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WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

December 4, 2001
TXR# 0050307

MEMORANDUM

SUBJECT: **Carbaryl**: Additional Morphometric Measurements, Analyses of Data, and an Age Related Sensitivity Study as supplements to the Developmental Neurotoxicity Study

TO: Anthony Britten, PM Team 53
Reregistration Branch III
Special Review and Reregistration Division (7508C)

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Carbaryl
PC Code 056801
DP Barcode: D276572

The purpose of this memorandum is to review three new submissions related to the Carbaryl Developmental Neurotoxicity Study, which provide additional morphometric brain measurements, re-analyses of other brain measurements, and an age related sensitivity and reliability study (MRID 45456701, 45456702, 45456703).

Background

In the review of the developmental neurotoxicity study (MRID 44393701), EPA concluded that a number of morphometric brain measurements seen at the high dose of 10 mg/kg were treatment related.

These included:

1. Statistically significant bilateral decreases in the length of the cerebella (Line F) of female pups;
2. Statistically significant bilateral increases in the width of the cerebella (Line G) of adult females (original data; now revised and no longer an effect);
3. A significant increase in thickness of the right forebrain (Line B) of male pups; and
4. A significant decrease in the thickness of the left forebrain (Line A) of adult males.

The definition of these measurements and a brief description of them is given below, as an aid for the reader.

Line A: a perpendicular line from the dorsal surface of the cerebral cortex to the cingulum; i.e., the thickness of the cerebral cortex (forebrain);

Line B: a perpendicular line from the surface of the cerebral cortex to the parietal cortex, somatosensory area; i.e., the thickness of the parietal cortex (forebrain);

Line F: the widest point of the long axis of the cerebellum; i.e., the length of the cerebellum;

Line G: the line perpendicular to and at the midpoint of the long axis of the cerebellum; i.e., the width of the cerebellum

The registrant, in the study report, and in an additional prior submission (MRID 44904204), has argued that none of the effects listed under 1-4 should be considered treatment related. In the review of the second submission (D259256, D261394), EPA agreed with the registrant that changes in the width of the cerebella (Line G) of adult female rats (2. above) should no longer be regarded as treatment related effects, based on their assertion that these measures were erroneous. In that submission (MRID 44904204), the registrant made additional measurements that it regards as reliable. These additional repeated measures are included in the attached DER as revised Table 18a.

The present submissions provide additional measurements of the cerebellum in female pups, where effects are still in dispute(item 1 above), re-analyzes the data on the forebrain (Line B) in male pups (item 3 above) and the forebrain (Line A) of adults (item 4 above), and the submitter concludes that neither area shows treatment related effects (MRID 45456701, 45456703). Data are also provided from a study comparing morphometric measures in untreated day 10 and day 13 pups as a way to demonstrate that their methods are sensitive enough to detect changes in brain size related to the age of the rat, and that the measurements are reproducible (MRID 45456702).

Review of this submission consists of an amendment to the original data evaluation record (DER) or EPA review (44393701.DER) and consists of several parts that:

1. Provide updated and corrected tables of the morphometric data;
2. Analyzes the additional data on the different layers of the cerebella in pups;
3. Reviews new statistical analyses of the forebrain measurements by the registrant;
4. Provides HED's statistical analyses of these and other data;

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5. Reviews the study on unexposed 10 day-old and 13 day-old rats; and
6. Provides a new executive summary for the DNT study.

Conclusions

HED concludes that there were no effects in the new measures of cell layers in the cerebellum made in this submission (MRID 45456701, 45456703) that it regards as treatment related.

HED disagrees with the registrant's analysis of the combined forebrain data and concludes that the bilateral decreases in the size of the forebrain (Line A) in adult males should be regarded as an effect of treatment, based, in part, on HED's new statistical analyses.

HED also concludes that, based on new statistical analyses of the revised data tables on the cerebellum in female adults, that there are statistically significant increases in the length (Line F) of the cerebella in 10 mg/kg females.

HED concludes that the age related sensitivity study is generally adequate to demonstrate reasonable sensitivity and reliability of the morphometric methods used in this study.

In light of its review of this submission, HED still concludes that the decreases in the size of the cerebellum (Line F) in female pups at 10 mg/kg are still considered an effect of treatment.

In summary, EPA finds that three morphometric measures were significantly affected by exposure to 10 mg/kg of Carbaryl:

1. A bilateral decrease in the size of the forebrain (Line A) in adult males (7.7-9.8%);
2. A bilateral decrease in the length of the cerebella (Line F) in female pups (15-22%);
3. A bilateral increase in the length of the cerebella (Line F) in female adults (7.4-15%).

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Carbaryl/056801 **Additional Morphometric Measurements and Statistical Analyses for DNT Study 2001 OPPTS 870.6300**

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TXR#: 0050307

> Supplement #1 to Data Evaluation Record #44393701.DER, that reviews additional morphometric data and statistical analyses, updates tables and results for morphometric effects, and provides a revised executive summary for the DNT study.

