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2D and 3D assessment of neuropathology in rat brain after prenatal exposure to methylazoxymethanol, a model for developmental neurotoxicty☆

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Abstract

To evaluate the ability of a tiered quantitative morphological approach to reveal developmental neurotoxicity, morphometric parameters were measured in the offspring of rats treated with methylazoxymethanol (MAM) during days 13–15 of pregnancy. Treatment was aimed at inhibiting the proliferation phase of hippocampal neurons while leaving cerebellar neurons unaffected. 2D and 3D assessment of brain morphology combined with straightforward measurement of brain size, weight and volume, and the usefulness of estimation of total neuron numbers were studied. Each tier indicated major effects of MAM, from macroscopic effects in the cerebrum (first tier) to a considerable loss of neurons in the hippocampal CA1 pyramidal layer (third tier). The cerebellum and the number of cerebellar granular neurons were not changed. Along with each step of the proposed tiered approach (brain size → linear morphometry → stereology), the discriminative strength of the endpoints, and thus the probability to pinpoint the extent and location of developmental brain lesions increased.
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1. Introduction

Susceptibility of the brain to chemicals and drugs appears to depend on its developmental stage at the time of exposure. Brain regions each have their own specific time-window for the proliferation, migration, differentiation and scheduled apoptosis of cells. During this specific time-window, a brain region may be adversely affected, often resulting in changes in cell numbers. Despite the fact that such changes may be considerable they can easily go unrecognised.

In a recent paper, we showed that preferably a quantitative morphological approach should be applied to study the effects of a neurotoxicant on developing brain morphology [1]. Slide reading, a major part of developmental neurotoxicity screening, is often inadequate as an isolated method. Linear morphometry – measurements of the width of major brain regions/layers – as a screening model may be sufficient, provided that homologous sections between individuals are used. Dissection of the brain along neuro-anatomical landmarks

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can to a large extent fulfil this requirement. However, for some regions this homology is much more difficult to accomplish. For example, in the rat brain the hippocampus, a key structure in learning and memory processes, is banana-shaped and shows a torsion. One end starts dorsally near the midline between the hemispheres about halfway along the cerebrum, whereas the other is ventro-caudally oriented. Systematic sections through the brain at the level of the hippocampus illustrate that. Although the outline of the hippocampus is, in general, clearly defined, one can also observe that its shape noticeably changes within short distances. Minor changes in hippocampus level may easily introduce considerable variation in 2D width measurements of a hippocampal region or layer. Consequently, the discriminative power of the 2D measures is limited due to the variation between individuals resulting from non-homologous sections.

This variation is likely to be reduced and the discriminative power increased, when the brain is sectioned systematically and homologous levels are selected from a series to carry out the measurements. In doing so, linear morphometry is likely to be more powerful and guiding in a tiered quantitative approach from straightforward measurement of brain weight towards more sophisticated modern stereology methods to estimate total numbers of neurons.

Whereas linear morphometry offers measurements in 2D, stereology provides methods for quantification of 3D structures based on unbiased principles. The discriminative power of stereology methods is high and they are likely to be powerful enough to detect smaller developmental changes in neuron numbers, which are not revealed with linear morphometry [2]. With modern stereology [3] the investigated structures, i.e. neurons, are directly counted in the 3D tissue. However, for these analyses, it is essential that the sampling is based on unbiased principles, usually applying systematic random sampling and thick sections.

Systematic (serial) sectioning is tedious. However, if necessary, efficiency can be improved by adapting the histology protocol, or indirectly, by making the sections accessible to various, even more sophisticated neuropathological approaches so that the sections can be adequately (re-)used. The goal of the present study was to test whether series of systematically sampled thick (40 μm) sections through the rat brain could serve as the starting point for combined 2D and 3D analysis of developmental neuropathology in a tiered morphological approach from brain weight (macroscopy) to total number of neurons (stereology) in selected regions of the rat brain.

In this study methylazoxymethanol (MAM), a well-known neurotoxic drug for the developing rat brain, was given to pregnant rats [4]. Prior to processing for microscopical investigation, the brains were quantitatively assessed at the macroscopical level for brain size, -weight, and -volume.

MAM is an anti-mitotic agent, interfering with cell proliferation. It kills dividing neurons through DNA damage [5]. Its effects on brain development are well characterized [4]. In the model, MAM was intentionally administered during gestation days 13–15. During this period the forebrain, cerebral cortex and hippocampus have their specific proliferation time-window. Although the duration of the proliferation phase of the main brain regions lasts up to 5 days, MAM was dosed during 3 days only, since dosing over longer periods was not possible due to cytotoxicity. Major brain regions such as the cerebellum and medulla oblongata also have main proliferation phases of 3–5 days, but their developmental time-frame is during the first postnatal days. Thus, it was hypothesized that, in the model, MAM would interfere with the proliferation of neurons in the hippocampus whereas neurons in the cerebellum would remain unaffected.

To study the advantages of stereology methods to estimate neuron numbers during early brain development, we counted neurons in the hippocampus and in the cerebellum. For stereology to be relevant for developmental toxicology, the estimates should clearly show the difference in susceptibility of these two regions, having different time-windows of development in relation to the dosing schedule. Key neurons in both brain regions, i.e. the pyramidal neurons in the CA1 region of the hippocampus and the granular neurons in the cerebellum were counted. Also the total volume of the reference layer of both types of neurons was estimated using stereology.

The same systematically randomly sampled sections used for stereology were used for 2D morphometric measurements of the width of main regions/layers through the brain. The results of the 2D and 3D approaches are evaluated relative to the effects observed on brain weight and are discussed for their power to detect early developmental neurotoxicity.

2. Materials and methods

2.1. Test substance

Methylazoxymethanol acetate (CAS Reg. no. 592-62-1) was obtained from the Midwest Research Institute, USA.

2.2. Test system

Nulliparous female Wistar outbred (Crl:(WI)WU BR) rats, approximately 11 weeks of age, were ordered. The rats were obtained from a colony maintained under SPF conditions at Charles River Deutschland, Sulzfeld, Germany. At the commencement of the mating period the females were approximately 12 weeks old. For mating, male rats from the same strain and supplier were used. These male rats were approximately 12–21 weeks old at mating. Pregnancy was confirmed by sperm cells in the vaginal smears.

Mated female rats (14 rats/group) were dosed intraperitoneally from GD 13–15 with 7.5 mg MAM/kg body weight/day, or saline (1 ml/kg body weight/day). F1-animals were selected for different purposes; one rat /sex/litter (10 l/group) was selected for extensive neuropathology on PN 22. The present study concerns the results on neuropathology

endpoints in the male F1 animal aged 22 days. The different measurements, i.e. sizeweight/volume (macroscopy), 2D linear morphometry and 3D stereology, were all obtained in the same subset of 10 animals in a group.

2.3. Histology

On PN 22, F1-animals (one animal/sex/litter; 10 l/group) were sacrificed under ether anaesthesia, by trans-cardial perfusion with neutral phosphate buffered formalin fixative (12 min; 100 mmHg), preceded by saline (approximately 30 s).

After the perfusion fixation the brains were removed from the skull and (post)fixed in formalin for 48 h (±30 min), before being transferred into 0.1 M phosphate buffer (pH 7.2–7.4). The brains were rinsed in buffer during a period of 11 days (±1 day) before further dissection and measurements of brain size, weight and volume started. During this period, the buffer was refreshed three times.

Dissection of the brain occurred along neuro-anatomical landmarks [1]. One brain half (alternating left/right to avoid lateralisation; the first one chosen randomly) was *split*, the other half was left *intact*, i.e. cerebrum and cerebellum attached.

The intact brain halves were used in the present histology study. Four brain halves together were uniquely orientated within one mould, resulting in a multi-organ block. On the bottom of the mould a paper line grid was placed, containing a set of parallel running 'orientation' lines (5–10 mm apart) and a 'front' line crossing the orientation lines perpendicularly (5–10 mm from the front façade of the mould). The line grid was used for consistent orientation of the brain halves during embedding using three predetermined axes: (1) medial side down (i.e. midline surface or cutting edge between the two hemispheres of one brain), (2) with the anterior pole of the hemisphere hitting the front line precisely, and (3) with the brain base oriented exactly parallel to the orientation lines. The brain base is defined by two neuro-anatomical landmarks at the ventral part of the brain, i.e. the chiasma opticum and the pons/medulla oblongata. The imaginary plane running through these two points is the third orientation axis.

The multi-organ blocks were sectioned systematically, from anterior to posterior, at a preset thickness of 40 μm. At regular intervals, every sixth section, three sections were sampled. The start of the first interval of a series of systematically sampled sections was always random. This start was determined by a number randomly chosen between 0 and 6 (since an interval of six sections was chosen). For example, if the random number is 4, then the first interval starts at the fourth section cut from the tissue-block.

