Chapter II

MATERIAL AND METHODS
Behaviour is the integrated final product of a highly complex nervous system. It may be altered in diverse ways by xenobiotic influences. Most behavioural alterations cannot be measured and interpreted easily. Laboratories the world over use tests such as the functional observational battery (FOB), the neurobehavioural test battery (NTB) and measures of motor activity accompanied by neuropathology, at the initial stage of neurobehavioural toxicity testing. At the later stage, tests are employed to measure and quantify more specific aspects of animal behaviour such as motor activity, sensory and cognitive functions.

This investigation was limited to the assessment of developmental and adult neurobehavioural toxicity of Al in rats.

**Animals and Housing**

Wistar rats were used for the experiments. The animals were obtained from the departmental animal house and bred in the laboratory. The animals were housed in plastic cages (4-5 rats in 25 × 35 cm sized cage). Food (Gold Mohur, Hindustan Lever, Bangalore, India) and tap water were provided *ad libitum*.
The animals were maintained on a 12 h light/dark cycle, with the lights switched on at 06:30 h and switched off at 18:30 h. Illumination was provided by two 40 W fluorescent lamps. The room temperature was 25±2°C.

Chemicals

The following chemicals were used in the experiments.

Aluminium chloride hydrated (AlCl₃·6H₂O; purity 98%, S.D. Fine-Chem Pvt. Ltd., India)
Sodium hydroxide (NaOH; S.D. Fine-Chem Pvt. Ltd., India)
Sodium chloride (NaCl; S.D. Fine-Chem Pvt. Ltd., India)
Neurobehavioural Toxicity of Aluminium

The neurobehavioural toxicity of Al on rats was investigated in two sets of experiments. In the first set, the developmental neurotoxicity and teratogenicity of Al was investigated wherein the offspring of treated female rats were tested, while in the second set of experiments, neurobehavioural toxicity of Al was assessed in adult male rats.

Dose determination

The choice of the Al doses was based on preliminary investigations and on previous studies on Al-induced behavioural changes in mammals (Colomina et al., 1998; Colomina et al., 1999a; Clayton et al., 1992; Yokel, 1985; Yokel, 1989; Rankin and Manning, 1993).
DEVELOPMENTAL NEUROTOXICITY AND TERATOGENICITY OF ALUMINIUM

Pregnant rats were administered Al and their offspring were tested using various behavioural tests. A slightly modified version of the Fox scale was employed; the behavioural endpoints used in the neurobehavioural test battery were righting reflex, placing reflex, suspension test, and auditory startle response. Other tests used were, the stress tolerance test, the motor coordination test (rotorod test), besides measurement of ultrasonic vocalizations, the open-field test, the elevated plus-maze test and the Morris water-maze test to assess activity, emotional reactivity and spatial learning deficits. The detailed schedule of the experiments has been provided in Table 2.1.

Treatment

Eight female rats aged about five months were mated with untreated males. The day when vaginal plug was observed was designated day 1 of gestation. The pregnant rats were individually housed in plastic cages. Food and tap water was provided ad libitum.
The pregnant rats were divided into four experimental groups with two rats in each group. On days 14, 15 and 16 of gestation, Groups I, II and III received aluminium chloride (AlCl₃·6H₂O) intraperitoneally (i.p.) in saline vehicle (0.9% NaCl) buffered with 0.1 N NaOH, at a dose of 5, 10 and 20 mg Al/kg body weight respectively, while the vehicle Control Group (Group IV) was administered only an equal volume of saline solution.

Maternal weights were recorded on days 14, 15 and 16 of gestation. Pregnant females were checked twice daily for the birth of pups. The day of birth of pups was designated as post-natal day 1 (PND 1). The litter size and body weight of the pups was recorded at birth. The rat pups born of the Treated and Control female rats were then put through a series of tests to assess different aspects of behavioural teratogenicity.

**Definitions/Descriptions of Behavioural Variables:**

- **Rear** (measure of vertical movement) – rat raising two forepaws off the ground

- **Lean** (measure of vertical movement) – rat leaning against maze wall with two forepaws
- Face wash – movement of paws over ‘face’
- Groom – movement of paws over body
- Freeze – rat suddenly remaining still with raised head for a brief period (less than a second to two seconds) before resuming its activities
- Ambulation/Line crossing (measure of horizontal movement) – rat crossing a line with fore and hind legs

Table 2.1. Schedule of developmental aluminium neurotoxicity experiments.