STUDY TYPE: Developmental Neurotoxicity Study (OPPTS 870.6300)

DP BARCODE: D276572 SUBMISSION CODE: S600895
PC CODE: 056801

CITATIONS: Robinson, K. and B. Broxup (1997) A Developmental Neurotoxicity Study of Orally Administered Carbaryl, Technical Grade, in the Rat. ClinTrials BioResearch, Ltd.. Quebec, Canada. Laboratory Project I.D. 97391, September 23, 1997. MRID 44393701. Unpublished

Robinson K, and Broxup B. (2001) Final Report Amendment No. 1 Supplement to MRID 44393701- A Developmental Neurotoxicity Study of Orally Administered Carbaryl, Technical Grade, in the Rat. ClinTrials BioResearch Ltd., Quebec, Canada. Laboratory Project I.D. 97391. July 6, 2001. MRID 45456703. Unpublished. 40 p.

Robinson K, and Broxup B. (2001) Final Report Amendment No. 2 Supplement to MRID 44393701- A Developmental Neurotoxicity Study of Orally Administered Carbaryl, Technical Grade, in the Rat. ClinTrials BioResearch Ltd., Quebec, Canada. Laboratory Project I.D. 97391. July 10, 2001. MRID 45456701. Unpublished. 34 p.

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Carbaryl/056801 **Additional Morphometric Measurements and Statistical Analyses for DNT Study 2001 OPPTS 870.6300**

Hamelin N, Yipchuck G. 2001. Morphometric Evaluation of Rat Brain Areas for Developmental Neuropathology. ClinTrials BioResearch Ltd., Quebec, Canada. Laboratory Project I.D. 99579. July 9, 2001. MRID 45456702. Unpublished 34p.

SPONSOR: Aventis CropScience, Research Triangle Park, NC 27709

EXECUTIVE SUMMARY:

In a developmental neurotoxicity study (MRID # 44393701, 45456701, 45456702, 45456703), 26 pregnant female Sprague-Dawley rats/group were administered carbaryl (99.1% a.i.) by gavage from Gestation Day (GD) 6 through Lactation Day (LD) 10 at doses of either 0, 0.1, 1.0 or 10 mg/kg/day. An additional 6 pregnant females/group were dosed at the same levels for the cholinesterase (ChE) phase of the study. ChE measurements were done pre-dosing (GD 6) and post-dosing at time of peak effect (1 hour post-dosing) on GD 6, 15 and 20 and LD 4 and 10. Functional Observational Battery (FOB) measurements were performed at approximately 0.5 and 2 hours post-dosing on the same days as body weight measurements during the dosing period (GD 0, 6, 9, 12, 15, 18 and 20 and LD 4, 7, 11, 13 and 21). Measures of reproductive performance were evaluated. Offspring were examined for body weight, physical development landmarks (tooth eruption and eye opening), FOB assessments (days 4, 7, 11, 13, 17 and 21) and motor activity (days 13, 17 and 21). On LD 11, 1 animal/sex/litter was sacrificed for brain weights; of these, six/sex were randomly selected for neuropathological evaluation. The eyes from all dose groups were examined. After LD 21, 3 animals/sex/litter were separated from the dams and constituted the F1 adult generation. These animals were evaluated for body weight, physical development (vaginal opening and preputial separation), motor activity (day 60), startle habituation response (days 22 and 60), passive avoidance (day 23) and water maze behavior (day 60). After completion of the behavior test period (at approximately 10 weeks of age), 12 animals/sex/group were anesthetized and perfused for post-mortem examination. Tissues from 6 animals/sex of the control and high dose group were processed for neuropathological evaluation and morphometric measurements; the eyes from the low and mid-dose group of all perfused animals were examined.

For the F0 generation animals, there were no carbaryl-associated deaths. No treatment-related clinical signs of toxicity were observed. There was a statistically significant decrease (92%) in body weight gain for females in the 10 mg/kg/day group for the period GD 6-9. Unfortunately, food consumption was not measured during the study. During the FOB measurements, the incidence of females in the 10 mg/kg/day group with decreased pupil size (pinpoint pupils) was increased on all occasions during the dosing period. An increased incidence of dams with slight tremors affecting the head, body and/or limbs was noted on the majority of assessment occasions in the dosing period. There were also occasional occurrences of ataxic gait/overall gait incapacity which was considered to be of toxicological significance due to other effects upon gait.

For the 10 mg/kg/day group, RBC and whole blood ChE levels were statistically significantly decreased (28% and 32-34%, respectively) on GD 20 and LD 10. Although the plasma ChE levels were not statistically significantly altered, the percentage decreases on GD 20, LD 4 and LD 10 were 32-39%. Brain ChE levels were statistically significantly decreased (42%). There were no treatment-related effects on gross necropsy findings for the F0 generation animals. There were no effects observed on maternal performance parameters of pregnancy rate, gestation index, length of gestation, numbers of live pups, dead or malformed pups, implantation scars, sex ratio or post-implantation loss. There was a slight ($P>0.05$) increase in the number of dead pups in the 10 mg/kg/day group, however the value was within the historical control range for this strain.