The sections were mounted dry to avoid undesirable changes in section size and average section thickness, i.e. collection of the sections on the microscopic glass slides occurred without intermediate floating of the sections on a water-bath to stretch the paraffin section and to get rid of possible wrinkles. Stretching of a section on water unavoidably introduces changes in average section thickness and in section size.

The average section thickness (actually, the microtome advance, MA) is simply calculated from the ratio of total block thickness and the total number of sections cut from the block. It is used in the estimation of brain region/layer volume according to the Cavalieri principle (see below Section 2.4.3). The volumes are uncorrected for shrinkage due to paraffin embedding.

A complete series of sampled sections was stained with haematoxilin and eosin and was used for microscopic examination.

2.4. Neuropathology methods

2.4.1. Macroscopy: measuring brain size, weight and volume

The intact brain was measured at distinct locations (Fig. 1A) after removal from the skull and rinsing in phosphate buffer for 11 days (±1 day). Distinct brain parts were weighed (Fig. 1B) after dissection of the brain along neuro-anatomical landmarks [1] and the volume of the same brain parts was measured (Fig. 2C) by Archimedes' principle, i.e. by measuring the increase in weight of the fluid (specific gravity of the fluid must be known) into which a brain part was completely submerged [6]. One has to be careful in drying/blotting fluid (buffer or fixative) from the complete surface of a brain part prior to submersing it for a volume measurement, or the procedure will result in an overestimate of the volume. This is particularly important in a small organ with a large, very folded surface as the cerebellum. Therefore, to minimize this bias, the different brain parts were blotted on paper napkins prior to submersion in the buffer for volume measurement.

The volume of a brain organ was calculated as

$$\text{volume(organ)} = \text{weight(displaced fluid)} / \text{specific gravity(displaced fluid)}$$

Body weight measured at the time of sacrifice, together with brain weight, were used to calculate the relative brain weight [brain weight/BW]. Brain volume and brain weight were combined to calculate the brain specific gravity [brain weight/brain volume]. A summary of the measured parameters is shown in Table 1 (compare also: Fig. 1).

2.4.2. Linear morphometry: measuring brain region/layer width

At seven different levels through the brain (Fig. 2) the widths of major regions and layers at representative locations within the olfactory bulb, neocortex, limbic cortex, hippocampus and cerebellum were determined. For these measurements a computer assisted counting grid (C.A.S.T., Olympus, Denmark[1]) enabling measurements in 2D and 3D tissue preparations, was used. Prior to assessment of linear

[1] Currently, C.A.S.T. is sold by Visiopharm, Denmark.

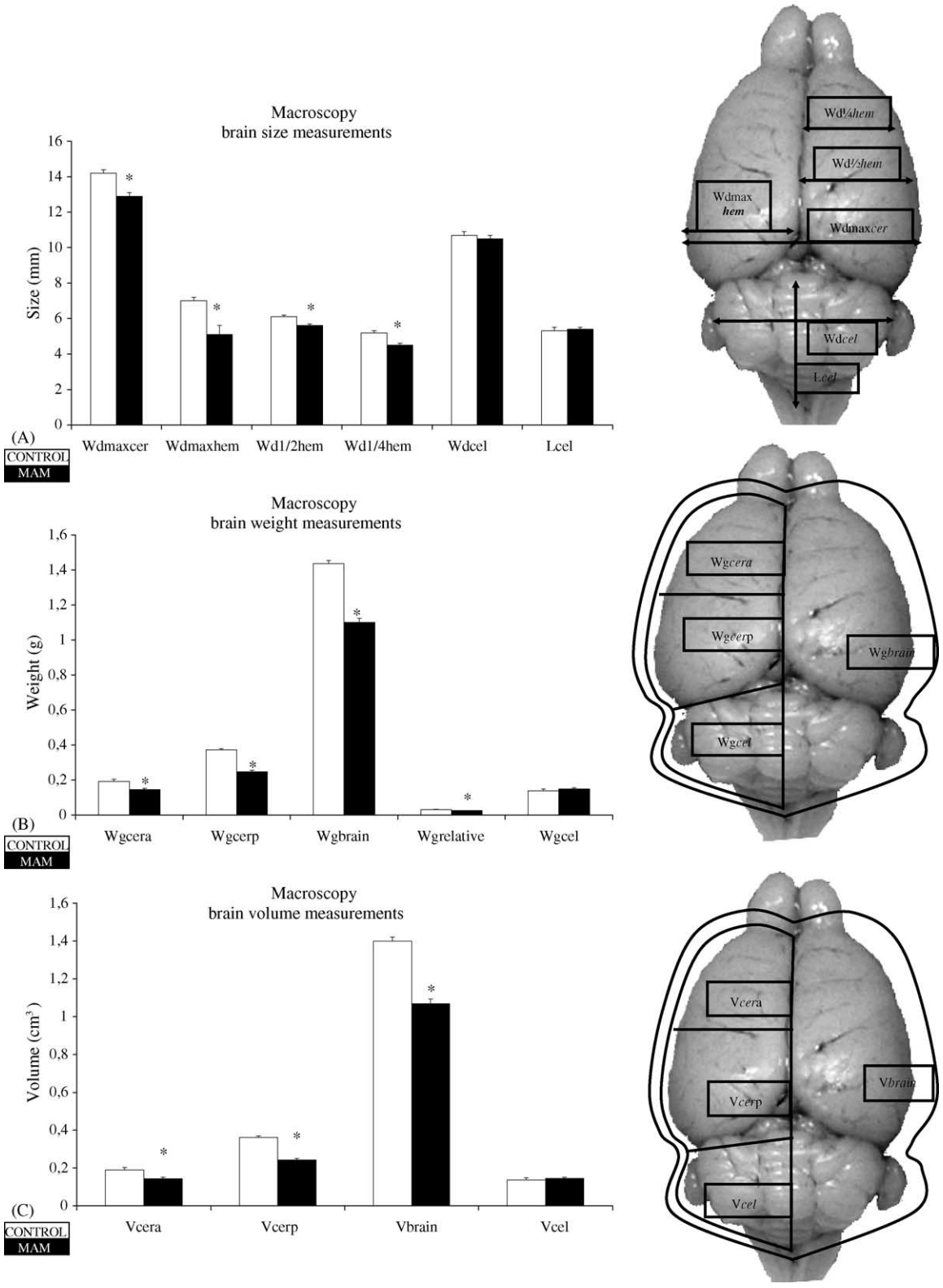

Fig. 1. Macroscopic measurements of brain size, weight and volume. Effects of prenatal exposure of male F1-animals (aged 22 days) to MAM (7.5 mg/kg body weight/day i.p., gestation days 13–15). For abbreviations, see Table 1. For group data, see Table 3. On the left: graphic representations of the results; on the right: the rat brain, indicating the different size parameters measured. (A) Brain size; (B) brain weight; (C) brain volume. Asterisk (*) denotes significantly different from controls. Statistical key: ANOVA (Bonferoni), $2p < 0.05$. Ten animals (one animal/litter)/group were analysed. (◯) Control animals (white); (●) MAM exposed animals (black).

morphometry, the seven sections per animal to be used for the morphometric analysis were identified and selected by comparing the sections with existing neuro-anatomy atlases [7,8]. Doing so, an optimal degree of homology between brain section levels of different individuals within a group was obtained.

After the selected sections were noted, the animals were renumbered so that measurements of the width of the different

