Available online at www.sciencedirect.com
SCIENCE DIRECT®
Environmental Toxicology and Pharmacology 19 (2005) 745–755
ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY ETAP
www.elsevier.com/locate/etap

Regulatory developmental neurotoxicity testing: a model study focussing on conventional neuropathology endpoints and other perspectives

Didima M.G. de Groot [a,*], Marga H.M. Bos-Kuijpers [a], Wolfgang S.H. Kaufmann [b], Jan H.C.M. Lammers [a], James P. O'Callaghan [c], Bente Pakkenberg [d], Manon T.M. Pelgrim [a], Ine D.H. Waalkens-Berendsen [a], Marloes M. Waanders [a], Hans-Jørgen J. Gundersen [e]

[a] *TNO Quality of Life, Utrechtseweg 48, P.O. Box 360, 3700 AJ Zeist, The Netherlands*
[b] *BASF, Ludwigshafen, Federal Republic of Germany*
[c] *Centers for Disease Control and Prevention-NIOSH, Morgantown, USA*
[d] *Research Laboratory for Stereology and Neuroscience, Copenhagen, Denmark*
[e] *Stereological Research Laboratory, University of Aarhus, Denmark*

Abstract

Our aim was to investigate a model of the morphologic approach proposed in guidelines for developmental neurotoxicity testing (DNT). Hereto, a limited DNT study [EPA Health Effects Test Guidelines OPPTS 870.6300, 1996a. Developmental Neurotoxicity Study "Public Draft", United States Environmental Protection Agency; Prevention, Pesticides and Toxic Substances (7101); EPA 712-C-96-239, June 1996. http://www.epa.gov/opptsfrs/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Drafts/870-6300.pdf] was carried out with different doses of methylazoxy methanol acetate (MAM), known to affect brain morphology and neuron numbers in the developing brain. After gross examination, the brains of F1-animals were further dissected along neuro-anatomical landmarks to ensure homology between tissues of different individuals. The (relative) weight of the brain (parts) was determined. One brain half (alternating left/right to avoid lateralization) was further used for microscopic slide reading and measurement of brain layer width (linear morphometry); the other was set aside for stereologic investigation in a later phase of the study.

In the offspring, a clear reduction in brain size (gross macroscopy) and weight (MAM high- and top-dose groups) was observed on postnatal days (PN) 22 and 62, but this reduction was hard to pinpoint in the microscope as the changes primarily appeared quantitative in nature, rather than qualitative. Linear measurements of brain layer width appeared very sensitive and efficient.

This first step of a project is presented and the perspectives of a further stereologic investigation are discussed.

Keywords: Rat; Methylazoxy methanol acetate (MAM); Regulatory developmental neurotoxicity (DNT) testing *(Guidelines OPPTS 870.6300, EPA*; *OPPTS 870.8600, EPA*; *OECD 426)*; Neuropathology endpoints; Microscopy and linear morphometry; Neuron numbers and stereology

1. Introduction

The developing brain can be affected by neurotoxic agents either as a primary target or indirectly. Such changes may have negative consequences for the mental and physical abilities of the individual. A morphological screening approach exists to examine the *adult* brain. The *developing* brain, however, may differ from the adult brain regarding susceptibility to the same toxicant and its effects on brain morphology. Hence, it is questionable whether a morphological approach designed for the adult brain, is adequate for the developing brain as well. After all, many *developmental* injuries manifest themselves as (subtle) *quantitative* changes in cell numbers in the absence of gliosis as most astrocytes (glial cells) are formed only after neurons during the development of the

* Corresponding author. Tel.: +31 30 694 49 54; fax: +31 30 696 02 64.
E-mail address: deGroot@chemie.tno.nl (D.M.G. de Groot).

1382-6689/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.etap.2004.12.039

nervous system. Even advanced neuropathological techniques like immunohistochemical detection of glial fibrillary acidic protein (GFAP) to examine the distribution of astrocytes and the hypertrophy of astrocytes in response to neural injury (Martin and O'Callaghan, 1995) or *linear* morphometry to measure the width of major layers at representative locations in the brain (Rodier and Gramann, 1979) may not be sufficient to detect such changes in neuron numbers. Cell numbers can only be determined by counting them. Counting neurons with the intention to achieve *unbiased* estimates of numbers with high precision can be performed, using *design-based stereological methods* (Gundersen et al., 1988). On the basis of their results of a comparison of linear morphometry and stereology, Duffell et al. (2000) have been suggesting that, if any morphometrical method is going to assist in the elucidation of potential effects of developmental neurotoxicants, unbiased *stereological* determination of neuron numbers offers the best chance.

It is very important to have an adequate testing strategy and diversity of tests available in the hazard identification process for potential human developmental neurotoxicants. In the context of *regulatory testing*, a compound will be considered for *developmental* neurotoxicity screening when there is an *indication* of neurotoxicity based on the numerous tests performed earlier. Compounds that induce overwhelming neurotoxic effects, or compounds that do not show any neurotoxic effect at all, will not be selected for further, more advanced testing.

To our knowledge, quantitative data regarding a change in neuronal numbers relative to other neuropathology measures in a *dose–response* relationship are not available in the literature. Our aim was to investigate a model of the conventional morphologic approach proposed in *regulatory developmental* neurotoxicity testing *(EPA Guidelines OPPTS 870.6300, 1996a and OPPTS 870.8600, 1996b;OECD 426, in preparation)*. *Conventional* neuropathology endpoints like brain gross macroscopy, brain weight, microscopic evaluation of brain sections and linear morphometric measurements of the width of distinct brain layers are evaluated on postnatal days (PN) 22 and 62 in a developmental neurotoxicity study *(EPA Guidelines OPPTS 870. 6300, 1996a*, with limited behavioural testing (motor activity)) with *methylazoxy methanol* (MAM) *acetate* (five dose groups, including vehicle control group), known to affect neuron numbers and brain development (Goldey et al., 1994). The dose–response relationship is examined for the different endpoints. The relative sensitivity and selectivity of the different endpoints for use in regulatory developmental neurotoxicity testing are reconsidered.