For the F1 generation pups, there were no treatment-related effects on pup weight, pup survival indices, developmental landmarks (tooth eruption and eye opening), FOB measurements or motor activity assessments. At sacrifice on LD 11, there were no treatment-related effects on brain weight and gross or microscopic pathology. Significant differences noted in the morphometric measurements included an increase in Line B of the right forebrain and Line F of the left cerebellum in the 10 mg/kg/day males. In the 10 mg/kg/day females, Line F through both the right and left cerebellum were significantly decreased (15% and 22%, respectively).

For the F1 generation adults, there were no treatment-related effects on clinical condition, body weight, physical development (vaginal opening and preputial separation), motor activity, auditory startle response, passive avoidance and water maze measurements. At sacrifice, there were no gross or microscopic neuropathological lesions observed for animals examined in this study that were attributable to treatment with the test article. There was an increased incidence of retinal fold/rosette in the 10 mg/kg/day group (1/12 for control vs. 4/12 for males; 0/12 for control vs. 2/12 for females). The finding was not considered of toxicological significance since the incidence was within the historical control range for males, occurred at a low rate and was not dose-dependent. For the morphometric measurements, there was a significant bilateral decrease in Line A through the forebrain (7.7-9.8%) and a significant increase in Line F through the right cerebellum of the 10 mg/kg/day males. Increases originally noted in 10 mg/kg adult females in Line G, width of the cerebellum, were found to be based on erroneous measurements, and additional measures were submitted. Now, for the 10 mg/kg/day females, there were significant bilateral increases in Line F through the cerebellum (7.4-15%). Measurements of the size of the thickness of lobes and of the granule cell layers of the cerebellum in high dose pups and adults did not differ from those of controls. While additional statistical analyses by the registrant indicated no treatment related effects, HED's additional statisical analyses did indicate treatment related effects.

The maternal toxicity LOAEL was 10 mg/kg/day based on decreased body weight gain, alterations in Functional Observational Battery measurements and RBC, plasma, whole blood and brain cholinesterase inhibition.

The maternal NOAEL was 1.0 mg/kg/day.

The developmental neurotoxicity LOAEL was 10 mg/kg/day based on a bilateral decrease in the size of the forebrain (Line A) in adult males (7.7-9.8%); a bilateral decrease in the length of the cerebella (Line F) in female pups (15-22%); and a bilateral increase in the length of the cerebella (Line F) in female adults (7.4-15%).

The developmental NOAEL was 1 mg/kg/day. Morphometric assessment at the mid and low doses could not be conducted due to inadequate tissue storage; however, based on the minimal findings at the LOAEL, it is HED's judgment that effects would be unlikely to occur at 1 mg/kg/day, which is 10% of the LOAEL.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Confidentiality Statements were provided.

Background

This document seeks to review the three submissions named above and to revise the review (44393701.DER) of the developmental neurotoxicity study (MRID 44393701) in light of these submissions and in light of previous submissions (MRID 44902204) and reviews (D259256; D261394) to result in one text that summarizes the current conclusions on these measurements. In the review of the developmental neurotoxicity study (MRID 44393701), EPA concluded that a number of morphometric brain measurements seen at the high dose of 10 mg/kg were treatment related. These included:

1. Statistically significant bilateral decreases in the length of the cerebella (Line F) of female pups;
2. Statistically significant bilateral increases in the width of the cerebella (Line G) of adult females (original data; now revised and no longer an effect);
3. A significant increase in thickness of the right forebrain (Line B) of male pups; and
4. A significant decrease in the thickness of the left forebrain (Line A) of adult males.

The definition of these measurements and a brief description of them is given below, as an aid for the reader.

Line A: a perpendicular line from the dorsal surface of the cerebral cortex to the cingulum; i.e., the thickness of the cerebral cortex (forebrain);

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Line B: a perpendicular line from the surface of the cerebral cortex to the parietal cortex, somatosensory area; i.e., the thickness of the parietal cortex (forebrain);

Line F: the widest point of the long axis of the cerebellum; i.e., the length of the cerebellum;

Line G: the line perpendicular to and at the midpoint of the long axis of the cerebellum; i.e., the width of the cerebellum

The registrant, in the study report, and in an additional prior submission (MRID 44904204), has argued that none of the effects listed under 1- 4 above should be considered treatment related. In the review of the second submission (D259256, D261394), EPA agreed with the registrant that changes in the width of the cerebella (Line G) of adult female rats (2. above) should no longer be regarded as treatment related effects, based on their assertion that these measures were erroneous. In that submission (MRID 44904204), the registrant made additional measurements that it regards as reliable. These additional repeated measures are included in this review as revised Table 18a.