<table>
<thead>
<tr>
<th>Event/Experiment</th>
<th>Post-natal Day/s (PND/s)</th>
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<tr>
<td>Treatment in pregnant rats</td>
<td>Days 14, 15 and 16 of pregnancy</td>
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<tr>
<td>Birth of offspring</td>
<td>PND 1</td>
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<tr>
<td>Recording of Ultrasonic vocalizations, Measurement of Line crossing, Face washes and Rearing</td>
<td>PNDs 4, 10, 15, 18</td>
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<td>Tests for Righting Reflex, Placing Reflex, Auditory Startle Reaction, and Suspension test, Stress Tolerance test</td>
<td>PNDs 32</td>
</tr>
<tr>
<td>Motor Coordination test</td>
<td>PNDs 32, 45 and 100</td>
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<tr>
<td>Acquisition of the Morris Water Maze Escape task</td>
<td>PNDs 89 to 95</td>
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<tr>
<td>Assessment of Open Field Behaviour</td>
<td>PNDs 96 to 100</td>
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<td>Assessment of the Elevated-Plus Maze task</td>
<td>PNDs 96 to 100</td>
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BEHAVIOURAL TESTS

ULTRASONIC VOCALIZATIONS (USVs)

Rat pups emit ultrasonic vocalizations (USVs) in the 40-50 kHz range between the ages of 1 and 20 days when isolated from its dam and littermates (Brunelli, et al., 1996, Okon, 1971). Ultrasonic calling can be used as a sensitive measure in the evaluation of behavioural teratogenicity (Rankin and Manning, 1993). The ultrasonic vocalization of rat pups (and of infants of other mammalian species) responds sensitively and specifically to anxiolytic and anxiogenic compounds. Anxiolytic compounds decrease ultrasonic isolation calling, whereas anxiogenic compounds increase isolation calling (Brunelli, et al., 1996).

Ultrasound detector

A bat detector (QMC Minibat Detector, England – Fig. 2.2) set to 40-50 kHz was used for the detection of ultrasonic emissions that were transduced to audible signals. Earphones were used to avoid feedback effects on the pups.
Ultrasonic vocalizations recording

Ultrasonic vocalizations were recorded on post-natal days (PNDs) 4, 10, 15 and 18. The testing was carried out in a test chamber (18 x 15 x 18 cm) painted black, with the floor marked with white lines into a grid of six equal rectangles (Fig. 2.1). The test chamber was open at the top. The USV detector was suspended 18 cm above the chamber floor.
Fig. 2.2. Ultrasound detector QMC Minibat Detector, England

Fig. 2.3. Rat pup in Ultrasonic vocalization test chamber
Ultrasonic vocalization testing was done between 1100 and 1400 h. The room temperature was 25.5±1°C. Each test trial was for a period of 2 min. Before testing, each pup was gently separated from the dam and littermates, and was kept isolated for the period of 5 min in an isolation cage whose temperature was maintained at normal nest levels (35-37°C). Then the pup was placed in the temperature regulated test chamber. The ultrasonic vocalizations were scored manually. Scoring was delayed for 30 s to allow any immediate handling response to die down.

**Measurement of Other Behavioural Variables**

The following behavioural variables were also measured in the pups at the same time in addition to ultrasonic vocalizations.

- Number of lines crossed (ambulations) in the test chamber as a measure of horizontal activity (Fig. 2.3),
- Number of rears and leans as a measure of vertical activity,
- Number of face washes.
NEUROBEHAVIOURAL TEST BATTERY

On post-natal day 32 (PND 32), tests for the righting reflex, placing reflex and auditory startle reaction, as well as the suspension test and the stress tolerance test were conducted. The motor coordination test was conducted on post-natal days 32, 45 and 100.

Test for Righting Reflex

The pup was placed on its back on a 30 × 30 cm level platform covered by a black paper. The righting response was defined as the ability of the pup to return to four feet from lying on the back within 2 s.

Test for Placing Reflex

The pup was lifted by holding its back and dropped from a height of 10 cm. The pup was considered to have successfully passed the test when it landed on its four feet.