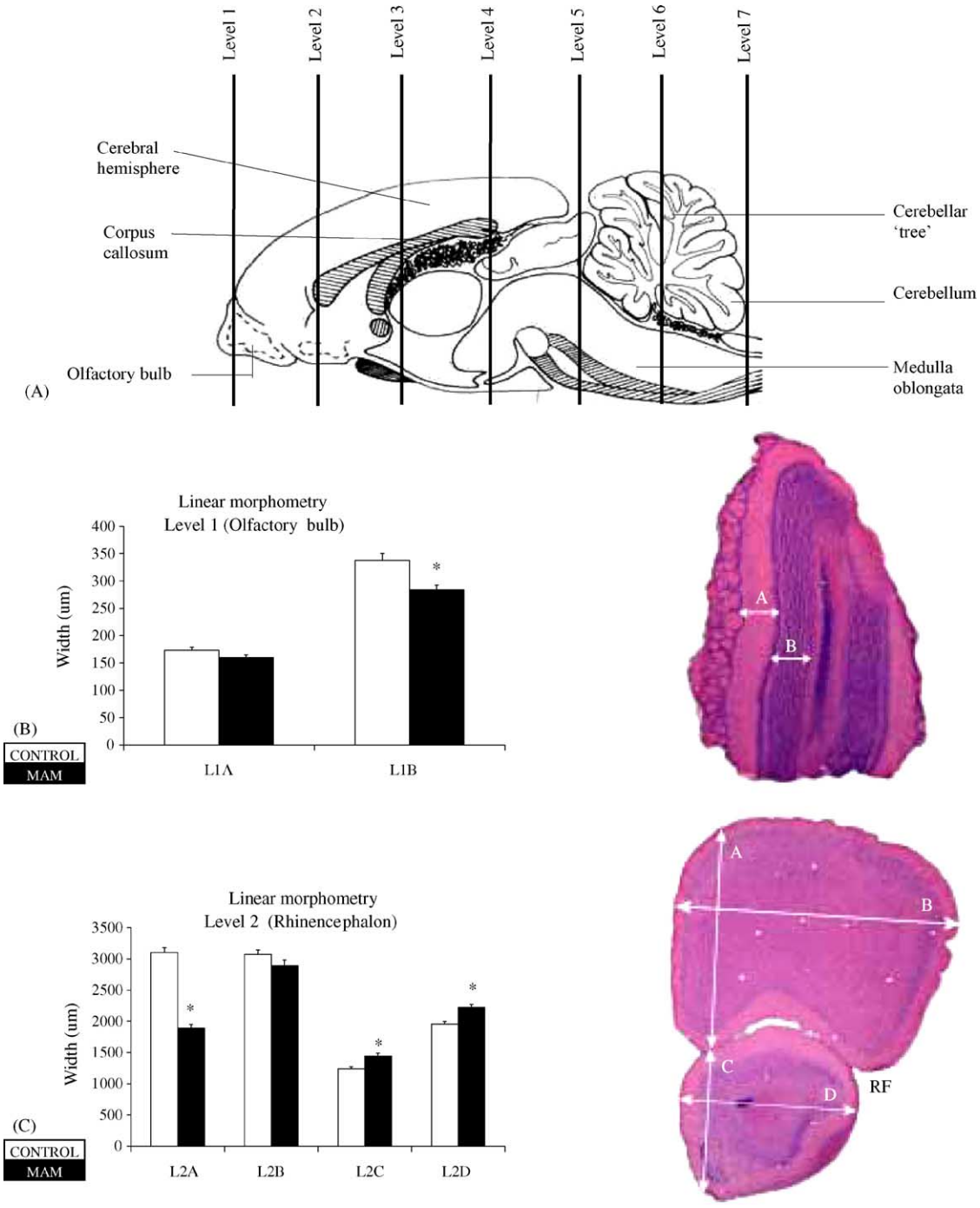

Fig. 2. Linear morphometry measurements at seven levels in the rat brain. Effects of prenatal exposure of male F1-animals (aged 22 days) to MAM (7.5 mg/kg body weight/day i.p., gestation days 13–15). For abbreviations, see Table 2. For group data, see Table 4. (A) Schematic drawing of a longitudinal section through the rat brain indicating the seven brain levels selected for 2D measurements of brain region/layer width (linear morphometry). One hemisphere is viewed from aside (midline between the two brain halves); (B)–(H) linear morphometry at Levels 1–7, respectively. On the left: graphic representations of the results (compare Fig. 2A). On the right: light-micrographs representing the parameters measured. Asterisk (*) denotes significantly different from controls. Statistical key: ANOVA (Bonferoni), $2p < 0.05$. Ten animals (one animal/litter)/group were analysed. (◯) Control animals (white); (●) MAM exposed animals (black).

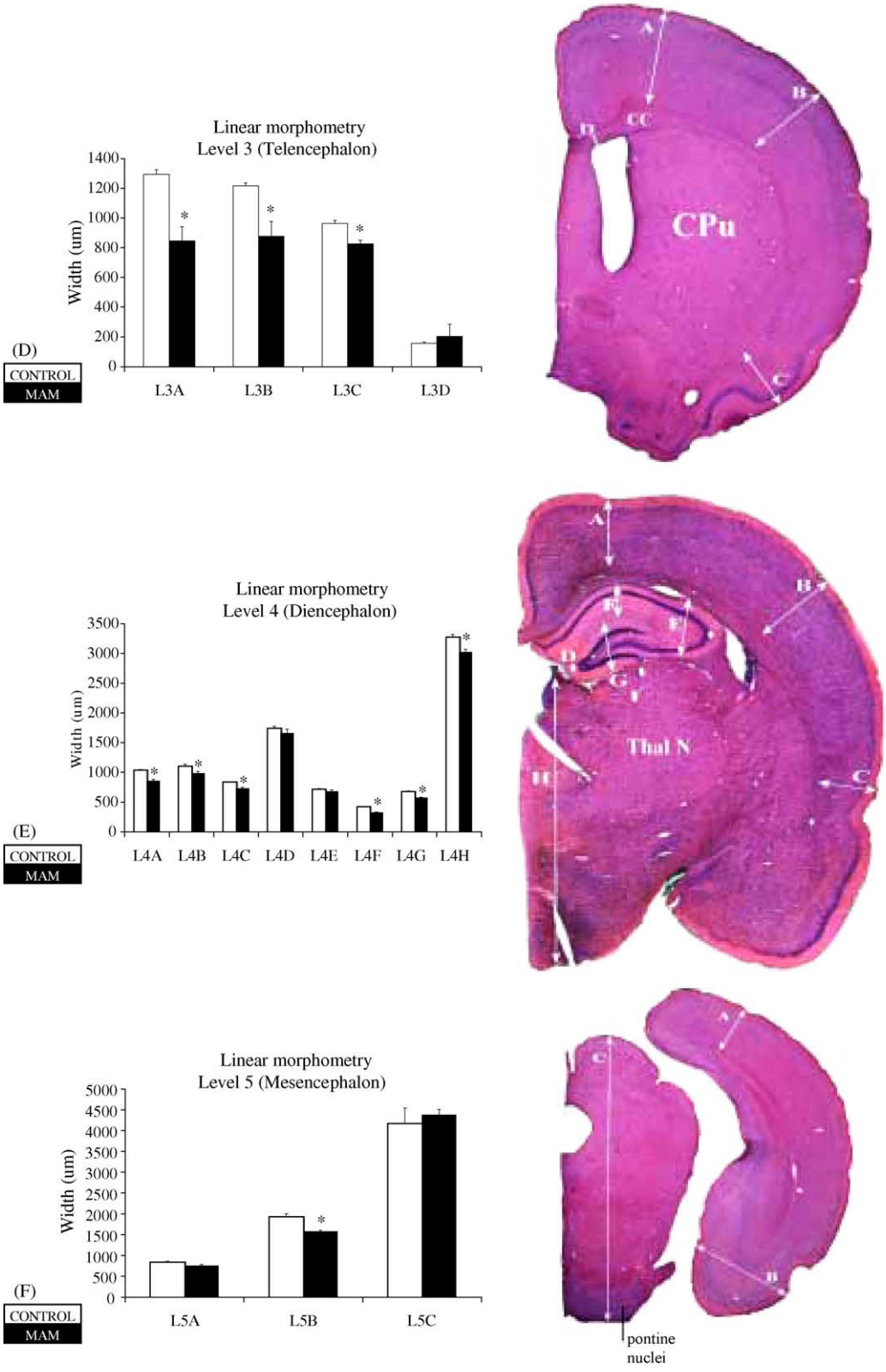

Fig. 2. (*Continued*)

