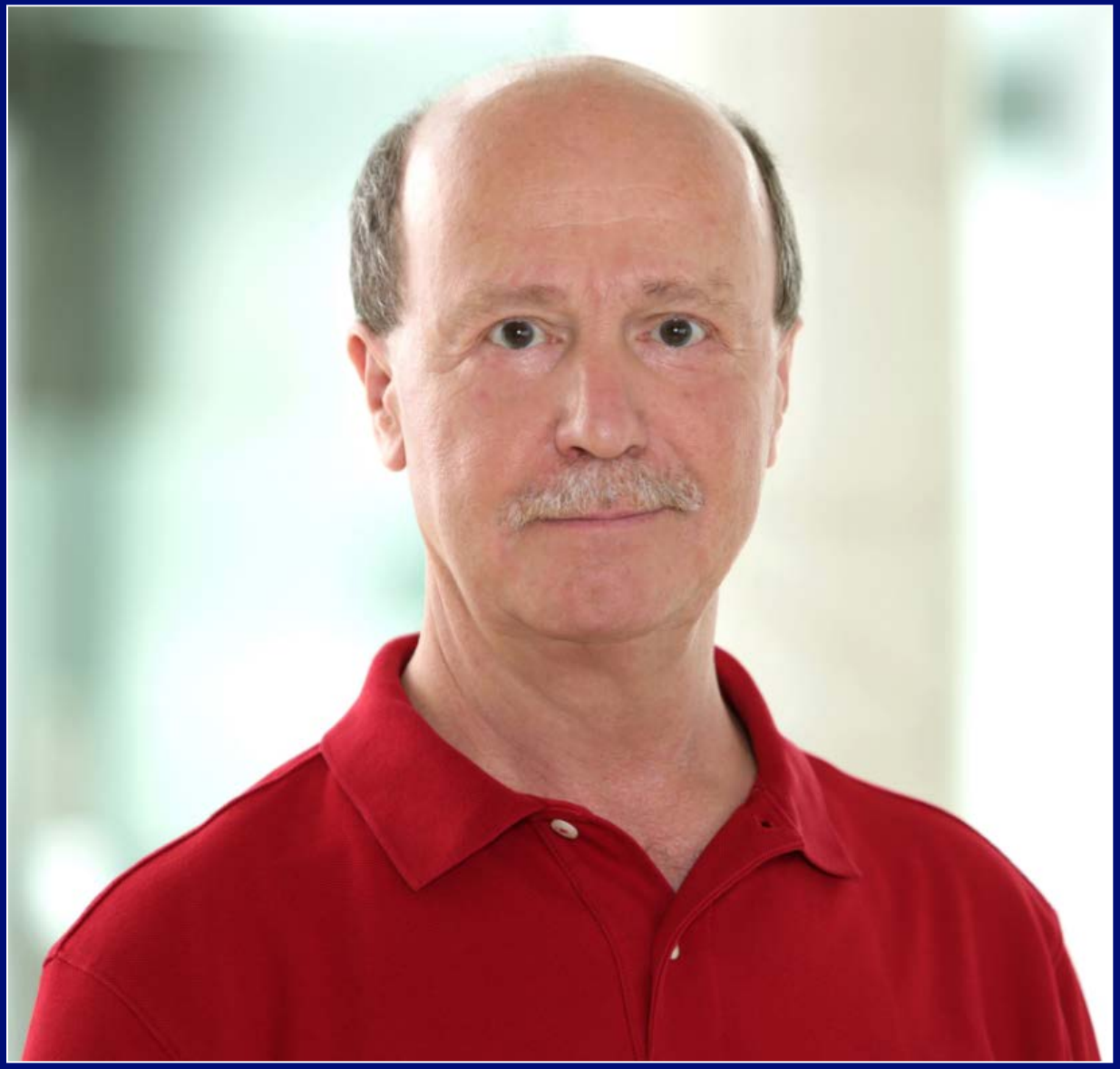
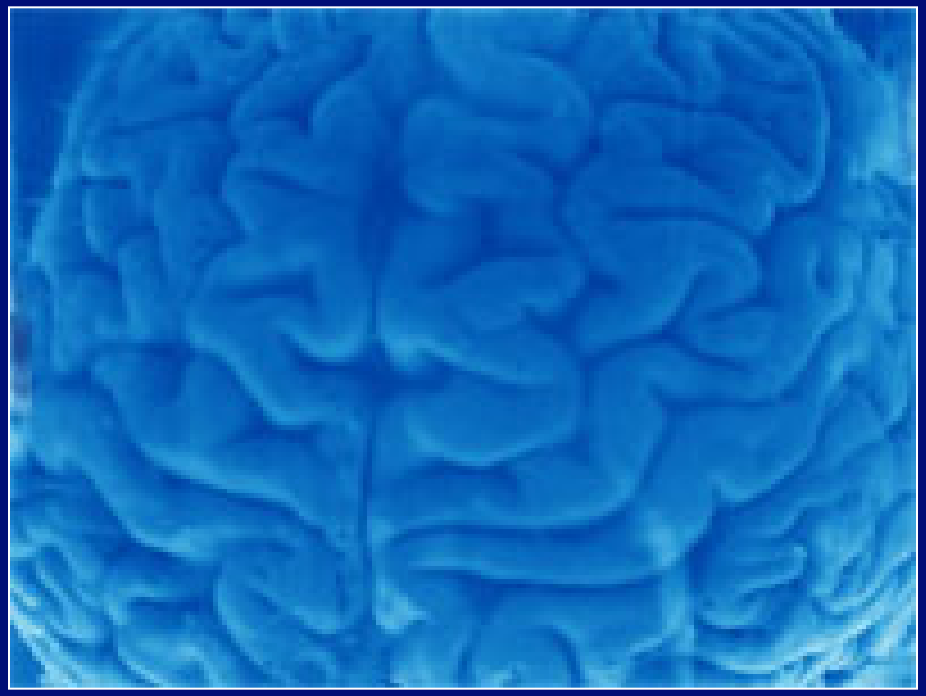
Developmental Neurotoxicity Testing – practical approaches and pitfalls in performing developmental neuropathology