The present submissions provide additional measurements of the cerebellum in female pups, where effects are still in dispute (item 1 above), re-analyzes the data on the forebrain (Line B) in male pups (item 3 above) and the forebrain (Line A) of adults (item 4 above), and the submitter concludes that neither area shows treatment related effects (MRIDs 45456701, 45456703). Data are also provided from a study comparing morphometric measurements in untreated day 10 and day 13 pups as a way to demonstrate that their methods are sensitive enough to detect changes in brain size related to the age of the rat, and that the measurements are reproducible (MRID 45456702).

Review of this submission consists of an amendment to the original data evaluation record (DER) or EPA review (44393701.DER) and consists of several parts that:

1. Provide updated and corrected tables of the morphometric data;
2. Analyzes the additional data on the different layers of the cerebella in pups and adults;
3. Reviews new statistical analyses of the forebrain measurements by the registrant;
4. Provides HED's own statistical analyses of these and other data;
5. Reviews the study on unexposed 10 day-old and 13 day-old rats; and
6. Provides a new executive summary for the DNT study.

Conclusions

HED concludes that there were no effects in the new measures of cell layers in the cerebellum made in this submission (MRID 45456701, 45456703) that it regards as treatment related.

HED disagrees with the registrant's analysis of the combined forebrain data and concludes that the bilateral decreases in the size of the forebrain (Line A) in adult males should

be regarded as an effect of treatment, based, in part, on HED's new statistical analyses.

HED also concludes that, based on new statistical analyses of the revised data tables on the cerebellum in female adults, that there are statistically significant increases in the length (Line F) of the cerebella in 10 mg/kg females.

HED concludes that the age related sensitivity study is generally adequate to demonstrate reasonable sensitivity and reliability of the morphometric methods used in this study.

In light of its review of this submission, HED still concludes that the decreases in the size of the cerebellum (Line F) in female pups at 10 mg/kg are still fairly considered an effect of treatment.

In summary, HED finds that three morphometric measures were significantly affected by exposure to 10 mg/kg of Carbaryl:

1. A bilateral decrease in the size of the forebrain (Line A) in adult males (7.7-9.8%);
2. A bilateral decrease in the length of the cerebella (Line F) in female pups (15-22%);
3. A bilateral increase in the length of the cerebella (Line F) in female adults (7.4-15%).

Revised Tables for DNT Study (Table numbers are the same as in the original DER)

This Table 15 from the original DER is included for convenience of the reviewers and is unchanged.

Table 15: Brain Morphometric Measurements in Pups[a]

		Line B Rt. Forebrain Mean SD (µm)	Line B Left Forebrain Mean SD (µm)	Line F Rt. Cerebellum Mean SD (µm)	Line F Left Cerebellum Mean SD (µm)
Group 1 Control	Male	1497.71 80.875	1549.47 44.996	4380.93 500.246	3929.16 322.175
Group 4 10 mg/kg/day		1613.64* 40.089	1555.19 83.458	4734.26 515.940 8%↑	4707.18* 563.820 20%↑
Group 1 Control	Female	1554.70 126.754	1598.07 98.205	4440.99 535.429	4601.68 440.127
Group 4 10 mg/kg/day		1553.15 50.665	1635.92 50.112	3753.68* 387.062 15%↓	3582.04** 417.496 22%↓

a Extracted from Table 25 (pages 163-164) of the study report.

* Significantly different from control ($P<0.05$, Dunnett's)

** Significantly different from control ($P<0.01$, Dunnett's)

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This revised Table 18 is revised to indicate that Line A, and not Line B as indicated in DER for MRID 44393701 is the data tabulated here.

Revised Table 18: Brain Morphometric Measurements in Adults[a]

		Line A Rt. Forebrain Mean SD (µm)	Line A Left Forebrain Mean SD (µm)
Group 1 Control	Male	1743.74 200.029	1819.05 74.852
Group 4 10 mg/kg/day		1608.81 135.278 8%↓	1641.06* 116.697 10%↓
Group 1 Control	Female	1818.30 102.888	1764.47 120.376
Group 4 10 mg/kg/day		1723.89 128.136	1636.13 129.172

a Extracted from Table 39 (pages 194-196) of the study report.
* Significantly different from control ($P<0.05$, Dunnett's)

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This revised Table 18a replaces erroneous measurements of Line F and G in females with those of the Repeated Addtional Evaluation from MRID 44904204 p.13.