Data from this first step of a project is presented here and the perspective of a further stereologic investigation is discussed. We present effects of MAM on the *male* offspring. A number of observations on the *dose–response relationship* obtained for MAM on *gross examination* of the brains, *brain weight, microscopic evaluation of the brains*, and *linear morphometry* are shown.

2. Materials and methods

2.1. Study design

Mated female rats (14 rats/dose group) were dosed intraperitoneally (i.p.) with 0, 1.25, 2.5, 5 or 7.5 mg MAM acetate/kg bw/day. MAM was administered daily from gestation day (GD) 13–15 only, since repeated daily dosing throughout longer periods during gestation and lactation as proposed in EPA's Guidelines OPPTS 870.6300, is not tolerated due to the cytotoxic effect of MAM. The dams, their pups and young adult F1-animals were examined for clinical abnormalities, body weight, food intake, mortality, and abnormalities at necropsy. F1-animals were selected and raised until PN 22 or 62. Two subsets of the selected F1-animals were assessed for motor activity (1 rat/sex/litter; 10 litters/dose group/subset). Subset 1 was tested on PN 13, 17 and 21 (sacrifice on PN 22); subset 2 on PN 13, 17, 21 and 61 (sacrifice on PN 62). Brains of both subsets of selected F1-pups and F1-young adult animals were preserved after perfusion fixation for detailed neuropathological examination.

2.2. Sacrifice of F1-animals, tissue preservation and gross examination of the brains

The selected F1-animals of subset 1 (PN 22) and subset 2 (PN 62) were sacrificed under ether anaesthesia, by transcardiac perfusion with neutral phosphate buffered formalin fixative. After the perfusion fixation the brains were removed from the skull and were examined for *gross abnormalities*. The brains were (post)fixed in formalin for 48 h (±30 min), before being transferred into 0.1 M phosphate buffer (pH 7.2–7.4). This material, of which the duration of perfusion fixation and immersion post fixation was kept constant for all individuals, was used for measurements of brain weight. The brains were kept in buffer until further processing and embedding in paraffin to minimize differences in tissue shrinkage due to differences in fixation among individuals. Also, during dehydration and embedding, the duration of the tissues in the different histological solutions was carefully controlled and kept constant to avoid procedural differences among individuals.

2.3. Dissection of the brains and brain weight measurements

Digital photographs were taken of all brains. Thereafter, each brain was further dissected. Specific neuro-anatomical landmarks, particularly of the ventral surface of the brain, were used to ensure uniform trimming of the brains (Fig. 1) and aiming at a high degree of homology between brain levels and tissue sections of different individuals. This is of prime importance for an objective comparison between individuals. Splitting of the brains into the two brain halves was exactly along the midline (Fig. 1). One half of the brain (alternating left or right; the first one chosen

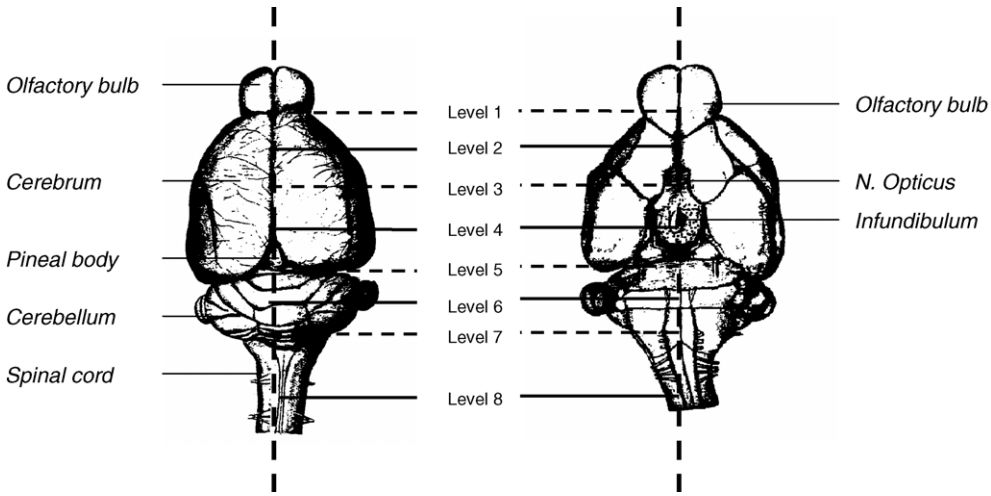


Fig. 1. Dissection of the rat brain along anatomical landmarks, resulting in one 'intact' brain half and a 'split' one, comprised of olfactory bulb, cerebrum anterior and posterior, cerebellum, and cervical spinal cord. The cerebrum anterior and posterior and the cerebellum are cut transversally into two equal slabs. The resulting six slabs as well as the cervical spinal cord from the split brain half are embedded within one and the same paraffin block, posterior face down, so that microtome sectioning occurs in anterior direction. The olfactory bulb is embedded horizontally in the same paraffin block.

randomly), i.e. cerebrum and cerebellum with underlying medulla oblongata/pons attached, remained 'intact', whereas the other, so-called 'split' hemisphere was further dissected into three parts: the cerebellum with underlying medulla oblongata/pons, the cerebrum posterior and the cerebrum anterior, also using neuro-anatomical landmarks (Fig. 1).

The various parts were weighed as proposed in the Developmental Neurotoxicity Screen EPA Guidelines OPPTS 870.8600, 1996b, except that the cerebellum was not separated from medulla oblongata/pons.

2.4. Histology and further dissection of the brains for embedding

Prior to embedding, the cerebellum, cerebrum posterior and cerebrum anterior of the split hemisphere were further dissected by dividing each of them, perpendicularly to the midline, into two equal transverse slabs (Fig. 1). Doing so, the dissection of the brain finally resulted in eight distinct parts or slabs at the side of the split brain half, i.e. (1) olfactory bulb; (2 + 3) cerebrum anterior (two transverse slabs); (4 + 5) cerebrum posterior; (6 + 7) cerebellum and (8) cervical spinal cord (see also Section 2.5).

The eight different slabs comprising the split brain half were embedded and carefully orientated in paraffin—eight slabs together—within one and the same mould. The olfactory bulb was embedded as a whole; the other brain parts/slabs and the cervical spinal cord were embedded posterior face down, so that microtome cutting occurred in the anterior direction. Paraffin sections (ca. 5 μm thick) were cut from this block. Each section of such a block, hence, included a transsection through *all* eight brain levels. Sections were stained with haematoxylin and eosin and were examined light microscopically.