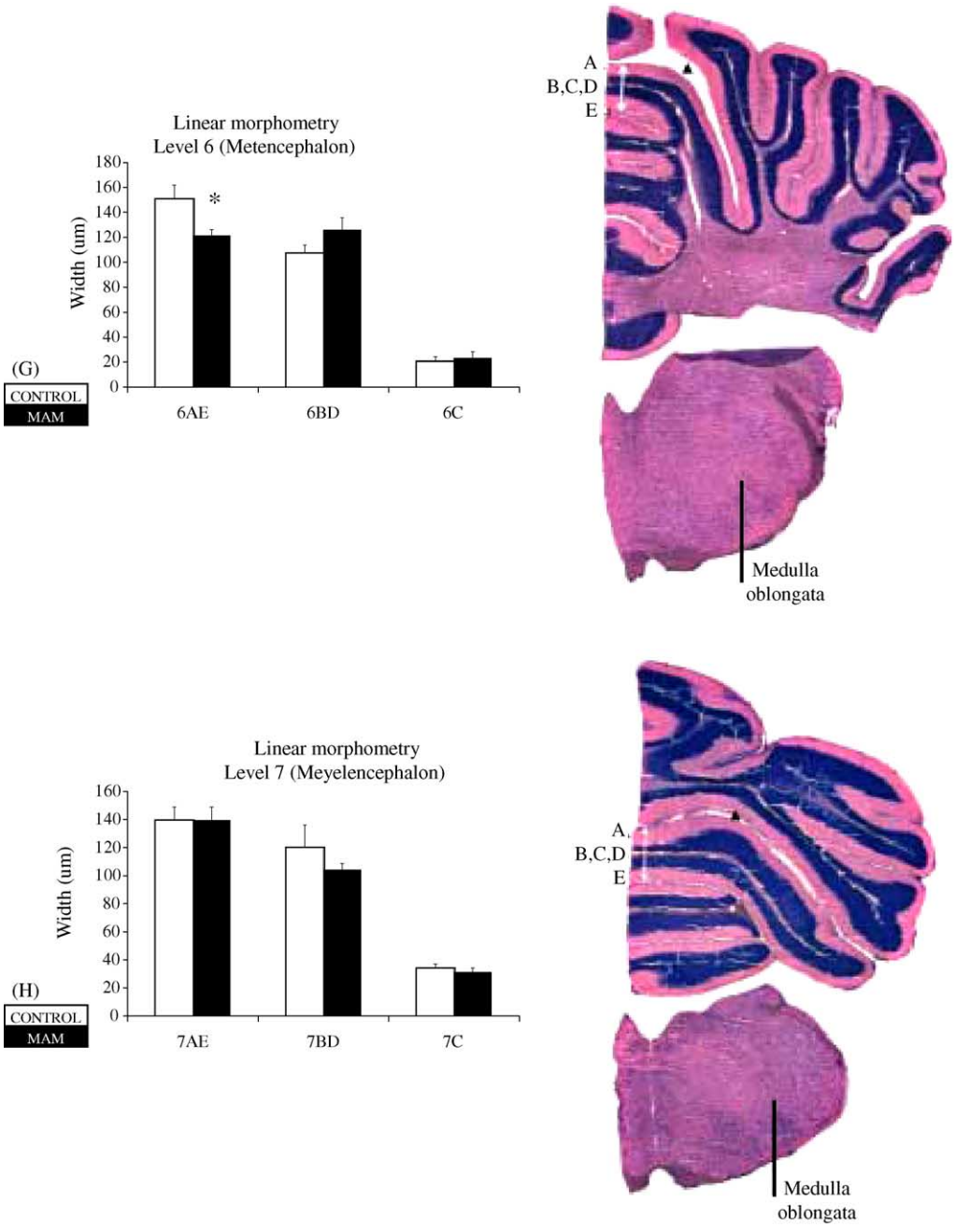

Fig. 2. (*Continued*).

brain regions/layers was blind, i.e. the C.A.S.T. operator was unaware of the treatment.

The brain levels described earlier (some with minor changes) [1] were analysed (Fig. 2): level 1, olfactory bulb; level 2, rhinencephalon; level 3, prosencephalon: telencephalon; level 4, prosencephalon: diencephalon; level 5, mesencephalon; level 6, rhombencephalon: metencephalon (mid cerebellum/medulla oblongata (pons)); level 7, rhombencephalon: myelencephalon (posterior cerebellum/medulla oblongata). A summary of the measured parameters is shown in Table 2 (compare also Fig. 2).

2.4.3. Stereology

2.4.3.1. Definition of neurons and brain regions for stereology. Prior to any stereological analysis, the hippocampal CA1 pyramidal layer, as well as the cerebellar granular layer were defined throughout the series of systematic sections, also by comparing the sections with published neuroanatomy [7,8]. To count neurons, the nucleus was used as the counting unit. The nucleus of both types of neurons can readily be identified. As a matter of fact, it is mainly the cell bodies of these neurons that comprise the CA1 pyramidal layer (Fig. 3) and the cerebellar granular layer (Fig. 4), respectively.

Table 1
Macroscopy: size, weight and volume measurements

Parameter (abbreviation)	Description of measured parameters (see also Fig. 1A–C)
Wdmax*cer*	Width posterior cerebrum: maximal position
Wdmax*hem*	Width posterior hemisphere: maximal position
Wd1/2*hem*	Width hemisphere: mid-position
Wd1/4*hem*	Width hemisphere (anterior): quarter position
Wd*cel*	Width cerebellum
L*cel*	Length cerebellum
Wg*cer*a	Weight cerebrum anterior
Wg*cer*p	Weight cerebrum posterior
Wg*brain*	Weight total brain
Wg*relative*	Relative brain weight (brain weight/body weight)
Wg*cel*	Weight cerebellum
V*cer*a	Volume cerebrum anterior
V*cer*p	Volume cerebrum posterior
V*brain*	Volume total brain
V*cel*	Volume cerebellum

2.4.3.2. Sampling design for number estimation. Neurons were counted in optical disectors [3] according to the optical fractionator sampling design. The optical fractionator involves counting of particles (neurons) with optical disectors in a uniform systematic sample that constitutes a known fraction of the volume of the region being analysed. In practice, this is accomplished by systematically sampling a known fraction of the section thickness, tsf, of a known fraction of sectional area, asf, of a known fraction of the sections that contain the region of interest, ssf, and a known fraction of the total brain, hsf, i.e. one brain half instead of two.

The total number of neurons N is estimated by multiplying $(\cdot)$ the number of neurons counted (Q^-) by the reciprocal of the overall fraction of the region sampled:

$$N = Q^- \cdot [1/\text{tsf}] \cdot [1/\text{asf}] \cdot [1/\text{ssf}] \cdot [1/\text{bsf}]$$

Focusing down through the 40 μm thick section, called an optical section as one passes through it in slow motion plane by plane, those nuclei that come into focus from one focal plane to the other are counted and are designated by Q^- (i.e. 'Q minus'). To avoid a problem of so called 'lost caps', i.e. nuclei that might fall off the section at the cutting surface and would be missed during counting, a guard area of 7 μm was included and counting of the neurons in a disector always started at 7 μm below the upper surface of the section. The height of the disector was set to 10 μm. The disector reference area is determined by an unbiased counting frame. Nuclei are counted only when they come into focus while passing through the optical disector from −7 to −17 μm (or 'plane by plane'). The total size of the optical disector was chosen such that, on average, 0–3 neurons appeared within the volume of one optical disector. The total number of disectors over all selected sections analyzed per brain was chosen such

Table 2
Linear morphometry: determination of brain region/layer widths

Parameter (abbreviation)	Brain level	Description of measured parameters (see also Fig. 2A–H)
L1A	Level 1	External plexiform layer
L1B	Level 1	From outside to inside: mitral cell layer, internal plexiform layer, internal granular layer until the germinal matrix
L2A	Level 2	Height upper 'lobe'
L2B	Level 2	Width upper 'lobe'
L2C	Level 2	Height lower 'lobe'
L2D	Level 2	Width lower 'lobe'
L3A	Level 3	Frontal cortex; perpendicular to pial surface
L3B	Level 3	Parietal cortex; perpendicular to pial surface
L3C	Level 3	Piriform cortex; perpendicular to pial surface
L3D	Level 3	Corpus callosum; perpendicular to its fibers and in line with the lateral ventricle
L4A	Level 4	Frontal cortex; perpendicular to pial surface
L4B	Level 4	Parietal cortex; perpendicular to pial surface
L4C	Level 4	Perirhinal cortex; perpendicular to pial surface
L4D	Level 4	Length hippocampus
L4E	Level 4	CA3 region hippocampus
L4F	Level 4	CA1 region hippocampus
L4G	Level 4	Dentate gyrus, from hippocampal fissure
L4H	Level 4	Height of lower 'lobe' (i.e. ventral part)
L5A	Level 5	Occipital cortex; perpendicular to pial surface
L5B	Level 5	Entorhinal cortex
L5C	Level 5	Height of pons
L6A	Level 6	Molecular layer 1; perpendicular to fissure
L6B	Level 6	Granular layer 1; perpendicular to fissure
L6C	Level 6	White matter; perpendicular to fissure
L6D	Level 6	Granular layer 2; perpendicular to fissure
L6E	Level 6	Molecular layer 2; perpendicular to fissure
L6AE	Level 6	Sum of molecular layers 1 + 2, divided by 2 ([L6A + L6E]/2)
L6BD	Level 6	Sum of granular layers 1 + 2, divided by 2 ([L6B + L6D]/2)
L7A	Level 7	Molecular layer 1; perpendicular to fissure
L7B	Level 7	Granular layer 1; perpendicular to fissure
L7C	Level 7	White matter; perpendicular to fissure
L7D	Level 7	Granular layer 2; perpendicular to fissure
L7E	Level 7	Molecular layer 2; perpendicular to fissure
L7AE	Level 7	Sum of molecular layers 1 + 2, divided by 2 ([L7A + L7E]/2)
L7BD	Level 7	Sum of granular layers 1+2, divided by 2 ([L7B + L7 D]/2)