Wolfgang Kaufmann, Merck KGaA, Darmstadt, Germany

6th STP-I Conference and Education Program, Pune, India, October 21 -23, 2016





„A behaviour – perceptual and motor acts - can be understood in terms of the properties of specific nerve cells and their interconnections in one region of the brain“

(Eric Kandel, 2000)

- **Background for the need of an appropiate animal model to test developmental neurotoxicity impacts**
- Developmental neuropathology in DNT-studies
- The quantitative brain analysis in DNT-studies

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

- The etiology of neurodevelopmental disorders is in parts or mostly unknown. A **multicausality** including genetic predispositions and environmental factors appear most likely.
- Though, **Morbus Parkinson**, **autism**, and **schizophrenia** are among other disorders (e.g. attention deficit hyperactivity disorder, mental retardation) which are shown to be induced by a perturbation of neurodevelopment
- Among the environmental factors, some **industrial chemicals and pharmaceuticals** are experimentally shown to perturb neurodevelopment

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Developmental neurotoxicants (1):

- Metals (lead, methyl mercury, manganese, cadmium)
- Drugs (nicotine, cocaine, marihuana, heroin)
- Antiepileptic drugs (phenobarbital, phenytoin, valproic acid, carbamazepine)
- Hormones (estrogenes, androgenes, hypothyreoidism, hypervitaminosis A)

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Developmental neurotoxicants (2):

- Virus infections (e.g. rubella, measles, influenza, zika)
- Some pesticides (DDT, organophosphates, pyrethroids)
- Chemical factors (e.g. solvents, dioxin, polychlorinated biphenyls)
- Physical factors (e.g. x-rays, heat)

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Developmental neurotoxicants (3):

Note:

- The most important factors for developmental neurotoxicity in children are two conditions:
  - **Undernutrition** and
  - Mothers consuming **drugs** (ethanol, smoking [O_2-deficiency], nicotine, and others)

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

The need for an **experimental study design** which addresses especially children as subpopulation in risk assessment of environmental chemicals is based on **fundamental different vulnerabilities of children** when compared with adults

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Different vulnerabilities (1):

- The metabolic capacities of children are still developing making them less able to metabolize, detoxify, and excrete exogenous substances
- Hence the *developing nervous system* of unborn, newborn and children may react on neurotoxic impacts differently to the adult brain.