Revised Table 18a: Brain Morphometric Measurements for Adults - First, Second, and Additional Evaluation Examinations[a]

		Line F Rt. Cerebellum Mean SD (µm)	Line F Left Cerebellum Mean SD (µm)	Line G Rt. Cerebellum Mean SD (µm)	Line G Left Cerebellum Mean SD (µm)
First Examination					
Group 1 Control	Male	6201.13 265.726	6139.81 298.621	4566.79 556.330	4602.84 491.233
Group 4 10 mg/kg/day		6718.66* 310.685	6186.32 422.015	4870.79 283.794	4885.12 465.128
Group 1 Control	Female	6217.21 222.922	6219.86 450.270	4724.84 149.327	4557.05 372.813
Group 4 10 mg/kg/day		6280.37 447.451	6287.54 440.619	4437.91 428.392	4473.14 334.580
Second Examination					
Group 1 Control	Male	6480.99 572.174	6643.26 363.806	5283.18 648.375	4971.00 314.405
Group 4 10 mg/kg/day		6778.12 642.408	6749.61 387.596	4961.01 421.550	4767.13 392.040
Repeated Additional Evaluation (from MRID 44904204)					
Group 1 Control	Female	5318.27 383.34	5875.14 340.80	4404.84 337.741	4380.54 251.196
Group 4 10 mg/kg/day		6128.98* 407.417 (15%↑)	6308.15 383.99 (7.4%↑)	4595.67 265.837	4732.73* 165.402 (8%↑)

a Extracted from Table 39 (pages 194-196) of study report MRID 44393701;and p 13 of MRID 44904204

* Significantly different from control (P<0.05, Dunnett's)

** Significantly different from control (P<0.01, Dunnett's)

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Additional measurements in the Cerebellum

METHODS

Morphometric measurements of the cerebellum were made using a Bioquant/TCW image analysis system in 6 pups/sex and 6 adults/sex from the high dose (10 mg/kg) and control groups. The slides were prepared previously as part of the developmental neurotoxicity study (MRID 44393701). These new measurements were made on one side of the brain based on the most "suitable" section. The same technician made all the measurements. Each datapoint represents the average of the number of measures made, which varied among the measures taken between 1 and 24 measurements/datapoint. Where more than one measure was averaged for a datapoint, the individual measurements were not reported.

In pups, three measurements were:

1) the thickness of lobule 5 taken at the base;

2) the thickness of the external granule layer measured at the base of lobule 5, made by a representative width from both external layers and the average calculated (2 measures); and

3) the thickness of the internal granule layer measured in lobules 4 and 5 at equidistant orthogonal widths (every 100 microns), with the central white tract as the referent, (roughly 24 measurements averaged).

In adults, two measurements were:

1) the thickness across all cell layers (i.e. molecular layer, granular layer, and the central white matter; and

2) the thickness of the granular layer in lobules 4 and 5, the average of equidistant orthogonal measurements made every 100 microns, starting at the base. [Adults only have one granular layer; roughly 24 measures averaged].

Changes in these regions were considered representative of the entire cerebellum, based on effects seen in a sensitivity study, that compared age related changes in these lobules to those of the entire cerebellum.

RESULTS

1. Cerebellar Measurements

The results presented are shown in Table 1 as group means and standard deviations, with one measure/rat/region, as described above. Individual measurements were not reported.

TABLE 1. Thickness of Lobules of the Cerebellum and Granular Cell Layers in Rats. (Mean ± S.D. N=6/sex/dose)

	Males		Females	
Measures (μm)	Controls	10 mg/kg	Controls	10 mg/kg
Pups				
Lobule Base Thickness	574.85±98.63	552.29±114.30	504.68±119.10	530.64±67.76
External Granule Layer	43.71±10.58	42.00±11.17	43.01±4.33	44.51±4.06
Internal Granule Layer	121.47±6.57	120.81±9.90	123.44±10.87	119.97±9.31
Adults				
Lobule Base	815.35±82.30	771.78±148.41	699.23±139.90	726.60±161.30
Granular Layer	148.80±10.81	148.62±8.79	135.77±5.93	143.68±7.58

There were no significant differences in either pups or adults in the thickness of the base of the lobules or the granular cell layers.

The coefficients of variation were roughly 20% for lobule thickness measures in pups and adults, 10% (females)- 25%(males) for external granule layer measurements in pups, and 5-7% for internal granule layer measures in pups, and 5-7% in granular layer in adults. These are roughly in line with the variation in the general measures of the cerebellum, i.e., length and width, in other studies.

2. Statistical Re-analyses of forebrain data in male pups and adult males

In EPA's review of the initial study, it concluded that a number of morphometric brain measurements that were seen at the high dose of 10 mg/kg were treatment related. These included a significant increase in thickness of the right forebrain (Line B) of male pups; and a significant decrease in the thickness of the left forebrain (Line A) of adult males. Measurements of these areas, (Line B) in male pups, and (Line A) in male adults were statistically re-analyzed by the registrant by averaging the measurement in each rat from each side, and analyzing the combined measures. The combined data are summarized in Tables 2 and 3. The registrant's statistical analyses of the combined data showed no statistically significant ($p<0.05$, Dunnet's test) differences for either measure.

Line B in Pups

The original data and the combined measurements are shown in Table 2. There was a statistically significant increase in the right forebrain seen.