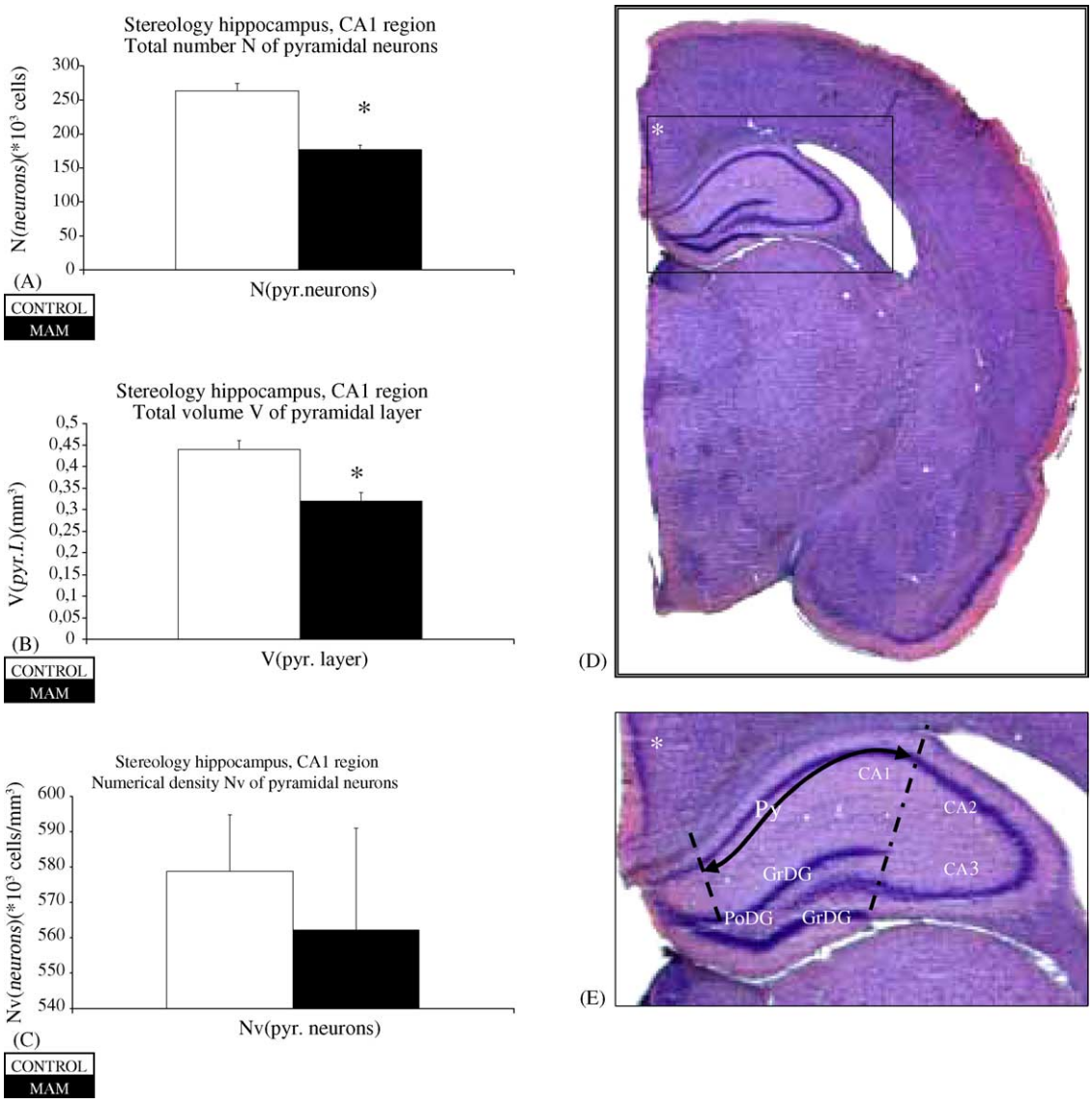

Fig. 3. Stereology in the hippocampus CA1 region. Graphic representation of total number *N* of neurons, total volume *V* of the reference layer and numerical density N_V of neurons. Effects of prenatal exposure of male F1-animals (aged 22 days) to MAM (7.5 mg/kg body weight/day i.p., gestation days 13–15). For group data, see Table 5. (A) Total number of hippocampal CA1 pyramidal neurons, *N* (pyr.neurons), estimated with optical fractionator; (B) total volume of the hippocampal pyramidal layer, *V* (pyr.layer), estimated with Cavalieri principle; (C) numerical density (number/volume) of hippocampal CA1 pyramidal neurons, N_V (pyr.neurons); (D) light micrograph of a paraffin section (thickness: 40 μm) through the rat brain (right hemisphere) used for stereological analyses and illustrating the location of the dorsal hippocampus (squared) (compare Fig. 2E). It should be borne in mind that for the stereological analyses series of systematically random sections through the hippocampus are used. For squared area (*): see (E) for enlargement (*); (E) enlargement of the dorsal hippocampus in (D). The dotted lines indicate the borders of the CA1 region. The arrow indicates the part of the pyramidal layer used for stereology. CA1: CA1 region of the hippocampus; CA2: CA2 region of the hippocampus; CA3: CA3 region of the hippocampus; Py: pyramidal layer; GrDG: granular layer of the dentate gyrus; PoDG: polymorphous layer of the dentate gyrus. Asterisk (*) denotes significantly different from controls. Statistical key: ANOVA (Bonferoni), $2p < 0.05$. Ten animals (one animal/litter)/group were analysed. (◯) Control animals (white); (●) MAM exposed animals (black).

that 150–200 neurons per brain would actually be counted. A 100/1.40 objective lens and oil immersion were applied. To avoid bias of tissue deformation for the number estimations, the section thickness (*t*) was measured locally and was corrected for tissue deformation (t_q^-) according to Dorph-Petersen et al. [9]:

$$t_q^- = \sum(t_i Q_i^-)/\sum Q_i^-$$

2.4.3.3. Cavalieri principle and point counting sampling design for volume estimation. The stereological approach applied to estimate the volume (*V*) of both layers was the Cavalieri principle [10], whereby the volume of the 3D brain region was estimated from analysis of the 2D areas of the region profile in the systematic microscopic sections. For this, the brain region is systematically sectioned down (section thickness (*T*) in a series must be known). At regular intervals (distance (*d*) between intervals must be known) a