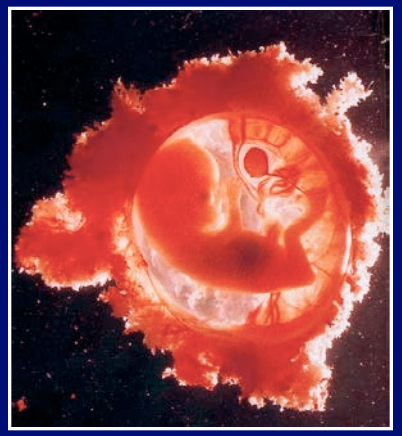
Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Different vulnerabilities (2):

Due to indirect exposure:

Possible protection by the placental barrier (gestational period)

Protection or potentiation by the blood - milk barrier (lactational period)



Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Different vulnerabilities (3):

Due to stage of maturation:

Developing brain

- immature blood-brain barrier (BBB)
- neurotoxic impacts at certain „time windows“ of development are critical for the kind of lesion

Adult brain

- mature blood-brain barrier (BBB)
- „time windows“ play no role for the kind of lesion

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Different vulnerabilities (4):

Due to stage of maturation:

Developing brain

- Receptor-mediated actions may have irreversible effects („irreversible imprinting“)

Adult brain

- Receptor-mediated actions have reversible character

Background for the need of an appropiate animal model to test developmental neurotoxicity impacts

Different vulnerabilities (4):

Due to stage of maturation:

Developing brain

- Receptor-mediated actions may have irreversible effects („irreversible imprinting“)

Adult brain

- Receptor-mediated actions have reversible character

- Background for the need of an appropiate animal model to test developmental neurotoxicity impacts
- **Developmental neuropathology in DNT-studies**
- The quantitative brain analysis in DNT-studies

Developmental neuropathology in DNT - studies

- U.S. EPA Health Effect Guidelines OPPTS 870.6300 Developmental Neurotoxicity Study (Final 1998)
- OECD 426 Developmental Neurotoxicity Study (Final 2009)

Both guidelines are the only guidelines that include functional, behavioral, and anatomical evaluations of the nervous system at multiple time points, in test subjects (as a rule rats) that were exposed to test substance during critical pre- and early postnatal periods of nervous system development

Developmental neuropathology in DNT - studies

- Storage in fixative of the brain at PD 11
- Morphological screening of the central nervous system (forebrain with frontal and parietal cortex, diencephalon with hippocampus, midbrain, pons, cerebellum, medulla oblongata, spinal cord) at PD 22 and 60 (+/-2)/ ~70.
- Simple morphometry from major brain areas at PD 22 and 60 (+/-2)/ ~70.
- Morphological screening of the peripheral nervous system (root ganglia, sciatic and tibial nerves, gastrocnemius muscle at PD 60 (+/-2)/ ~70.

Developmental neuropathology in DNT - studies

- The more *dynamic character* of the developing brain with a continuum of changing structures over time makes it necessary to develop a morphological view of what is a
  - *Normal neurodeveloping process* or a *variation* from that, or what is a
  - *Pathological neurodeveloping process* and how the neuromorphological correlate is then characterized

Developmental neuropathology in DNT - studies

Detection of impacts on:

- Process of growth
  - Proliferation, migration, and settling of cells (neurogenesis, gliogenesis)
- Process of maturation
  - Differentiation
  - Synaptogenesis
  - Selection (apoptosis)
  - Myelination