Three parts were left at the side of the *intact* brain half, namely: olfactory bulb, cerebrum plus cerebellum attached (i.e. 'intact'), and the cervical spinal cord. The intact brain half is kept in formalin until further use for counting numbers of neurons in the brain.

2.5. Microscopic examination, identification of brain levels and section homology

The eight embedded brain slabs of the split brain half included the following brain levels (compare Fig. 1): *Level 1*, olfactory bulb; *Level 2*, rhinencephalon; *Level 3*, prosencephalon: telencephalon; *Level 4*, prosencephalon: diencephalon; *Level 5*, mesencephalon; *Level 6*, rhombencephalon: metencephalon (mid cerebellum/pons); *Level 7*, rhombencephalon: myelencephalon (posterior cerebellum/medulla oblongata); *Level 8*, cervical spinal cord.

Prior to neuropathologic evaluation and linear morphometry, all sections were viewed in the light microscope. The brain levels observed in the sections were identified by comparing each of them with those, published in the neuroanatomy atlas of Paxinos and Watson (1986). The level indicated in the atlas, showing most resemblance with the observed level in the microscopic section, was noted down to allow deviations from the desired brain level, resulting from imprecise dissection of the brain, to be recognized. It should be borne in mind that *homologous* sections are a prerequisite especially when linear morphometric measurements of e.g. brain layer width are used to detect effects of a putative neurotoxicant on developing brain morphology (see below Section 2.6).

After identifying the brain levels in the sections, the sections were examined light microscopically—'dose down', as proposed in current guidelines for DNT testing - for possible effects of the neurotoxicant on the morphology of the brain.

Microscopical slides were viewed by the pathologist. Since this study was carried out for regulatory purposes, the regulations were followed as nearly as possible. This implied that in first instance, the top-dose group was compared with the control group. The pathologist was aware of having only these two groups under investigation; however, the allocation of the individual animals to the groups was not known. In addition, light micrographs were taken of the MAM top-dose and control groups for a mutual comparison between controls and exposed brains. When examining these micrographs, the pathologist was aware of the allocation of the animals to the dose groups.

2.6. Linear morphometry

Simple linear morphometry was carried out within the microscopical sections as outlined in the Developmental Neurotoxicity Study (EPA Guidelines OPPTS 870.6300, 1996a). For this, the widths of major layers at representative locations within the neocortex and the hippocampus (Fig. 2) and cerebellum were determined (Rodier and Gramann, 1979; Duffell et al., 2000). Measurements were always carried out per brain level, all levels per animal, all animals per group on PN 22, followed by PN 62. Control groups were analysed first, followed by the MAM dose groups (top-dose down). The linear measurements were carried out with the CAST Grid system (Olympus, Denmark), which is capable of measuring in 2D and 3D scenarios. Each individual measurement was immediately stored by the system, and it was only *after* completion of *all* measurements that the evaluation of the data of the individuals in the different groups took place. This was done to keep the CAST-operator unaware of the results during measuring.

2.7. Statistics

Evaluation of the test results included the relationship between the doses of the test substance and the presence or absence, incidence and extent of any neurotoxic effect. The incidence of neuropathological changes were evaluated by Fisher's exact probability test. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was the statistical procedure used in the evaluation of the quantitative data (*brain weight* and *linear morphometry measurements*).

The tests were two-sided. As a level of significance, $2p < 0.05$ was considered.

3. Results

Examination of the brains of the selected F1-animals showed significant effects of MAM on the various neuropathology endpoints mentioned under Materials and Methods. However, as discussed below, the sensitivity and efficacy of the different approaches to detect developmental morphological changes, at least as a stand-alone method, clearly differed.

Effects were found on PN 22 and on PN 62. Pronounced sex differences were not observed. Here, we show results in the *male* F1-animals.

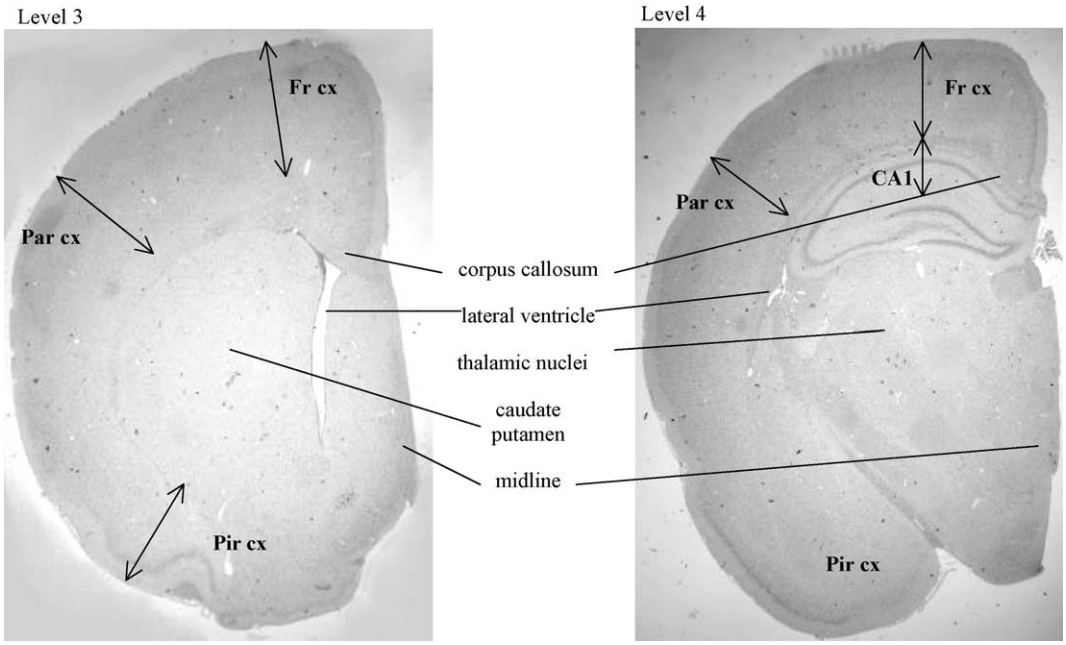


Fig. 2. Linear morphometry is used to measure the width of relevant brain layers/regions. A number of morphometric measures are determined using the eight brain levels sampled for microscopic examination. In the presented levels 3 (telencephalon) and 4 (diencephalon), particularly the neocortex and hippocampus are measured.