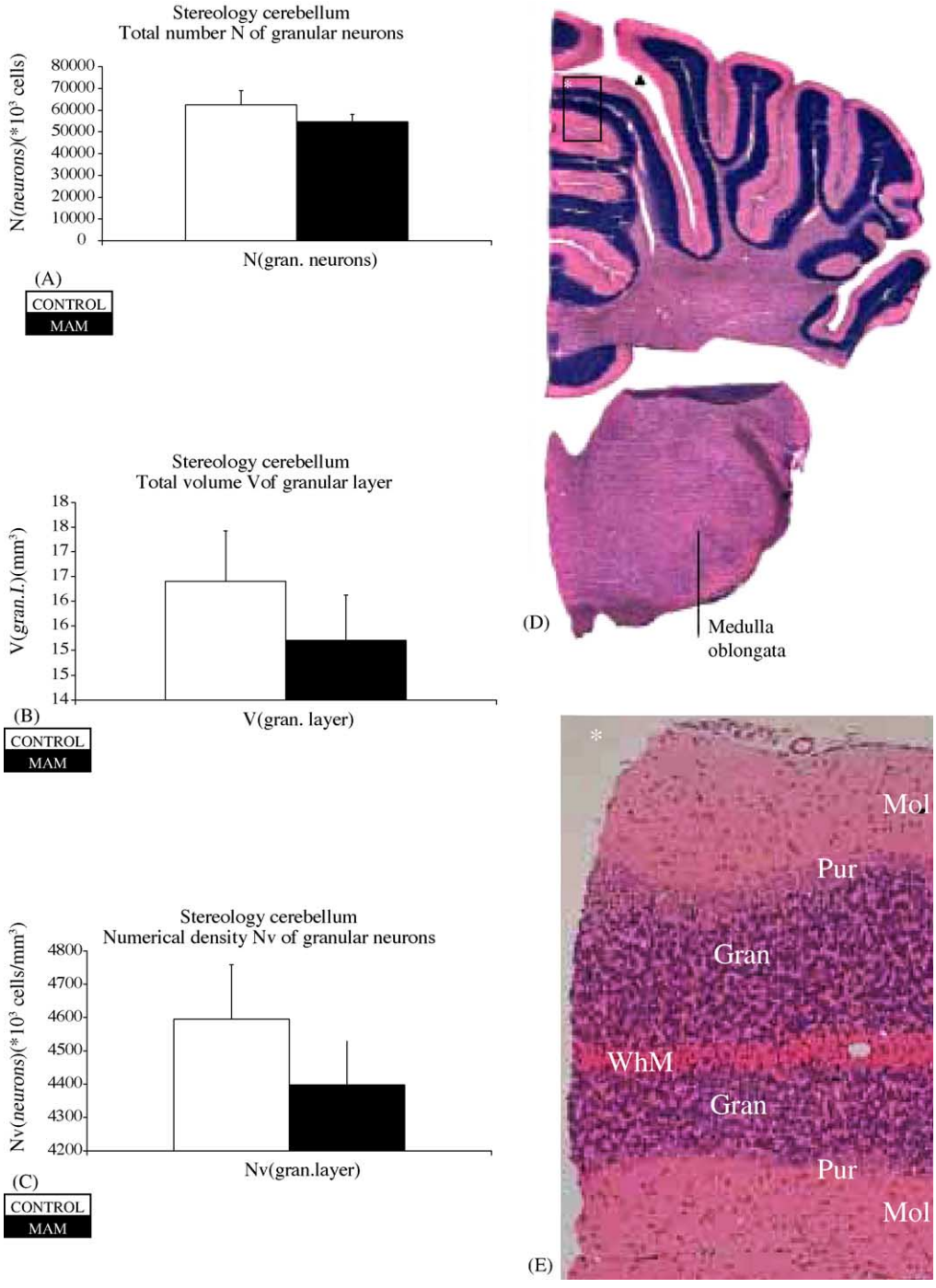

Fig. 4. Stereology in the cerebellum (granular layer). Graphic representation of total number N of neurons, total volume V of the reference layer and numerical density N_V of neurons. Effects of prenatal exposure of male F1-animals (aged 22 days) to MAM (7.5 mg/kg body weight/day i.p., gestation days 13–15). For group data, see Table 5. (A) Total number of granular neurons, N (granular neurons), estimated with optical fractionator; (B) total volume of the cerebellar granular layer, V (granular layer), estimated with Cavalieri principle; (C) numerical density (number/volume) of cerebellar granular neurons, N_V (granular neurons); (E) light micrograph of a paraffin section (thickness: 40 μm) through the rat brain (right hemisphere) used for stereological analyses illustrating the cerebellum with underlying medulla oblongata (squared) (compare Fig. 2G). It should be borne in mind that for the stereological analyses series of systematically random sections through the cerebellum are used. For squared area (*): see (E) for enlargement (*); (E) enlargement of the squared area in (D) illustrating the different layers seen in a transsection through a cerebellar lobule. Stereology was carried out in the granular layer. Mol.: molecular layer; Pur: Purkinje cell layer, Gr: granular layer; WhM: white matter. Asterisk (*) denotes significantly different from controls. Statistical key: ANOVA (Bonferoni), $2p < 0.05$. Ten animals (one animal/litter)/group were analysed. (◯) Control animals (white); (●) MAM exposed animals (black).

section is sampled. In a sampled section, the profile area of the brain region of interest is measured. The volume of the brain regions of interest is finally estimated by multiplying the total profile area in all section ($\sum a$) with mean section interval (d):

$$V = d \sum a$$

The profile areas were estimated by point counting using C.A.S.T. In these 22-day-old rats, a combination of a point counting grid and a section interval was used, aiming (as near as possible) at a point counting sampling design whereby about 10 systematic sections through the region of interest were analyzed and a total number of points, say 50–200, was falling on the region. This point counting was carried out simultaneously with counting of the neurons in optical disectors, which was done at high magnification using the C.A.S.T. system.

Point counting to finally estimate the volume of both neuron layers, and also counting of the neurons, was blind, i.e. prior to counting, the animals of both groups were renumbered (MAM exposed brains and vehicle controls) so that the C.A.S.T. operator was unaware of the treatment of the animal under investigation.

2.5. Qualitative microscopic examination

The thick (40 μm) sections selected for linear morphometry were examined by the pathologist for qualitative morphological alterations such as ectopic neurons and/or hypoplasia, known to result from exposure to high doses of MAM.

2.6. Statistical analysis of the results

One-way analysis of variance (ANOVA) with Bonferroni was the statistical procedure used in the evaluation of the data. The tests were two-sided. As a level of significance, $2p \leq 0.05$ was considered.

3. Results

3.1. Brain size, volume, weight

Effects of MAM on brain size, weight and volume are shown in Table 3. Graphic representation of the results is shown in Fig. 1.

MAM significantly affected brain size (Fig. 1A), brain weight (Fig. 1B) and brain volume (Fig. 1C). The cerebrum, the anterior part as well as the posterior part, was significantly affected. The cerebellum was not. Also the relative brain weight [brain weight/body weight] was significantly affected by MAM; the body weight itself was reduced as well. Specific gravity of the brain tissues [weight/volume] was not affected.

3.2. Brain region/layer width

Fig. 2A shows a schematic drawing of the rat brain (one hemisphere) indicating the seven brain levels used for 2D morphometric analysis. Fig. 2B–D shows for the seven different brain levels examined the effects of MAM on the different

Table 3
Macroscopic measurements of brain size, weight and volume

Macroscopic measurements[a]	Control				MAM			
	N[b]	Mean	S.E.M.	CE%	N[b]	Mean	S.E.M.	CE%
Brain size (mm)								
Wdmax*cer*	10	14.2	0.2	1.3	10	12.9*	0.2	1.3
Wdmax*hem*	10	7.0	0.2	2.3	10	5.2*	0.5	9.2
Wd1/2*hem*	10	6.2	0.1	1.3	10	5.6*	0.1	1.8
Wd1/4*hem*	10	5.2	0.1	2.1	10	4.5*	0.1	2.3
Wd*cel*	10	10.7	0.2	1.4	10	10.5	0.2	1.7
L*cel*	10	5.3	0.2	3.3	10	5.4	0.1	1.9
Brain weight (mg)								
Wg*cera*	10	192.0	12.6	6.6	10	146.2*	7.7	5.3
Wg*cerp*	10	372.1	8.1	2.2	10	248.4*	7.8	3.2
Wg*brain*	10	1437.7	16.9	1.2	10	1102.4*	23.3	2.1
Wg*relative*	10	32.1	0.6	1.9	10	25.4*	0.5	2.1
Wg*cel*	10	138.1	10.6	7.7	10	147.9	7.2	4.8
Brain volume (ml)								
V*cera*	10	189.1	13.1	6.9	10	142.4*	8.6	6.0
V*cerp*	10	360.5	9.0	2.5	10	242.8*	7.8	3.2
V*brain*	10	1398.5	21.9	1.6	10	1068.8*	24.3	2.3
V*cel*	10	136.8	10.5	7.6	10	143.5	6.6	4.6

Effects of prenatal exposure of F1-ANIMALS (aged 22 days) to MAM (7.5 mg/kg body weight/day i.p., gestation days 13–15).

[a] For abbreviations, see Table 1; For graphic presentation, see Fig. 1 Notice that some parameters refer to a brain half whereas others refer to a total brain as indicated in Fig. 1.

[b] $N = 10$ animals/group (one animal/sex/litter; 10 litters per group). S.E.M.: standard error of the mean; CE%: coefficient of error.

* Significantly different from controls; statistical key: ANOVA (Bonferoni); level of significance: $2p \leq 0.05$.

Table 4
Linear morphometry measurements at seven levels in the rat brain

Linear morphometry[a] (μm)	Control				MAM			
	N[b]	Mean	S.E.M.	CE%	*N*[b]	Mean	S.E.M.	CE%
Olfactory bulb level 1								
A	9[b]	172.9	6.1	3.6	9[b]	160.1	5.3	3.3
B	9[b]	338.0	12.2	3.6	9[b]	284.3*	8.1	2.9
Rhinencephalon level 2								
A	10	3104.8	75.9	2.4	10	1891.8*	55.4	2.9
B	10	3070.7	69.8	2.3	10	2890.0	91.1	3.2
C	10	1240.5	29.4	2.4	9[b]	1445.9*	41.0	2.8
D	10	1957.6	37.4	1.9	10	2222.7*	48.4	2.2
Telencephalon level 3								
A	10	1293.4	32.4	2.5	10	847.3*	94.1	11.1
B	10	1214.4	21.0	1.7	10	877.1*	98.1	11.2
C	10	964.2	18.2	1.9	8[b]	825.2*	24.0	2.9
D	9[b]	156.2	10.0	6.4	10	202.9	82.3	40.6
Diencephalon level 4								
A	9[b]	1030.6	17.3	1.7	9[b]	855.1*	24.0	2.8
B	10	1102.2	31.5	2.9	10	986.5*	27.2	2.8
C	10	834.7	13.0	1.6	9[b]	726.6*	19.1	2.6
D	10	1739.8	37.8	2.2	10	1658.5	67.4	4.1
E	10	708.0	22.1	3.1	10	680.5	25.7	3.8
F	10	417.5	13.4	3.2	10	326.8*	6.0	1.8
G	10	672.7	18.5	2.8	10	570.8*	15.4	2.7
H	7[b]	3270.9	47.7	1.5	8[b]	3018.5*	52.9	1.8
Mesencephalon level 5								
A	10	830.5	28.0	3.4	8[b]	755.5	31.1	4.1
B	10	1924.5	82.1	4.3	9[b]	1575.0*	29.8	1.9
C	10	4158.7	384.2	9.2	10	4378.6	129.2	3.0
Metencephalon level 6								
AE	10	150.7	11.8	7.4	10	121.4*	6.2	5.1
BD	10	107.2	7.6	7.3	10	125.8	10.1	8.8
C	9[b]	22.7	3.4	15.1	9[b]	25.7	4.8	18.8
Myelencephalon level 7								
AE	10	139.4	10.4	7.5	10	139.3	10.4	7.0
BD	10	119.9	17.4	15.5	10	104.1	6.4	6.9
C	10	34.0	3.1	9.2	10	31.1	3.1	10.1