Test for Auditory Startle Reaction

The pup was placed on a 30 × 30 cm level platform. Auditory stimulus was administered in the form of a clap 30 cm behind
the animal's back. The pup was considered to have successfully passed the test when it showed an instant startled response, that is, a jerk of the head and extension of the hind limbs.

**Suspension Test**

The pup was placed on the 30 x 30 cm level platform. Its forefeet were touched with a wood stick (diameter = 0.8 mm) held horizontally, which elicited an immediate grabbing reaction. The pup was considered to have successfully passed the test if it hung on to the stick for 5 s.

**Stress Tolerance Test**

A cylindrical glass container (diameter = 26 cm) containing 15 cm column of water (temperature = 26°C) was used for this test. A naïve rat offspring was placed in the water for 5 min. After swimming around in the container searching for an exit, the naïve animal became immobile for sometime before searching again for the exit. The total duration of immobility was recorded.
**Motor Coordination Test**

The motor coordination test was conducted on post-natal days 32, 45 and 100. A kymograph set (INCO, Elect E-8, Instruments and Chemicals Pvt. Ltd., India.) was used. The animal was administered three trials on each testing day. For each trial, the rat was placed on the kymograph rod which was rotating at the rate of 40 rpm. The interval between placement and falling of the animal was recorded.

**Morris Water Maze Task**

The Morris water maze (Fig. 2.4 and Fig. 2.5) consisted of a galvanized circular tank (diameter = 150 cm, height of wall = 50 cm) filled with water up to 31.5 cm (distance between the edge of tank and water level = 18.5 cm). The inside walls and the floor of the tank were painted black. The temperature of the water was 22±3°C. The escape platform (diameter = 12 cm) was submerged 1 cm below the water level. A visual cue was affixed to the platform. The escape platform was placed 25 cm from the wall of the tank. Illumination was provided by a 40 w fluorescent tube light 180 cm above the water level.
The rats were tested on the water maze from post-natal days 89 to 95. All testing was carried out between 0900 h to 1700 h. The rats were first subjected to a habituation session on PND 89, while testing for acquisition was conducted from PND 90 to PND 95.

In the habituation session, the animals were twice allowed to swim in the tank for 60 s and climb onto an unmarked hidden platform located at any one of the predetermined positions marked 1 – 8 (Figs. 2.4 and 2.5). The rat was guided to the escape platform if it was unable to locate it in 60 s.
Fig. 2.5. Morris water maze with escape platform and the visible cue on it.

Fig. 2.6. Morris water maze test: Rat on the hidden escape platform marked with visible cue.
Acquisition testing

The animals were administered five trials a day for 5 days (PND 90 to PND 94) and three trials on the sixth day (PND 95). The body weights were recorded before beginning of the first trial each day. At each trial, the rat was gently placed into the water from a predetermined starting position (marked 1 - 8) from near the tank wall (Figs. 2.4 and 2.5), facing the center. The amount of time (escape latency) the rat took to locate and climb on to the escape platform marked by a visible cue (Fig. 2.6) was recorded. The escape platform was located at one of the remaining seven positions.

The maximum length of each trial was 60 s and the inter-trial interval was 40 min. As in the habituation session, the rat was guided to the escape platform if it was unable to locate it in 60 s. For each trial, the platform location and the starting position of the rat were independently varied in a semi-random manner. (There was no change in the platform location and the starting position for the rats of all the groups - for a given trial, all the rats had the same platform location and starting position). After the third trial on PND 95, a single no cue probe (NCP) trial was
administered during which the visible cue was absent. The purpose was to investigate whether the rats were guided to the escape platform by the visible cue or by other spatial cues.

The escape latencies were recorded with the aid of a stopwatch by the experimenter standing at the 'N' position of the Morris water maze (Fig. 2.4).

Escape latencies measured at individual trials were analyzed by Kruskal-Wallis non-parametric ANOVA to identify group differences. Treated Groups were independently compared with the Control Group using Mann-Whitney U test.

**Open-Field Behaviour Test**

The open-field apparatus was made of plywood. It consisted of a square chamber (100 × 100 × 50 cm) painted black. The floor of the open-field was subdivided into 36 equal squares by white lines (Fig. 2.7 and Fig. 2.8). Illumination was provided by 40 w fluorescent tube light 160 cm above the open-field floor.
Testing was carried out on five consecutive days from PND 96 to PND 100. The duration of each testing session was five minutes. 

