 3.11 Nissl stained cerebrum of rat (40X).

(A) control section with darkly stained Nissl body by cresyl violet, inset photograph of viable brain cell with Nissl body (B) Treated section showing necrotic cell (black arrow) and pyknotic cell (green arrow) at hippocampal region in inset photograph.
Figure 3.12 Transmission electron micrograph of nerve stained by lead uranyl acetate.

Control A(A and B): control animals, (C-F): thiacloprid treated animals. (A) Normal rats showing axon (Ax), myelin (M) and Schwann cell (Sc) (4000x). (B) Thickness and appearance of myelin in control rat (22000x). (C) Dysplasia of myelin sheath, disordered myelin layer structure, and vacuolar degeneration in thiacloprid treated rat (4000x) (D) Axon retraction (shrinking) and myelin degeneration (E) Detachment of myelin layers from axons (arrow), and severe loss of compact myelin. (E) Axonal separation from the myelin(8000x) (F) Crystallization of axoplasm, loss of mitochondrial membrane potential (arrow) (b) and complete separation from the myelin (14000X).
Figure 3.13 Light micrographs of the control and treated rat brain tissues.

Control group (I and II). Thiacloprid treated group (III and IV). Tissue sections were stained with H&E. I: Control showing the various cells of cerebrum (A: Astrocytes, N: Neuron cell body, P: Purkinje cells, Py: Pyramidal cells) [40X]; II: Control showing the Thalamic region of brain [40X]; III: Treated rat brain showing minimal Haemorrhages [10X]; IV: Treated rat brain showing Gliosis (G) and Vacuolar degeneration (V) [10X].
Figure 3.14 Light micrographs of the brain tissue of Thiacloprid treated group.

V to VIII (Thiacloprid treated group): Tissue sections were stained with H&E. V: Vacuolar degeneration (V) [20X]; VI: Axonal swelling (AS) [20X]; VII: Spongiform degeneration (SP) [100X]; VIII: Pyknotic nuclei (PN).
Figure 3.15 Florescent microscopy images of IMR 32 cells (AO/EB staining, 40X).

(A) Cell with green fluorescent is for viable cells of control; (B) Thiacloprid treated cells-Viable cells are with green fluorescent and nonviable are with red fluorescent (Blue arrow).
Figure 3.16 IMR 32 neuroblastoma cells (40X).

(A) Control cells: elongated neurite processes and healthy growth of cells; (B) Thiacloprid exposed cells with less neurite process elongation by inhibition of growth (black arrow).