Effects of prenatal exposure of male F1-animals (aged 22 days) to MAM (7.5 mg/kg body weight/day i.p., gestation days 13–15).
[a] For abbreviations, see Table 2; for graphic presentation, see Fig. 2B–H.
[b] $N = 10$ animals/group (one animal/sex/litter; 10 litters per group); sometimes, relevant tissues are missing. S.E.M.: standard error of the mean; CE%: coefficient of error.
* Significantly different from controls; statistical key: ANOVA (Bonferoni); level of significance: $2p \leq 0.05$.

parameters. The reducing effects of MAM were most pronounced in the cerebral cortex: frontal cortex (L2A, L3A, L4A); parietal cortex (L3B, L4B); piriform cortex (L3C), perirhinal cortex (L4C) and entorhinal cortex (L5B). The occipital cortex (L5A) was not significantly affected. In the olfactory bulb (level 1) the sum of mitral cell layer, internal plexiform layer and internal granular layer (L1B) was significantly reduced. In the rhinencephalon (level 2), a significant *increase* is observed in the size of the 'lower brain lobe' (L2C, L2D) which is dominated by the anterior olfactory nuclei. In the diencephalon (level 4) also the width of the hippocampal CA1 region (L4F) and dentate gyrus (L4G) are reduced, as well as the total height of the basal regions (L4H) including, a.o. the thalamic nuclei. In the cerebellum (levels 6 and 7), no effects were found except for an inconsistent finding that was demonstrated for the molecular layer, being significantly reduced at level 6 but not at level 7.[2]

3.3. Total number of neurons, brain layer volume, numerical density of neurons

MAM clearly affected the total number of neurons *N* in the hippocampal CA1 pyramidal layer (Fig. 3A), but not in the cerebellar granular layer (Fig. 4A). The volume *V* of the corresponding layers (Figs. 3B and 4B) was reduced in accor-

[2] It should be noted that in the present study using systematic coronal sections, the different layers in the cerebellar cortex on both sides of the white matter were measured individually, and the mean of the two measurements of a layer per individual was used for statistical analysis (see Table 2).

Table 5
Stereology in the hippocampus CA1 region (pyramidal layer) and stereology in the cerebellum (granular layer)

Stereology measurements[a]	Control				MAM			
	N[b]	Mean	S.E.M.	CE%	N[b]	Mean	S.E.M.	CE%
Hippocampus (CA1)								
Total number N of pyramidal neurons[c] ($\times 10^3$ cells)	10	263.1	11	4.2	10	176.6*	6.5	3.7
Total volume V of pyramidal layer[d] (mm^3)	10	0.44	0.01	4.1	10	0.32*	0.01	5.3
Numerical density N_V of pyramidal neurons ($\times 10^3$ cells/mm^3)	10	578.8	15.9	2.7	10	562.2	28.8	5.1
Cerebellum								
Total number N of granular neurons[c] ($\times 10^3$ cells)	10	62461.8	6613.4	10.6	10	54686.8	3438.3	6.3
Total volume V of granular layer[d] (mm^3)	10	16.4	0.5	6.3	10	15.2	0.5	6.1
Numerical density N_V of granular neurons ($\times 10^3$ cells/mm^3)	10	4594.9	163.6	3.6	10	4398.0	130.4	3.0

Total number (N) of neurons, total volume (V) of the reference layer numerical density (N_V) of the neurons. Effects of prenatal exposure of male F1-animals (aged 22 days) to MAM (7.5 mg/kg body weight/day i.p., gestation days 13–15).

[a] For graphic presentation see Fig. 3 (hippocampus CA1 region) and Fig. 4 (cerebellum).

[b] $N = 10$ animals/group (one animal/sex/litter; 10 litters per group); S.E.M.: standard error of the mean; CE%: coefficient of error.

[c] Estimated with optical fractionator.

[d] Estimated with Cavalieri principle.

* Significantly different from controls; statistical key: ANOVA (Bonferoni); level of significance: $2p \leq 0.05$.

dance with the relative reduction in neuron numbers which is in line with the finding that the numerical density N_V of the neurons was not affected by MAM either in the hippocampus or cerebellum (Figs. 3C and 4C, respectively).

3.4. Qualitative microscopic examination

During blind microscopic examination of the selected thick (40 μm) sections, clear evidence for qualitative alterations was not observed at the brain levels studied. For example at the level of the diencephalon (level 4) with the cerebral cortex and the dorsal hippocampus, both regions where effects of MAM could be expected at the dosing regime used, differences from the controls were not observed in the MAM treated rats, not even when light micrographs were mutually compared (Fig. 5). Comparison of light micrographs of thin sections (5 μm) from the contra-lateral brain halves of these rats sometimes indicated a reduction of the thickness of the frontal cortex in some individuals but not in others; ectopic neurons were never observed [1].

4. Discussion

The aim of this study was to evaluate the power of different, mainly quantitative morphological approaches to reveal possible developmental brain damage due to methylazoxymethanol (MAM) using a rat model. A number of morphometric parameters ranging from brain size to total number of neurons was measured. MAM, an alkylating agent killing dividing cells, was administered during gestation days 13–15, aiming at inhibiting the proliferation phase of hippocampal neurons while cerebellar neurons would remain unaffected. The hippocampus is known to undergo development at that period of gestation whereas the cerebellum develops mainly after birth. It was hypothesized that the model could provide insight in the ability of each tier of the approach to detect early developmental neurotoxicity. 2D and 3D assessment of brain morphology combined with straightforward measurement of brain size, brain weight or brain volume, and the usefulness of estimation of total cell numbers were studied.

The macroscopic measurements of brain size showed that the size of the cerebrum, in particular its posterior part, was reduced in the MAM exposed rats, whereas the cerebellum was not affected. The brain weight measurements showed a similar tendency: a major effect on the posterior cerebrum and no effect on the cerebellum. Absolute brain weight and relative brain weight were both significantly reduced. The volume measurements corroborated the outcome of the weight measurements. This was confirmed by the finding that the specific gravity [weight/volume] of the brain tissues did not differ between control and treated groups. Thus, all three types of macroscopic measurements, i.e. brain size, weight, and volume, are fully in line with each other and are in agreement with our hypothesis that in the current design of the MAM model, the cerebellum will not be affected whereas the cerebrum will. The observation that the posterior part of the cerebrum was primarily affected could be indicative for a possible effect on the hippocampus as the hippocampus forms a major part of the posterior cerebrum.