TABLE 2. Re-analysis of Initial (Right and Left Forebrain)[A] and Combined Forebrain Data[B] (Line B) in Male Pups.

	Right Forebrain	Left Forebrain	Combined Forebrain (Line B) Measures
Controls	1497.91± 80.875	1549.47± 44.996	1523.59 ±39.346
10 mg/kg Carbaryl	1613.64± 40.089* (7.7%)	1555.19± 83.458 (0.37 %)	1584.42 ± 56.916 (4 %)

* p <0.05, Dunnet's Test A from MRID 44393701; B from MRID 45456703

Line A in Adult Male Rats

These measurements are shown in Table 3 below. In the initial DER, EPA noted decreases in both sexes (females, 5-7%), bilaterally, were seen in forebrain measurements (Line A, dorsal surface of the cerebral cortex to the cingulum), although these changes only achieved statistical significance in the left forebrain measures in males (by the registrant's original analyses).

Table 3.Re-analysis of Initial (Right and Left Forebrain)[A] and Combined Forebrain Data[B] (Line A) in Male Adults

	Right Forebrain (Line A)	Left Forebrain (Line A)
Males, controls	1743.74 ± 200.29	1819.05± 74.852
10 mg/kg Carbaryl	1608.81± 135.278 (-7.7%)	1641.06 *± 116.697 (-9.8%)
	Combined Forebrain (Line A) Measurements	
Males, controls	1781.40 ± 127.81	
10 mg/kg Carbaryl	1624.94± 117.74 (-8.8 %)	

(% decrease relative to controls) A from MRID 44393701; B from MRID 45456701

Coefficient of variation on these measurements were less than 10%.

EPA conducted 2 analyses, that use each datapoint (i.e., left and right side), by a MANOVA, which treats the two sides as separate variables, and by a 2 way repeated measures analysis of variance, which regards each side as a repeated measure.

For the decrease in the frontal cortex thickness (Line A) in adult males, the MANOVA had a p value of 0.04, for the overall dose effect. The 2 way analysis of variance with side as a repeated measure, had a p value of 0.05. In the ANOVA, there was no significant interaction noted between the sides, i.e., they were not different. It is concluded then, that the combined decrease (-8.8%) in the thickness of the cerebral cortex (Line A) in male adult rats given 10 mg/kg achieved statistical significance. The significant overall decrease in this measure in both sides in adult males adds support to the view that it should reasonably be regarded as an effect of treatment.

The MANOVA for the increase in the thickness of the forebrain in the pup (line B) shows an overall p of 0.04, indicating a significant overall difference in this measure as well. When these data were included in a repeated measure ANOVA, however, there was a significant dose by side interaction, indicating that the effect is due primarily to changes in one side, as can be seen from the % changes shown in the data table. This is more difficult to interpret as clearly treatment related, as no rationale is apparent about why such an effect should apparently involve only one side of the brain.

Age Comparison and Reliability Study (MRID 45456702)

METHODS

Test animals:
Species: *Rattus norvegicus*
Strain: CRL: CD® SD/BR rats
Age at study initiation: 10 days old or 13 days old
Source: Charles River Canada, Quebec, Canada

20 male and 20 female rat pups that were 10 days old and 20 male and 20 female rat pups that were 13 days old, each from a separate litter, served as subjects.

Necropsy and Tissue Preparation

Pups were euthanized by CO_2 asphyxiation followed by exsanguination from the abdominal aorta. The calvarium with meninges was removed (when possible) to expose the brain which was then immersion fixed *in situ* in 10% formalin.

Brains from 6 rats/sex/age (and 4 spares) were kept for 2 weeks before trimming. Remaining brains were kept for 10 weeks in neutral buffered formalin(10%) in tissue bags.

Sections from the left and right forebrain and cerebellum were prepared, processed, and slides prepared for 10 rats/sex/age. Tissues from remaining animals were brought to the block stage.

Tissues were embedded in paraffin wax, and ten 6 micron serial sections/block for forebrain were cut and stained with hematoxylin and eosin (H&E). Five 6 micron serial sections/block for cerebellum were cut and stained with Klüver-Barrera stain.

Tissue Examination and Morphometric Evaluation

Sections were examined grossly and by light microscopy to verify the brain levels and the comparability of anatomical sites between animals.

Measurements of 6 pups/sex/age were made of forebrain (left *and* right) and cerebellum (left *or* right) by the same operator.

Forebrain measurements were:

the thickness of the frontal cortex;
the thickness of the parietal cortex; and
the thickness of the corpus callosum.

Cerebellar measurements were:

internal granular layer thickness (all lobes);
internal granular layer thickness (lobes 4/5);
external granular layer thickness (all lobes);
external granular layer thickness (lobes 4/5);
lobule base thickness (lobes1/2/3; 4/5; 6/7/8; 9; and 10)

These measurements were reported for individual animals and group means, as average measurements for the areas noted above (e.g., one measurement/rat for internal granular layer, all lobes). Individual lobe measurements were not reported.