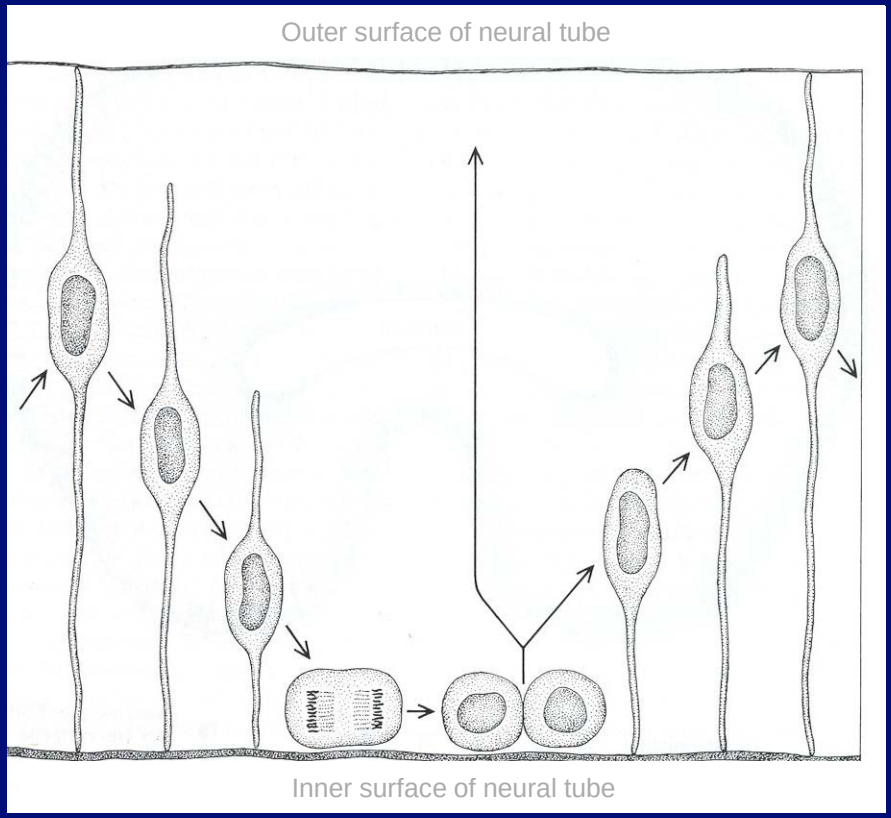
Developmental neuropathology in DNT - studies

Proliferation

In rats, neuro- and gliogenesis are observed until weaning (PD 21). Periventricular population of stem cells belong proliferative abilities a life-long time

Principle of neurogenesis:

Specific neuron populations are generated at specific sites of the neuroepithelium according to strict timetables during development.

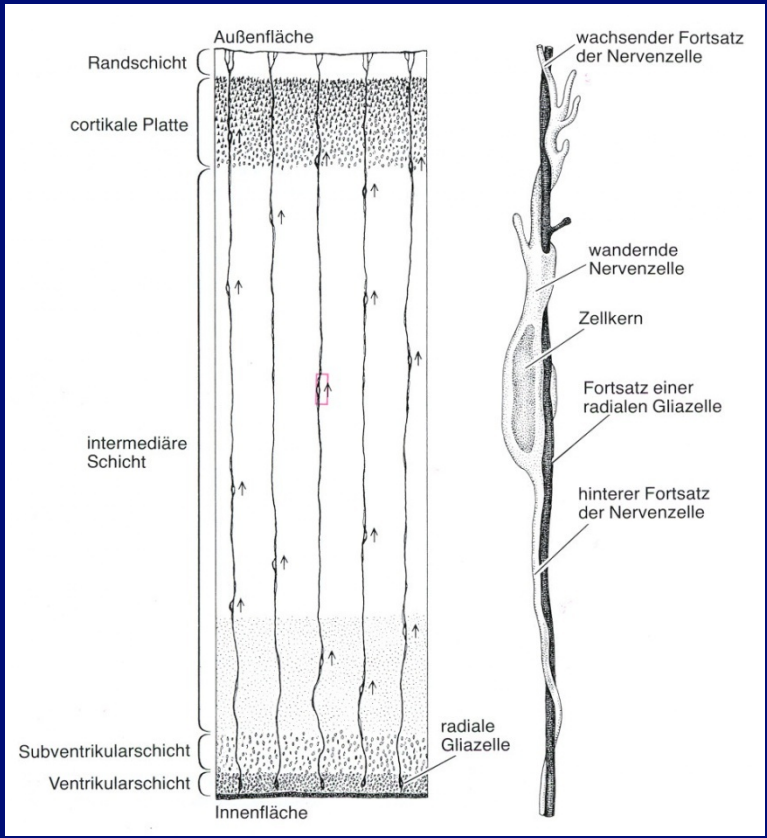


Developmental neuropathology in DNT - studies

Migration

Neuronal migration is characterized by a radial glia-guided translocation of nerve cells from their primary or secondary germinal matrix layers to their adult location

„Radial glia“ are considered to be a transitional stage of astrocytes.