The 2D linear morphometric measurements not only confirmed what was indicated by the first step in the tiered quantitative approach, i.e. the macroscopic measurements, but, in addition provided more detailed information on the developmental neurotoxicity of MAM. Brain layer width in the posterior part of the brain, in particular at the level of the dorsal hippocampus showed a significant reduction of the total width of the CA1 region and total width (inner and outer blade) of the dentate gyrus of about 20%. In the cerebellum, the molecular layer was significantly reduced at level 6 but not at level 7. The granular layer (including the Purkinje cell layer) and white matter were not significantly

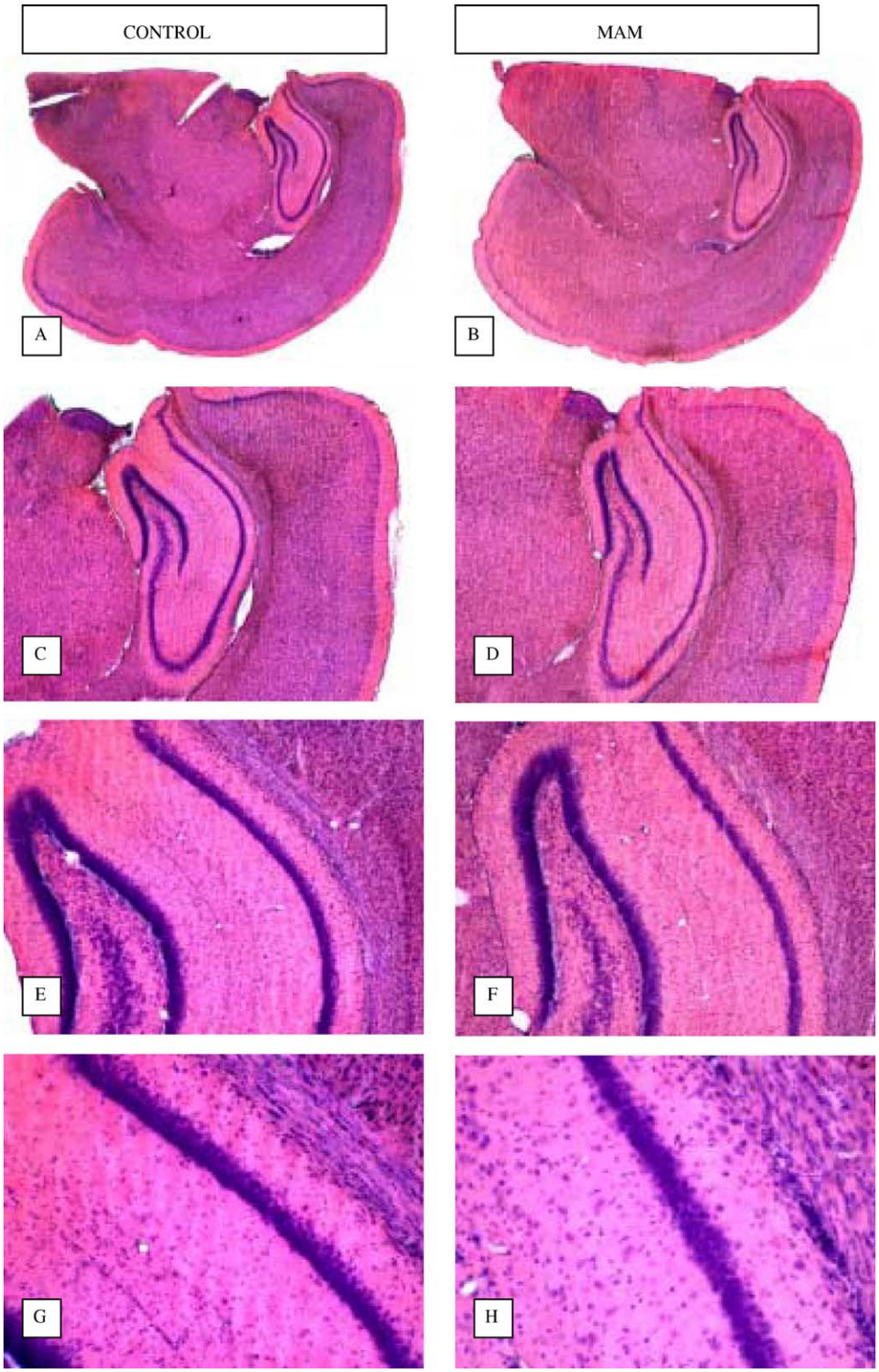

Fig. 5. Light micrographs of level 4, diencephalon (compare Fig. 2E of a 22-day-old control rat and one of the 7.5 mg/kg MAM group. No *qualitative* differences are observed between controls and MAM dosed animals at this level, either in the overview (Fig. 2A and B) or after zooming in on the dorsal hippocampus (Fig. 2C and D), the hippocampal CA1 region (Fig. 2E and F) and the CA1 pyramidal layer (Fig. 2G and H) in which the pyramidal neurons were counted (compare also Fig. 3D and E). The micrographs are taken with different objective lenses: magnification 2.5× (Fig. 2A and B), 5× (Fig. 2C and D), 10× (Fig. 2E and F) and 20× (Fig. 2G and H). Notice that layers of cells and cell concentrations are well outlined in these thick sections. (Left) Saline control animal. (Right) Prenatal exposure to MAM 7.5 mg/kg body weight/day (gestation days 13–15). Paraffin sections (40 μm), stained with heamatoxilin and eosin.

affected at either level. Considerable effects were observed in the neocortex. Especially the frontal cortex, situated in the anterior part of the brain was affected. In posterior direction, the effects of MAM on neocortical regions were less pronounced although still significant.

The ventral brain regions in the posterior part of the brain, mainly comprising thalamic nuclei, were also reduced in size by MAM. In the anterior part, however, the size of the ventral brain areas, mainly comprising olfactory nuclei, was *increased*. The latter finding is difficult to explain, but some kind of tissue deformation resulting from the substantial reduction in size of the overlying frontal cortex cannot be ruled out. It may as well be a fortuitous finding; however, from the macroscopic results of the in toto posterior and anterior cerebrum one could deduce that the observed increase in volume of the ventral brain regions was actually real and 'masked' the extensive reduction in volume of the frontal cortex by MAM in the outcome of the macroscopical measurements of the anterior cerebrum.

Using modern stereology and direct counting of neurons in the same systematic optical sections as used for the 2D measurements, a significant loss of CA1 pyramidal neurons of more than 30% was demonstrated. The volume of the hippocampal CA1 pyramidal layer was reduced accordingly, which was in agreement with the observation that the numerical density had not changed. The 2D changes in the total width of the hippocampal CA1 region were indicative for developmental neurotoxicity changes in the hippocampus and are likely due to changes in the number of neurons. Apparently MAM, as an anti-mitotic agent, has interfered with cell proliferation processes thereby killing dividing cells through DNA damage. This, in turn, has finally led to a lower number of neurons in the hippocampus and hypoplasia in other regions of the brain undergoing cell proliferation at the time of exposure to MAM: the forebrain, cerebral cortex and hippocampus. Other regions not under development at this time-point, e.g. the cerebellum, proved to remain unchanged.

The morphometric alterations in brain morphology induced by MAM could not be demonstrated during qualitative microscopic examination (slide reading). This result confirmed our earlier findings in thin paraffin sections [1].

For conventional qualitative light microscopy the resolution of the present thick paraffin sections is small compared to that of a thin section. Nevertheless, to detect characteristic neurodevelopmental pathology like dislocated (ectopic) neurons and hypoplasia, alterations that are observed after exposure to doses of MAM higher than the present one [11–15], photographs of thick sections as shown in Fig. 5 may provide useful probes.

Selection of homologous brain levels from the systematically random, dry sampled sections improves the discriminative power of the 2D measurements, especially in regions like the hippocampus and cerebellum. The fact that the paraffin sections are sampled dry, i.e. without intermediate floating on a water bath to stretch the section, prevents unknown variations in section size coming from this stretching to occur. This indirectly reduces variations in linear measurements and enhances the ability of linear morphometry to detect early developmental neurotoxicity.

For screening, the total width of a brain region proved to be a good indicator towards the next step in a tiered approach. As was shown in this study, the total width of regions in, e.g. the hippocampus and cerebral cortex provided relevant information during the linear morphometry tier. The inconsistent findings obtained for the different cerebellar layers probably resulted from the large variations in width of the distinct layers of the cerebellar cortex in the different lobules. When using systematic coronal sections exposing transects through cerebellar lobules, the total width of a lobule actually includes twice the width of the molecular layer, Purkinje cell layer, granular layer and the white matter. From the current results it was concluded that, for 2D linear screening of the cerebellum, measurements of the total width of a particular cerebellar lobule at a constant site, i.e. near the midline, probably are more efficient and effective than measuring all layers individually, as was done in the present study.

In summary, the results of the tiered approach showed that each tier contributed to the refinement of the search for the predilection areas of MAM. In accordance with the exposure time-frame in the MAM model, each level of the tiered approach indicated that major effects of MAM were to be found in the cerebrum, including a considerable loss of neurons as was demonstrated for the hippocampal CA1 pyramidal layer; the cerebellum and more specifically the number of granular neurons was not changed. It was demonstrated that, along with each step of the tiered quantitative approach, the location and extent of the brain lesions are drawn up closer. The use of the proposed tiered approach (brain size → linear morphometry → stereology) increases the probability to pinpoint the extent and location of developmental brain lesions. The discriminative strength of the endpoints used increased along with each step of the tiered approach, whereas their use as apical test decreases. The results also show that qualitative microscopic examination may be insufficient to demonstrate developmental neurotoxicity. As was demonstrated here by 2D and 3D morphometry, prenatal exposure to MAM (7.5 mg/kg body weight/day) during gestation days 13–15 causes substantial effects on brain morphology which go unrecognized during slide reading [1].

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