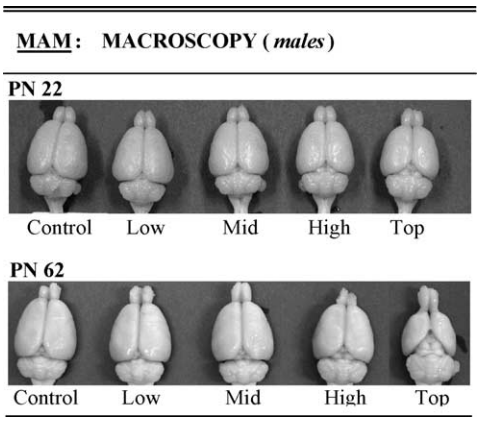


Fig. 3. Representative examples of brains of male pups on PN 22 and 62, of control and MAM dose groups. MAM exposed brains show a reduction in size of the cerebrum (posterior end) in the top-dose particularly on PN 62.

3.1. Macroscopy: gross examination of the brains after removal from the skull

During sacrifice on PN 22, gross abnormalities were observed in a number of animals in the 7.5 mg/kg MAM top-dose group. This effect appeared more pronounced during sacrifice on PN 62. Sometimes, it was observed in the 5 mg/kg MAM high-dose group. The changes included a considerable reduction of the size of the cerebral cortex, particularly noticeable at the posterior end near the midline between the two cerebral hemispheres at the site of the pineal body. In contrast, an increase in size of the cerebellum was observed (Fig. 3).

3.2. Macroscopy: brain weight measurements

Significant reduction in brain weight was observed in the offspring of MAM exposed rats, both on PN 22 and 62. The effects were dose-related and appeared significant in the 7.5 mg/kg MAM top-dose and in the 5 mg/kg MAM high-dose groups (Fig. 4A). Also the relative brain weight showed significant effects (Fig. 4B). The cerebrum (the anterior as well as the posterior part) was primarily affected (Fig. 4D and E). The weight of the cerebellum was *not* affected, either on PN 22 or PN 62 (Fig. 4C).

3.3. Microscopy: light microscopic screening or 'slide reading' and reading micrographs

The results of the comparison of the topography of each microscopic section with that of the brain atlas of Paxinos and Watson (1986) indicated that for most individuals a large degree of homology between sections was obtained (individual data not shown). Apparently, the dissection of the brain using neuro-anatomical landmarks forms a solid basis to guarantee the essential homology between sections of different individuals.

Detection and identification of effects of MAM on brain morphology during light microscopic screening appeared more difficult, even with the knowledge that homologous sections were screened and differences in size of the sections resulting from an actual decrease in brain size were observed with the naked eye (see above Sections 3.1 and 3.2). At the *microscopical* level, this decrease in brain size did not manifest itself as qualitatively observable changes in the composition of certain cell layers and/or the presence of e.g. ectopic cells. As a matter of fact, the changes at the *microscopical* level appeared hard—or not at all—to be distinguished by the human eye during conventional slide reading. Apparently, the macroscopically observable reduction in brain size rather reflects *quantitative* (numerical) changes in cells and cell structures that go unrecognised by the human eye during examination through the microscope. Therefore, the use of light micrographs not only appeared very helpful, but, in many cases was felt *essential* to ensure proper comparison and enable detection of test substance related effects on the *developing* brain. Fig. 5 shows such an example at brain *Level 2*, the rhinencephalon, where differences in section size were observed with the naked eye, and comparison of the photomicrographs demonstrates differences in the size of certain brain regions. Still, such effects may go unrecognised during microscopic screening, i.e. conventional slide reading, since the morphological changes are more quantitative than qualitative in nature. Especially here, the use of homologous sections appears essential. The micrographs show that the size of the fore-brain in the MAM top-dose is reduced due to hypoplasia of the frontal and piriform cortex. Micrographs of the cerebellum levels did not point at significant effects of MAM.

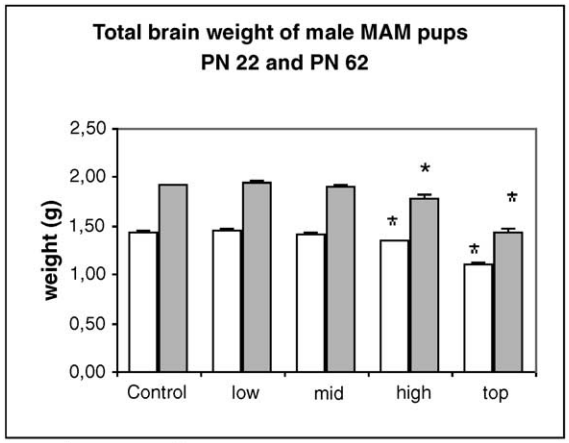
The significance of apparent changes in the hippocampus (Fig. 6) appeared difficult to pinpoint from examination of only one or two hippocampus levels; the shape of the hippocampus and its position in the rat brain near the lateral ventricle, which in itself may dilate during processing or as a result of treatment, is such that even in homologous sections minor differences in orientation of the hippocampus within a section may occur.

3.4. Microscopy: layer width measurements (linear morphometry)

Significant test substance related reduction in the width of the cerebral cortex and hippocampus was observed. The parietal cortex, for example, appeared significantly affected in the MAM top-dose group on PN 22 and on PN 62 (Fig. 7). Also, in the hippocampus (Fig. 8) the effects were significant in the 5 mg (PN 22) and 7.5 mg MAM top-dose groups (PN 22 and 62. The effects on the cerebellum, if at all significant, were considered fortuitous findings.