Repeated measurements were made of the slides of the cerebellum of pups and adults from study 97391 (main DNT study of Carbaryl MRID 44393701) to assess the reproducibility of the measurements. These measurements were made in this study by one operator who was "blind" to the identity, e.g, dose group, of each rat.

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These measurements and their bases were:

Lobule 5 base thickness in pups and adults;
External Granular thickness in pups;
Internal Granular layer thickness in pups;
Granular layer thickness in adults (Further details were described earlier in this review).

Statistical Analyses

Means and standard deviations were calculated for the measurements made, and comparisons between day 10 and day 13 pups made using Dunnet's t test.

RESULTS

1. Forebrain Measurements

Forebrain measurements are shown in Table 4.

TABLE 4. Forebrain Measurements

	MALES			FEMALES		
	DAY 10 Mean ± S.D.	DAY 13	CVs DAY 10 DAY 13	DAY 10	DAY 13	CVs DAY 10 DAY 13
Right Frontal Cortex	1673.7 ± 71.2	1813.6** ± 66.9 8.4%	4.3% 3.7%	1755.5 ± 90.55	1812.1 ± 111.8 3.2 %	5.2% 6.2%
Left Frontal Cortex	1669.1 ± 55.2	1755.9* ± 46.7 5.2%	3.3% 2.7%	1778.6 ± 104.4 1.32%	1804.2 ± 61.5 1.4%	5.9% 3.4%
Right Parietal Cortex	1545.7 ± 54.6	1664.8* ± 87.8 7.7%	3.5% 5.3%	1644.1 ± 82.4	1730.1 ± 89.6 5.2 %	5% 5.2%
Left Parietal Cortex	1555.1 ± 25.2	1675.8* ± 97.5 7.8%	1.6% 5.8%	1619.4 ± 116.8[a] -1.5%	1700.2 ± 81.7 5 %	7.2% 4.8%
Corpus Callosum	410.2 ± 45.3	381.3 ± 20.3 -7%	11% 18.8%	413.8 ± 35.9	396.4 ± 33.8 -4.2%	8.7% 8.5%

* $p < 0.05$; ** $p < 0.01$; Dunnet's Test. [a] $n = 5$ (μm; %- difference between day 10 and 13) CV = coefficient of variation, standard deviation/mean.

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Coefficients of variation (CVs) were 3-7 % for cortical measures, and 9-19 % for the corpus callosum, which is generally acceptable. The general expectation is that as the rat ages, its brain size will increase. Cortical thickness measurements increased as a function of age, while the corpus callosum decreased. Only the increases in males in cortical thickness measures in both frontal and parietal cortex were statistically significant by Dunnet's test. These changes ranged from 5.2-8.4 % in males, and 1.4-5.2 % in females. Thus, the statistically significant increase in size in demonstrates the sensitivity of the method while the lack of significant effect in females bounds the sensitivity of those changes, which were smaller.

There was generally good agreement between the right and left measurements made, though the extent of growth was greater on one side of the frontal cortex. Differences in growth between left and right side cortical measures in frontal cortex were 38% for males (8.4 vs. 5.2%), and 56% for females (3.2 vs. 1.4%); in parietal cortex 1.3% in males (7.7 vs. 7.8%) and 3.8% in females (5.2% vs 5%). In some cases, then, one might expect that chemical changes of this magnitude might not show bilateral statistical significance, and supports the view that the direction and magnitude of changes should also be examined, not just the statistical significance.

Cerebellar measurements are shown in Table 5.

Table 5. Cerebellar Measurements

	MALES			FEMALES		
	DAY 10 Mean ± S.D.	DAY 13	CVs DAY 10 DAY 13	DAY 10	DAY 13	CVs DAY 10 DAY 13
Internal Granular Thickness (4/5)	134.3 ± 5.7	157.3**± 6.3 17.1 %	3.4% 4 %	134.6 ± 7.2	159.6** ± 5.0 18.6 %	5.3 % 3.1 %
External Granular Thickness (4/5)	48.8 ± 3.8	30.2** ± 6.7 -38.1 %	7.8 % 22.2 %	47.7 ± 4.9	29.1** ± 6.6 -39 %	10.3 % 22.7 %
Internal Granular Thickness (all)	140.0 ± 3.5	157.1** ± 3.2 12.2%	2.5 % 2 %	139.8 ± 3.7	159.7** ± 7.3 14.2 %	2.6 % 4.6 %
External Granular Thickness (all)	50.8 ± 2.9	33.3** ± 5.0 -34.4%	5.7 % 15 %	50.0 ± 3.6	34.1** ± 5.6 -31.8 %	7.2 % 16.4 %
Lobule 1,2,3	496.4 ± 55.5	532.2 ± 153.5 7.2 %	11.2 % 28.8 %	521.6 ±118.6a	555.4 ± 109 6.5 %	22.7 % 19.6 %
Lobule 4,5	470 ± 63.9	519.7 ± 55.6 10.6 %	13.6 % 10.7 %	492 ± 47.9	540.4 ± 51.4 9.8 %	9.7 % 9.5 %
Lobule 6, 7,8	471.3 ± 44.5	458.1 ± 28.7 -2.8 %	9.4 % 6.3 %	428 ± 33.5	487.9* ± 33.9 14 %	7.8 % 6.9 %
Lobule 9	580.4 ± 60.7	579.4 ± 45.3 -0.17%	10.5 % 7.8 %	520.7 ± 53.7	599* ± 61.8 15 %	10.3 % 10.3 %
Lobule 10	414.1 ± 51.1	439.7 ± 21.7 6.2 %	12.3 % 4.9 %	430.5 ± 20.6b	424.3 ± 29.5 -1.4 %	4.8 % 7 %