The rat was weighed before testing. It was placed in the center of the open-field and allowed to explore. The following behavioural variables were recorded.

- Number of lines crossed (ambulation) in the open-field as a measure of horizontal activity (Fig. 2.8),
Fig. 2.8. The open field testing arena with the experimental rat in the central squares.

Fig. 2.9. The elevated plus-maze test with the rat in the closed arms.
- Number of leans and rears as a measure of vertical activity,
- Total time spent in the twenty corner squares

The behavioural variables were scored manually by the experimenter sitting in front of the open-field. After each test, the open-field was wiped clean with alcohol.

**Elevated Plus-Maze Test**

The elevated plus-maze consisted of four arms arranged in the shape of a plus sign. Each arm was 50 cm long and 12 cm wide. Two opposite arms were open platform having no walls, whereas the other two were closed with 40 cm high walls. It had an open roof (Fig. 2.10). The central square measured 12 × 12 cm.

The plus-maze was elevated 35 cm above the ground. The plus-maze was made of plywood and painted black on the inside walls and the floor. Illumination was provided by a by 40 w fluorescent tube light about 180 cm above the floor of the elevated plus-maze.
Fig. 2.10. The elevated plus-maze

Testing was carried out on five consecutive days PND 96 to PND 100. The duration of each testing session was five minutes. The rat was weighed and placed in the center of the maze and allowed to explore (Fig. 2.9). The following behavioural variables were recorded.

- First open arm entry latency – the latency to enter an open arm timed from the start of the test (An arm entry is defined as the rat having all four paws in an arm)
- Number of attempts to enter open arm (an attempt is defined as the rat entering an open arm with only the fore paws and returning to the central platform or closed arm)
- Number of open arm entries
- Number of closed arm entries
- Time spent in open arms
- Time spent in closed arms
- Number of protected head-dips (a protected head-dip is defined as the rat scanning over the side of the maze toward the floor from the relative security of closed arm exit and the center platform)
- Number of unprotected head-dips (an unprotected head-dip is the scanning over the side of the maze toward the floor from the open arms)
- Number of rears
- Number of groomings
- Number of freezes

The behavioural variables were scored manually by the experimenter sitting in front of the elevated plus-maze. After each test, the maze floor and walls were wiped clean with alcohol.
NEUROTOXICITY OF ALUMINIUM IN ADULT MALE RATS

Adult male rats approximately nine months of age were used for the experiments. They were administered Al to investigate its neurobehavioural toxicity soon after administration and again in the ninth month after administration. The rats were tested on the open-field maze, the elevated plus-maze and the Morris water-maze. The schedule of the experiments has been provided in Table 2.2.

Treatment

Aluminium chloride was administered to the rats intraperitoneally in saline vehicle (0.9% NaCl) buffered with 0.1 N NaOH, for 10 days. The rats were divided into four experimental groups – Group I was administered 5 mg Al/kg/day; Group II was administered 10 mg Al/kg/day; Group III was administered 20 mg Al/kg/day and the Control Group (Group IV) was administered only an equal volume of saline solution. The rats were individually housed in plastic cages. Food and tap water was provided *ad libitum*. Testing commenced on the twelfth day (designated as Day 1) that is, on the second day after Al administration had ended.
Table 2.2. Schedule of aluminium neurotoxicity experiments in adult male rats.

<table>
<thead>
<tr>
<th>Event/Experiment</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
</tr>
<tr>
<td>Morris Water Maze Escape task</td>
<td>Days 1 to 265</td>
</tr>
<tr>
<td>Habituation</td>
<td>Day 1</td>
</tr>
<tr>
<td>Acquisition Phase</td>
<td>Days 2 to Day 7</td>
</tr>
<tr>
<td>Retention Phase</td>
<td>Days 14, 21, 35, 49, 70, 75, 260 and 265</td>
</tr>
<tr>
<td>Assessment of</td>
<td></td>
</tr>
<tr>
<td>Open Field Behaviour; and</td>
<td>Phase 1 – Days 1 to 5;</td>
</tr>
<tr>
<td>Elevated-Plus Maze task</td>
<td>Phase 2 – Days 260 to 264</td>
</tr>
</tbody>
</table>

**MORRIS WATER MAZE TASK**

The experiment consisted of two phases: an acquisition phase and a retention phase.