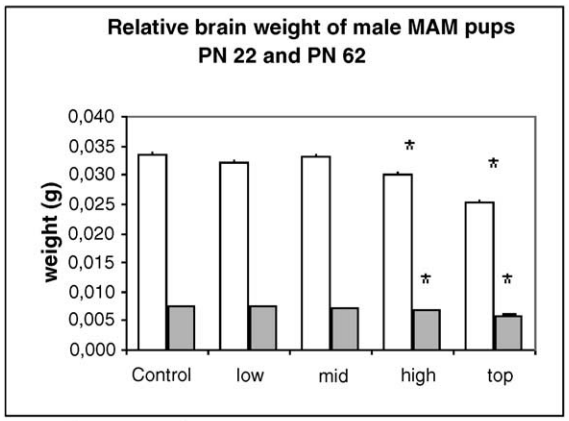
4. Discussion

The present paper forms part of a developmental neurotoxicity study with methylazoxy methanol acetate (MAM) (EPA Guidelines OPPTS 870.6300, 1996a, with limited



(A)

Dose group	PN	N	Total brain weight Mean	SEM	CE%
control	22	10	1,4377	0,0169	1,2
low	22	10	1,4513	0,0152	1,0
mid	22	10	1,4202	0,0160	1,1
high*	22	10	1,3491	0,0119	0,9
top*	22	10	1,1024	0,0233	2,1
control	62	10	1,9203	0,0157	0,8
low	62	10	1,9424	0,0180	0,9
mid	62	10	1,9020	0,0194	1,0
high*	62	10	1,7799	0,0382	2,1
top*	62	10	1,4432	0,0413	2,9



(B)

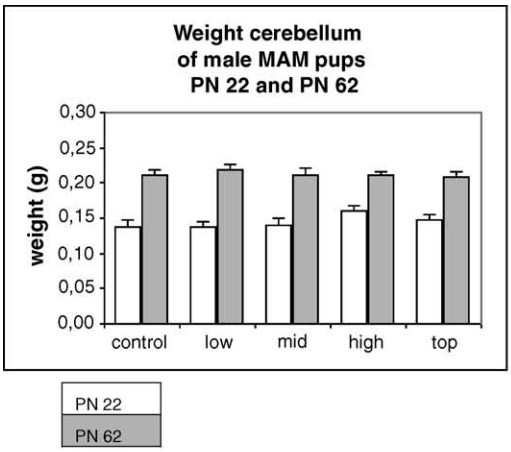
Dose group	PN	N	Relative brain weight Mean	SEM	CE%
control	22	10	0,0336	0,0005	1,9
low	22	10	0,0322	0,0007	1,9
mid	22	10	0,0332	0,0007	2,4
high*	22	10	0,0302	0,0004	1,4
top*	22	10	0,0254	0,0006	2,2
control	62	10	0,0074	0,0002	1,2
low	62	10	0,0074	0,0001	16,4
mid	62	10	0,0071	0,0001	1,6
high*	62	10	0,0069	0,0001	3,7
top*	62	10	0,0059	0,0003	4,4

Fig. 4. Brain weight measurements of male F1-animals at PN 22 and 62 of control and MAM dose groups. (A) Total brain-weight; (B) relative brain weight; (C) weight cerebellum (of one brain half); (D) weight cerebrum anterior (of one brain half); (E) weight cerebrum posterior (of one brain half). MAM exposed brains show a dose-related reduction in (*relative*) brain weight. The cerebrum appears significantly affected, but not the cerebellum. Notice that the values in C, D and E account for means (sem and CE%) of brain *halves*! (*) Significantly different from controls. Statistical key: ANOVA followed by Dunnett's multiple comparison test, $2p < 0.05$, significant.

behavioural testing (motor activity)) on the re-evaluation of neuropathology endpoints for developmental neurotoxicity testing. It summarizes conventional neuropathology endpoints and is used as a baseline for the evaluation of a potential new neuropathology endpoint, i.e. absolute neuron numbers, to be estimated by stereological means.

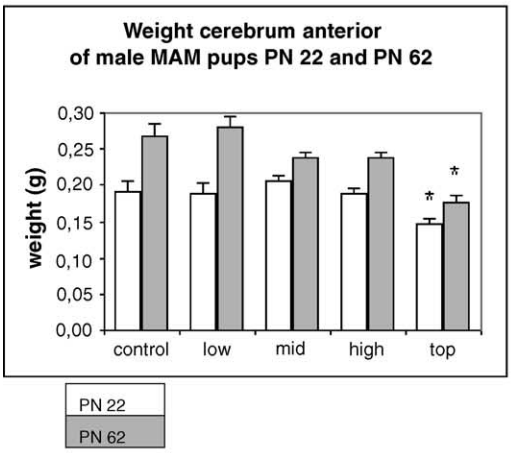
Mated female rats (14 rats/dose group) were dosed intraperitoneally from GD 13–15 with MAM acetate at the following dose levels: 0, 1.25, 2.5, 5 or 7.5 mg MAM/kg bw/day. Clear (dose-related) effects of MAM on developmental brain morphology were demonstrated for all neuropathology endpoints so far evaluated, both on PN 22 and 62. The effects involved the cerebrum (particularly the cerebral cortex), but *not* the cerebellum, although gross *qualitative* examination of the brains demonstrated a suggestive increase in cerebellar size.

In general, these findings are in good agreement with the results of other investigators studying the effects of MAM on the developing rat brain. Goldey et al. (1994) used MAM before to evaluate testing procedures for identification of potential developmental neurotoxicants. Prenatal administration of MAM (gestation day (GD) 10 or 15) appeared to



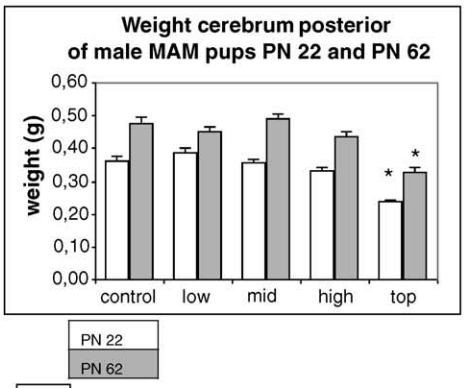
(C)

Dose group	PN	N	Weight cerebellum		
			Mean	SEM	CE%
control	22	10	0,1381	0,0106	7,7
low	22	10	0,1376	0,0080	5,8
mid	22	10	0,1407	0,0098	7,0
high	22	10	0,1598	0,0088	5,5
top	22	10	0,1479	0,0072	4,8
control	62	10	0,2104	0,0084	4,0
low	62	10	0,2194	0,0070	3,2
mid	62	10	0,2112	0,0112	5,3
high	62	10	0,2107	0,0054	2,6
top	62	10	0,2082	0,0079	3,8