* $p < 0.05$; ** $p < 0.01$; Dunnet's Test. a, $n = 4$; b, $n = 5$. CV = coefficient of variation, standard deviation/mean.

The coefficients of variation (CVs) was 2-5 % for the internal granular layer, 8-23% for the external granular layer, with much greater variability in the day 13 measurements. For the lobule thickness measurements, the CVs ranged from 5-14 % for lobules 4-10, but were somewhat higher, 11-29% for lobules 1-3.

It is expected that as the pups age, there is migration of cells from the external to the internal granular layers. (In adults, there is only one (internal) granular layer). The data show a significant increase in the internal granular layer in both sexes, while the thickness of the external granular layer shows significant decreases. Differences between granular layer measurements in Lobules 4 and 5 (38-39%) and all lobules (32-34 %) were comparable. Differences between the sexes were comparable as well. CVs for granular layer measures were higher for the external granular layer at day 13 in both sexes than at day 10 (15-20% vs. 5-10%).

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There were no significant increases in lobule thickness among male rats, with the range of changes from -2.8% to + 10.6 %. Among females, the range of changes were similar, from -1.4% to +15%, but the increases in lobules 6,7,8 (one measure) and lobule 9 (14-15 %) were significant at the .05 level. So the growth between days 10 and 13 in these groups had less impact on the growth of the cerebellum, although the larger CVs played a role in the general lack of statistical significance.

DISCUSSION

Measurements in the cerebellum of the thickness of major cell layers are recommended in the DNT guideline as part of a minimal set of morphometric measurements, and have now been conducted for this study. These additional measures describe more completely the thickness of a representative lobule of the cerebellum (comprised of several cell layers and white matter) and of granule cell layers. No differences were seen in these measures between high dose and control pups or between control and high dose adults. The lack of changes seen here suggest that the decreases in overall length (Line F) is not correlated with differences in base lobe thickness or cell layer thicknesses.

Statistical analyses by this reviewer found that while there was an overall significant increase in the thickness of the forebrain in pups, it was due primarily to effects on one side. These findings are inconsistent, and so undermine any conclusion that they are treatment related.

Statistical analyses by this reviewer also found that there was an overall significant decrease in the thickness of the frontal cortex in adult males, that was bilateral, and in which there was no interaction between dose and side. It is therefore reasonable to conclude that this bilateral effect is treatment related.

New statistical analyses by this reviewer also found that there were significant increases in the length of the cerebellum(Line F) in adult high dose females. This could be a compensatory response to the decreases seen in the pups in this measure. Recall that for neither pups nor adults were changes seen in the other cerebellar measures. In the original review, there has been discussion of the lack of changes in these tissues in terms of microscopic pathology, and it has been noted that while there were some decreases seen in the cerebellar weights of the pups, on the order of 5-10%, these lacked statistical significance and dose dependence. (On the last point, the inability to examine low and mid dose animals for morphometry limits any conclusions relating weight to effect).

My overall judgment is that it is prudent to consider the high dose changes in the frontal cortex and the cerebellum as treated related, but to regard these as likely minimum effect levels. That is, the consistent but small changes seen here would likely not have been present at the mid dose. Many arguments have been put forward as to why all the effects seen should be discounted, including only in one sex, not correlated with functional changes or other morphometric or microscopic pathology. Changes in one sex are possible and common in toxicology and not a basis for discounting effects. The clinical/pathological correlation in neurotoxicity studies is quite poor in general, so this should not be considered sufficient. Lack of changes in thickness of cell layers or cellular pathology provides detail that do contribute to the weight of evidence. But these measures are small samples of linear measurements of three dimensional structures, that only faintly approximately the volume of the tissue involved. In addition, there are many ways that tissue size could be adversely impacted in the absence of such changes, e.g. by disturbances in fluid balance. So when changes in these simple measures are seen that are not well understood, the sufficiency of the bases for discounting them, e.g., not correlated with other simple measures, are also weak approximations of the underlying processes related to them.