**Acquisition phase**

The animals were first subjected to a habituation session on Day 1. Testing for acquisition phase was conducted from Day 2 to Day 7. Five trials a day were administered for five days (Day 2 to Day 6) and three trials on the sixth day (Day 7). At each trial,
the rat was gently placed into the water from a predetermined location from near the tank wall, facing the center. The amount of time (escape latency) the rat took to locate and climb on to the platform marked by a visible cue was recorded. The maximum length of each trial was 60 s and the inter-trial interval was 40 min. As in the habituation session, the rat was guided to the escape platform if it was unable to locate it in 60 s. For each trial, the platform location and the starting position of the rat were independently varied in a semi-random manner. (There was no change in the platform location and the starting position for the rats of all the groups; for a given trial, all the rats had the same platform location and starting position).

After the third trial on Day 7, a single no cue probe (NCP1) trial was administered during which the visible cue was absent. The purpose was to investigate whether the rats were guided to the escape platform by the visible cue or by other spatial cues.

Retention phase

Trials in the retention phase of the experiment were administered in conditions similar to that of the acquisition
phase. Single daily trials were administered with an inter-trial interval of 1 week (two trials on days 14 and 21), 2 weeks (two trials on days 35 and 49), 3 weeks (one trial on Day 70) and 190 days (one trial on Day 260) to assess medium and long-term memory. Two no cue probe trials (NCP 2 and NCP 3) were administered on Day 75 and Day 265 respectively.

**Open-Field Behaviour Test**

The details of the open-field apparatus and the test conditions have been described earlier (Open-Field Behaviour Test in "Developmental Neurotoxicity and Teratogenicity of Aluminium" section).

Testing was carried out for a total of ten days – first for five days from Day 1 to Day 5 in Phase 1, and then from Day 260 to Day 264 in Phase 2. The duration of each testing session was five minutes. The body weight of the rat was recorded before testing. It was placed in the center of the open-field and allowed to explore (Fig. 2.8).
The following behavioural variables were recorded.

- Number of lines crossed (ambulation) in the open-field as a measure of horizontal activity,
- Number of leans and rears as a measure of vertical activity,
- Total time spent in the twenty corner squares

The behavioural variables were scored manually by the experimenter sitting in front of the open-field. After each test, the open-field was wiped clean with alcohol.

**ELEVATED PLUS-MAZE TEST**

The details of the elevated plus-maze apparatus and the test conditions have been provided earlier (Elevated Plus-Maze Test in “Developmental Neurotoxicity and Teratogenicity of Aluminium” section).

Testing was carried out for a total of ten days – first for five days from Day 1 to Day 5, and then from Day 260 to Day 264. The
duration of each testing session was five minutes. The body weight of the rat was recorded before testing. The rat was weighed and placed in the center of the maze and allowed to explore.

The following behavioural variables were recorded.

- First open arm entry latency - the latency to enter an open arm timed from the start of the test (An arm entry is defined as the rat having all four paws in an arm)
- Number of attempts to enter open arm (an attempt is defined as the rat entering an open arm with only the fore paws and returning to the central platform or closed arm)
- Number of open arm entries
- Number of closed arm entries
- Time spent in open arms
- Time spent in closed arms
- Number of protected head-dips (a protected head-dip is defined as the rat scanning over the side of the maze toward the floor from the relative security of closed arm exit and the center platform)
- Number of unprotected head-dips (an unprotected head-dip is the scanning over the side of the maze toward the floor from the open arms)
- Number of rears
- Number of groomings
- Number of freezes

The behavioural variables were scored manually by the experimenter sitting in front of the elevated plus-maze. After each test, the maze floor and walls were wiped clean with alcohol.

**Statistical Analysis**

Body weights were analyzed using analysis of variance (ANOVA) and Post-hoc Tukey-HSD Test. All other measures were analyzed using Kruskal-Wallis non-parametric ANOVA to identify group differences. Treated Groups were independently compared with the Control Group using Mann-Whitney U-tests. Two-way (repeated measure) non-parametric test (Kruskal-Wallis) was used to compare the no cue probe trials in the Morris water maze experiment.