(D)

Dose group	PN	N	Weight cerebrum anterior		
			Mean	SEM	CE%
control	22	10	0,1920	0,0126	6,6
low	22	10	0,1873	0,0162	8,6
mid	22	10	0,2061	0,0080	3,9
high	22	10	0,1892	0,0074	3,9
top*	22	10	0,1462	0,0077	5,3
control	62	10	0,2689	0,0174	6,5
low	62	10	0,2810	0,0136	4,8
mid	62	10	0,2373	0,0085	3,6
high	62	10	0,2372	0,0091	3,8
top*	62	10	0,1752	0,0101	5,8



(E)

Dose group	PN	N	Weight cerebrum posterior		
			Mean	SEM	CE%
control	22	10	0,3643	0,0116	1,8
low	22	10	0,3849	0,0147	3,5
mid	22	10	0,3569	0,0094	2,8
high	22	10	0,3346	0,0058	3,1
top*	22	10	0,2370	0,0074	2,8
control	62	10	0,4782	0,0196	3,8
low	62	10	0,4511	0,0171	2,4
mid	62	10	0,4928	0,0109	4,6
high	62	10	0,4360	0,0166	3,8
top*	62	10	0,3257	0,0163	2,8

Fig. 4. (*Continued*.)

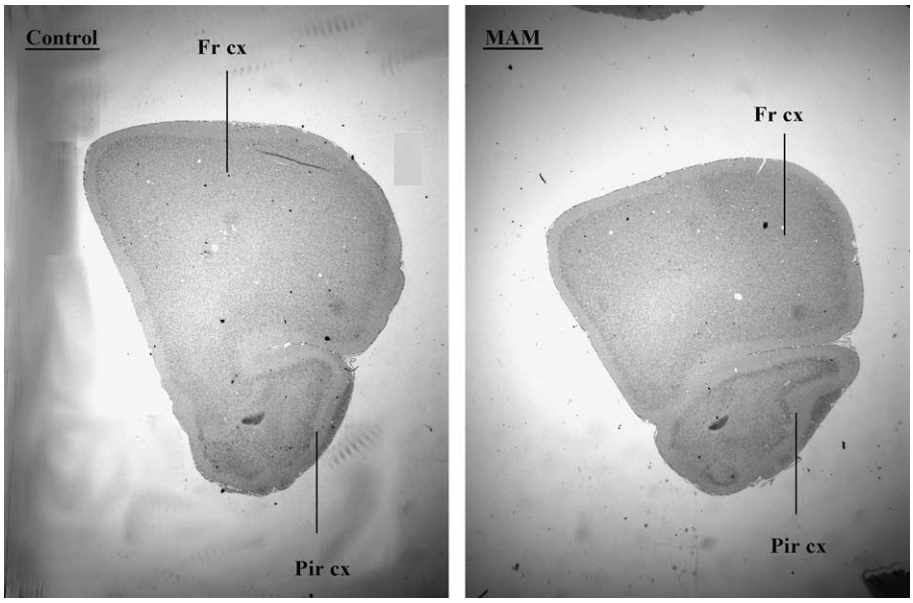


Fig. 5. Light micrographs of brain *Level 2* (rhinencephalon) of a control animal and one of the 7.5 mg/kg MAM top-dose. Notice the reduction in size; particularly the height of upper part of the brain section, i.e. the frontal cortex (Fr cx) is reduced and hypoplasia of the piriform cortex (Pir cx) can also be observed.

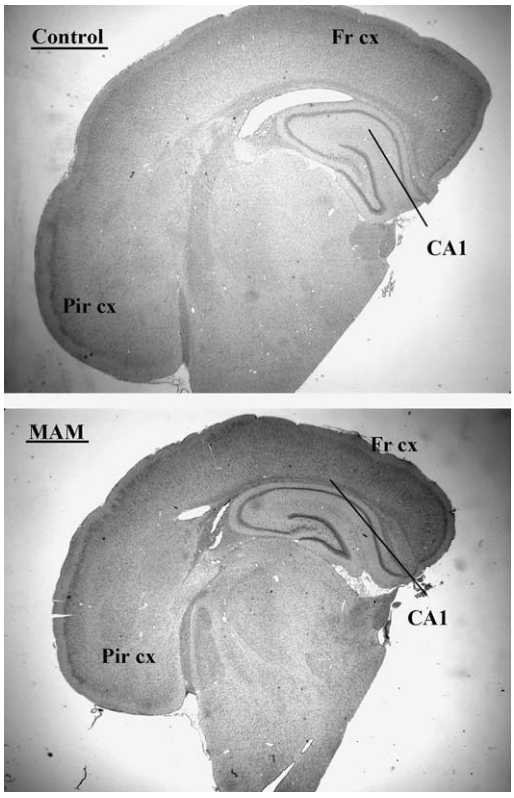
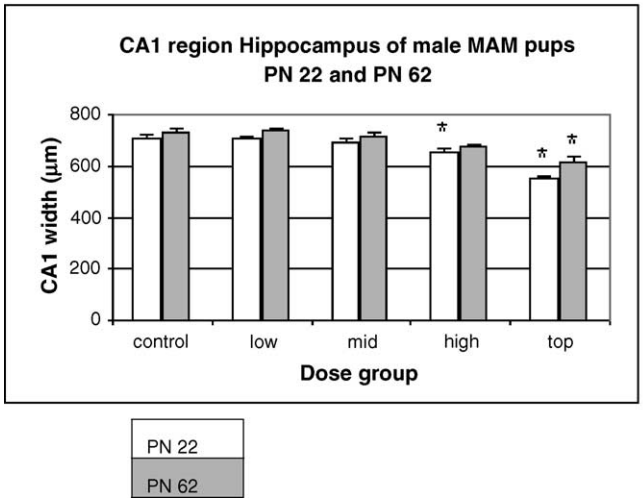