Developmental neuropathology in DNT - studies

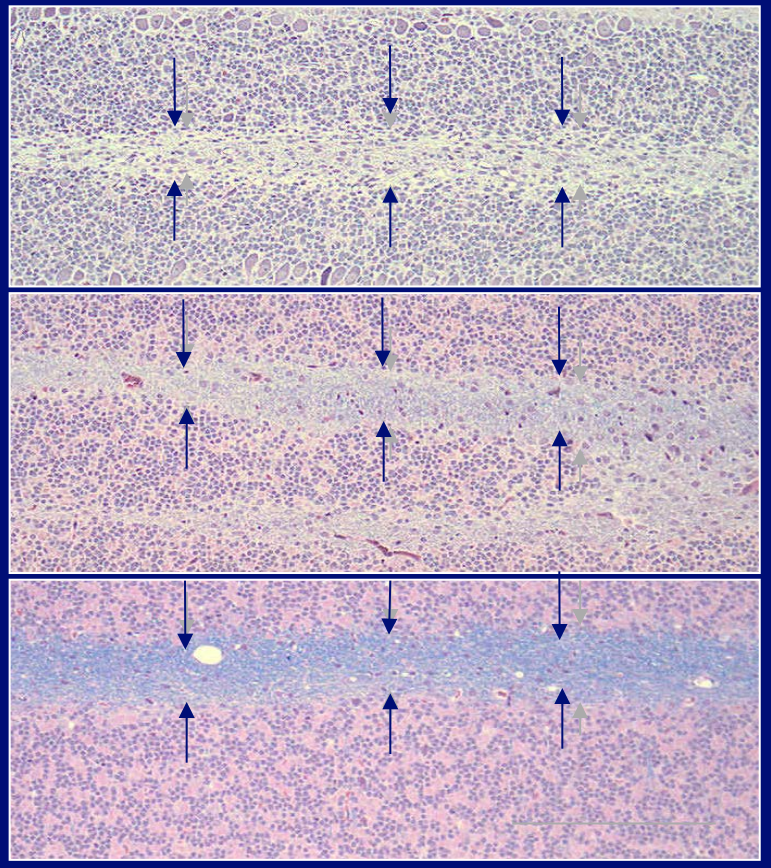
Myelination

Rat cerebellar cortex, the white matter is indicated with arrows

at PD 11

at PD 22

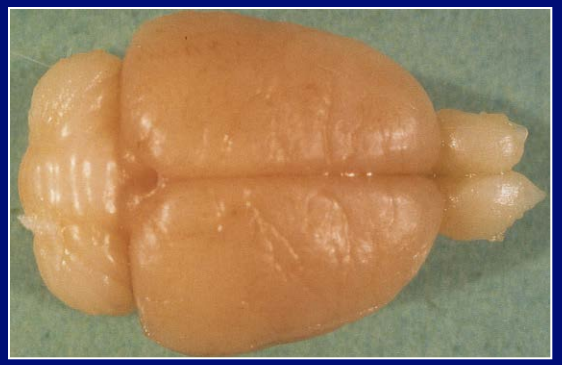
at PD 62



Klüver Barrera stain for myelin

Developmental neuropathology in DNT - studies

Brain „growth spurt“



at birth
(500 mg)

at PD 11
(1050 mg)

at PD 22
(1250 mg)

the rat brain

Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes are:

  (Macroscopically)

- Deviations of normal size and shape of cerebral hemispheres or normal pattern of foliation of the cerebellum
  - Hydrocephalus
  - Abnormal proliferations

Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes (examples):

  (Macroscopically)

Brain from a GD 20 rat fetus:

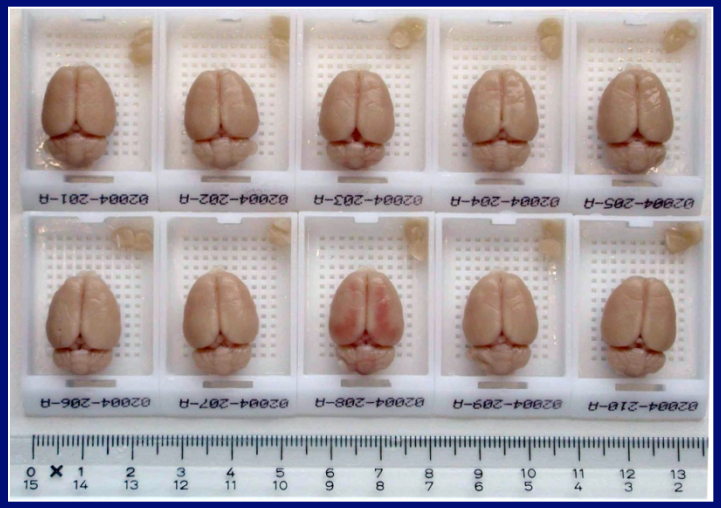
Abnormal proliferations: ”periventricular overgrowth”

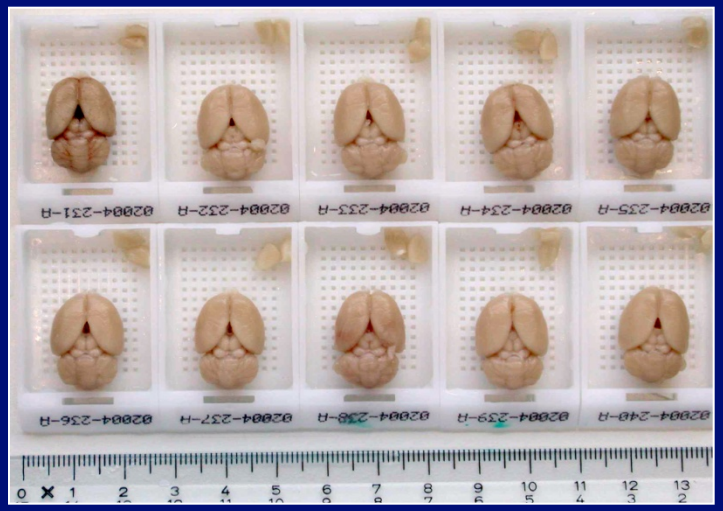
Bouin fixative

Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes (examples):

  (Macroscopically)





Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes are:

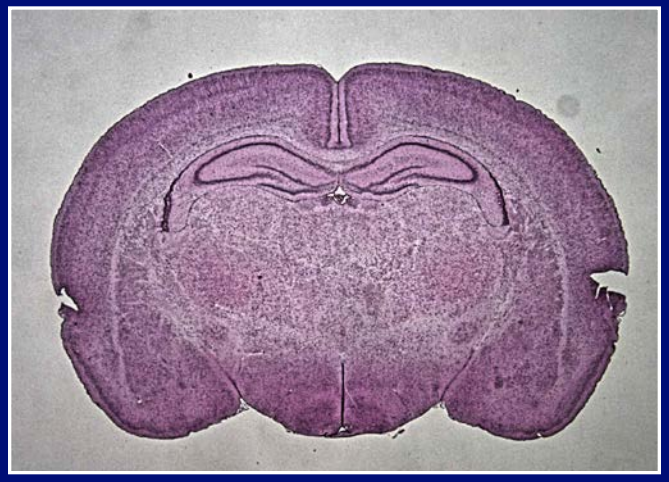
  (Neurohistologically)

  - Hypoplasia of certain brain areas
  - Neuronal ectopias/ heterotopias
  - Secondary dilation of brain ventricles
  - Hypomyelination