Fig. 6. Light micrographs of brain *Level 4* (diencephalon) of a control animal and one of the 7.5 mg/kg MAM top-dose. Notice the reduction in size of the neocortex (particularly the frontal cortex (Fr cx), and also the piriform cortex (Pir cx)) in the MAM top-dose group. The apparent difference in size of the hippocampus might be the result of MAM treatment. CA1: hippocampal CA1 region.

cause profound neurotoxic effects in the off-spring: reduced neonatal body and brain weight, hypoplasia of the neocortex and hippocampus, striatum and colliculi (cerebellum seems to be unaffected) and defects in a number of behavioural tests. Yet, viability and survival of the off-spring is not affected by MAM. MAM seems, therefore, to be a suitable model neurotoxicant for the purpose of this study. Kaufmann (2003) recently showed ectopic neurons in the hippocampus and neocortex using a *single* dose of 30 mg MAM i.p./kg bw (GD 15), but not in the next lower 15 mg MAM dose group. This effect was not observed in the 7.5 mg MAM top-dose group of the present study, given to the dams on *three consecutive days* during gestation (GD 13–15).

Regarding the efficacy and sensitivity of the different neuropathology endpoints used, the following is discussed. Most detailed and precise information was obtained from *microscopic linear morphometry*. It was demonstrated that the effects of MAM mainly concerned hypoplasia of the neocortex and hippocampus.

During *microscopic screening of the brain sections* (i.e. conventional 'slide reading') these effects were observed in the top-dose group, but direct comparison of *homologous brain sections* of control and MAM exposed animals and additional use of light micrographs appeared essential to detect the changes, as they were quantitative, rather than qualitative in nature. Therefore, conventional slide reading, which is considered primarily a qualitative microscopic screening approach, was felt incomplete and as a stand-alone method not suitable to detect test substance related effects on *developmental* brain morphology. It was also experienced that it is of vital importance to compare homologous sections of control and MAM exposed brains during microscopic screening.



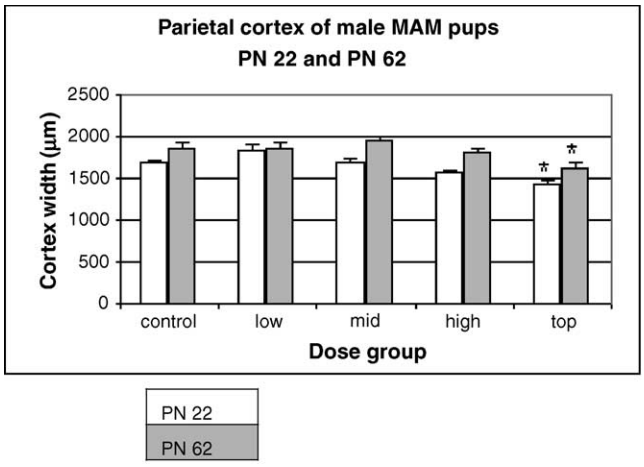
Dose group	PN	N	CA1 region Hippocampus		
			Mean	SEM	CE%
control	22	10	706,87	13,34	1,89
low	22	6	708,17	7,37	1,04
mid	22	9	692,74	14,60	2,11
high*	22	10	648,85	16,03	2,47
top*	22	8	553,49	8,75	1,58
control	62	10	726,95	15,61	2,15
low	62	9	735,70	13,74	1,87
mid	62	9	716,19	14,66	2,05
high	62	10	675,06	11,37	1,68
top*	62	8	614,22	23,78	3,87

Fig. 7. Graphic representation of the dose–response relationship of linear morphometric measures of the width of the parietal cortex for male F1-animals of MAM exposed rats, for PN 22 and 62. Dose-related effects of MAM are observed. The effects appear significant on PN 22 in the high-(5 mg/kg) and top-dose (7.5 mg/kg) groups, and on PN 62 in the top-dose MAM group (see asterisks). (*) Significantly different from controls. Statistical key: ANOVA followed by Dunnett's multiple comparison test, $2p < 0.05$, significant. *N*: number of available measurements/parameter; one measurement/parameter/animal. Ten animals/group were analysed, but measurements were only carried out in homologous sections. Otherwise, the measurement was considered 'missing' and was not included in the group means.

Therefore, the topography of each section was checked by comparing it with those published in the atlas of Paxinos and Watson (1986). From the results, it was concluded that dissection of the brains along *neuro-anatomical landmarks* forms a good basis to guarantee homology between sections of the different brain levels of both control and MAM exposed brains.

Brain weight measures appeared to give *relevant information* in a minimum amount of time with the brain still intact for further investigations. By weighing the total brain and straightforward dissected brain parts (cerebrum anterior and posterior parts, and cerebellum part) as proposed in EPA Guidelines OPPTS 870.8600, 1996b, it was demonstrated that (1) MAM affected the brain in a dose dependent way (significant in the MAM high- and top-dose groups) on PN 22 and 62; (2) the effects were most pronounced in the cerebrum; (3) the cerebellum was not affected. The suggestive increase in cerebellar size observed during gross examination either represented a relative change in the *shape* of the cerebellum and/or was an optical illusion, resulting from the decrease in size of the cerebrum.

These findings were confirmed by the results of *microscopic linear morphometry*, i.e. the measurement of the width of relevant brain layers. The morphometry data, moreover,



Dose group	PN	N	Parietal cortex		
			Mean	SEM	CE%
control	22	9	1682,63	40,37	2,40
low	22	10	1845,16	68,60	3,72
mid	22	9	1688,43	54,58	3,23
high	22	10	1564,76	32,39	2,07
top*	22	9	1434,39	50,52	3,52
control	62	9	1853,52	73,51	3,97
low	62	9	1862,86	62,39	3,35
mid	62	10	1959,04	35,80	1,83
high	62	9	1804,46	57,58	3,19
top*	62	7	1629,88	51,26	3,15

Fig. 8. Graphic representation of the dose–response relationship of linear morphometric measures of the width of the hippocampus (CA1 region) for male F1-animals of MAM exposed rats, for PN 22 and 62. Treatment-related effects are observed on PN 22 and 62; significant in the 7.5 mg/kg MAM top-dose (see asterisks). (*) Significantly different from controls. Statistical key: ANOVA followed by Dunnett's multiple comparison test, $2p < 0.05$, significant. *N*: number of available measurements/parameter; 1 measurement/parameter/animal. Ten animals/group were analysed, but measurements were only carried out in homologous sections. Otherwise, the measurement was considered 'missing' and was not included in the group means.

gave information with regard to the morphological predilection areas for MAM-induced developmental neuropathology (neocortex and hippocampus).

Regarding time and costs of the different endpoints the following points should be emphasized: Simple linear measurements of the width of major brain layers can be done rapidly, especially with present-day computers. However, for useful and correct linear measurements *only* highly homologous sections should be used, which—as shown in this study—can be obtained by dissection of the brain according to well-defined neuro-anatomical landmarks, and verification with the atlas of e.g. Paxinos and Watson (1986). It appeared worthwhile to invest some extra time in proper dissection of the brain according to anatomical landmarks and to check the homology of the sections prior to neuropathology screening; the benefit of this investment for *quantification* both at the macroscopic and microscopic level is by far larger than the extra time investment.

Conventional slide reading *per se*, proposed also in current guidelines *(EPA, OPPTS 870.6300, 1996a and 870.8600, 1996b;OECD 426, in preparation)*, appeared to be the least discriminative method to detect the quantitative changes in developmental brain morphology caused by MAM. The use of light micrographs turned out to be helpful, but very time-consuming as well, even with modern digital cameras and computer-assisted archiving.

Bilateral symmetry is a frequent hallmark of neurotoxicant-induced brain lesions. In the present histology protocol, one brain half was used for initial screening and linear morphometry, whereas the other brain half was left intact for future examinations, in this case for counting neurons in the brain. To avoid bias in left/right differences (lateralization), the brain halves were sampled, left/right systematically, randomly alternating, so that lateralization effects on averages are *a priori excluded*.

Whether lateralization does at all exist in the context of neurotoxicants and the *developing* brain, remains unknown. To our knowledge, this was not reported up till now. For MAM, we do not expect to find lateralization effects in the present study. Kaufmann (2003) studied the effects of MAM in the developing rat brain and carried out linear measurements of brain layer width, distinguishing between measurements in the left and the right hemispheres. Their results did not point at left/right differences (Kaufmann, personal observation). Whatever the case, a bias resulting from lateralization is *a priori* ruled out by the *random* allocation of the left/right hemispheres. So far, our results obtained in one brain half appear to be in good agreement with those in the literature for total brains. The present approach, however, has the advantage that the contra-lateral brain halves remain intact and can be used for whatever further investigations.

In this study, the remaining intact brain halves will be used in a second step of the project to study *absolute neuron numbers* by stereological means (Gundersen et al., 1988). The comparison with the results of the first step (conventional neuropathological approach) will show whether this method can contribute to a better understanding of relevant morphologic changes in the developing brain. Also, brain region volumes will be estimated using stereology. The nature and precision of such estimates are expected to enable detection of early quantitative neuropathological changes. In case of positive results, this method may be a candidate to be used as new endpoint besides conventional endpoints.

Summarizing the results obtained for the conventional neuropathology endpoints, it was concluded that for an adequate morphological approach to study effects of a neurotoxicant on the *developing* brain, primarily those endpoints that provide *quantitative* information (such as brain weight and linear morphometry) are useful to detect effects of the neurotoxicant on the developing brain. Microscopic screening of brain sections *only*, is incomplete and as a stand-alone procedure not suitable to detect the kind of changes induced by developmental neurotoxicants.

Acknowledgements

The authors are grateful to Anja Dijkstra (TNO Zeist, NL), Linda van de Horst-Groeneveld BSc (TNO Zeist, NL), Maj-Britt Lundorf (Stereological Research Laboratory, Arhus, DK), Lidy van Oostrum (TNO Zeist, NL), Marlies Otto BSc (TNO Zeist, NL) and Susanne Sørensen (Stereological Research Institute, Copenhagen, DK) for their skilful assistance regarding tissue preservation, brain dissection, macroscopic measurements, embedding and sectioning, linear morphometry and statistics.

This Project is financially supported by the American Chemistry Council (ACC Contract No. 1847). The views given in this paper are not necessarily the views of the sponsor but are solely the responsibility of the authors.

References

Duffell, S.J., Soames, A.R., Gunby, S., 2000. Morphometric analysis of the developing rat brain. Toxicol. Pathol. 28 (1), 157–163.

EPA Health Effects Test Guidelines OPPTS 870.6300, 1996a. Developmental Neurotoxicity Study “Public Draft”, United States Environmental Protection Agency; Prevention, Pesticides and Toxic Substances (7101); EPA 712-C-96-239, June 1996. http://www.epa.gov/opptsfrs/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Drafts/870-6300.pdf.

EPA Health Effects Test Guidelines OPPTS 870.8600, 1996b. Developmental Neurotoxicity Screen “Public Draft”, United States Environmental Protection Agency; Prevention, Pesticides and Toxic Substances (7101); EPA 712-C-96-258, June 1996. http://www.epa.gov/opptsfrs/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Drafts/870-8600.pdf.

Goldey, E.S., O’Callaghan, J.P., Stanton, M.E., Barone, S., Crofton, K.M., 1994. Developmental neurotoxicity: evaluation of testing procedures with methylazoxy methanol and methylmercury. Fundam. Appl. Toxicol. 23, 447–464.

Gundersen, H.J.G., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Sørensen, F.B., Vesterby, A., West, M.J., 1988. The new stereolog-

ical tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96 (5), 379–394.

Kaufmann, W., 2003. Developmental neuropathology in DNT-studies: Practical approaches and considerations on morphometric data. In: Seeber, A., Fechter, L. (Eds.), Book of Abstracts of the 9th Meeting of the International Neuroscience Association (INA-9), June 22–27.

Martin, P.M., O'Callaghan, J.P., 1995. A direct comparison of GFAP immunocytochemistry and GFAP concentration in various regions of ethanol-fixed rat and mouse brain. J. Neurosci. Methods 58, 181–192.

OECD Guideline for testing of chemicals, in preparation. Proposal for a new Guideline 426, Developmental Neurotoxicity Study.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates, second ed. Academic Press, New York, London.

Rodier, P.M., Gramann, W.J., 1979. Morphological effects of interference with cell proliferation in the early fetal period. Neurobehav. Toxicol. 1, 129–135.