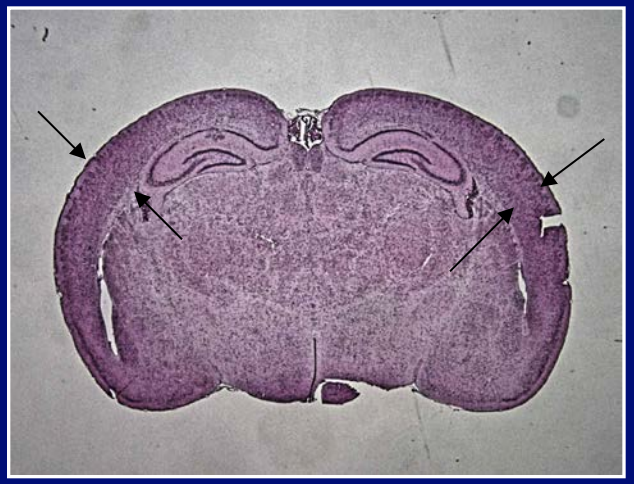
Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes are:

  (Neurohistologically)



Control rat brain, PD 22 offspring,
HE-stain

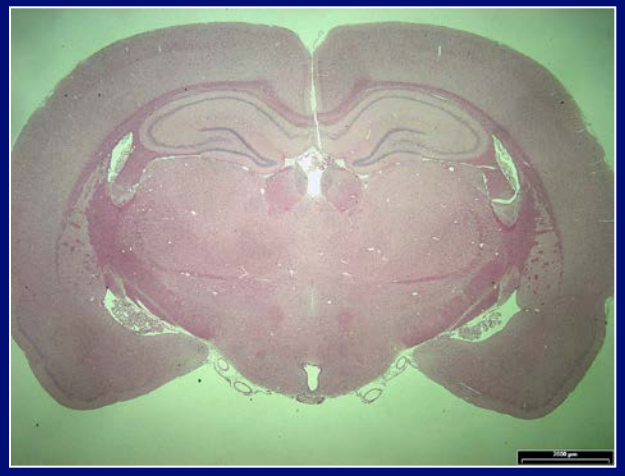


MAM-treated dams, PD 22 offspring, Note:
Hypoplasia of cortex cerebri (arrows),
HE-stain

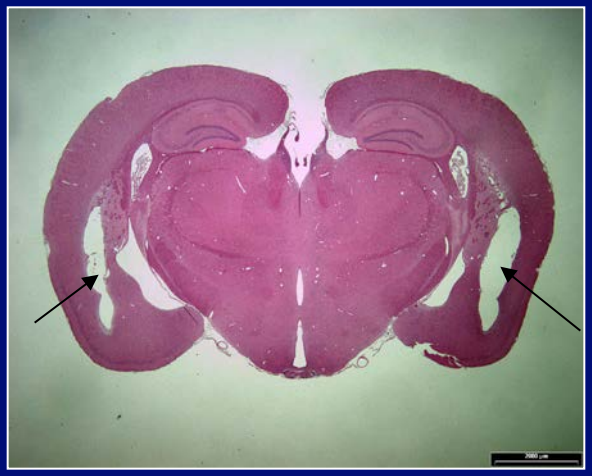
Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes are:

  (Neurohistologically)



Control rat brain, PD 60 offspring,
HE-stain

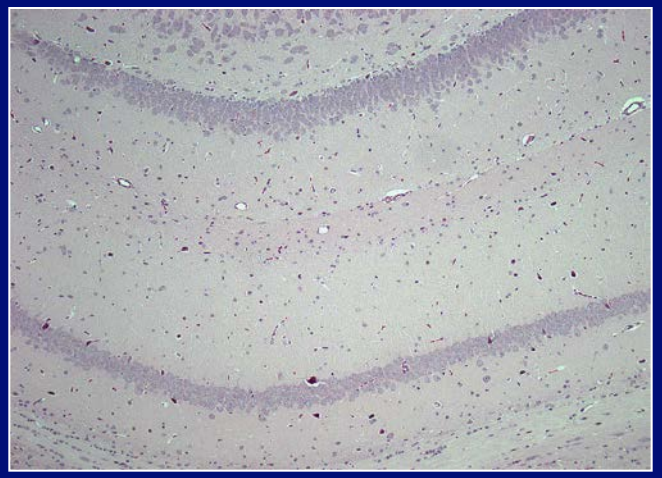


MAM-treated dams, PD 60 offspring, Note:
Ventricle dilation (arrows), HE-stain

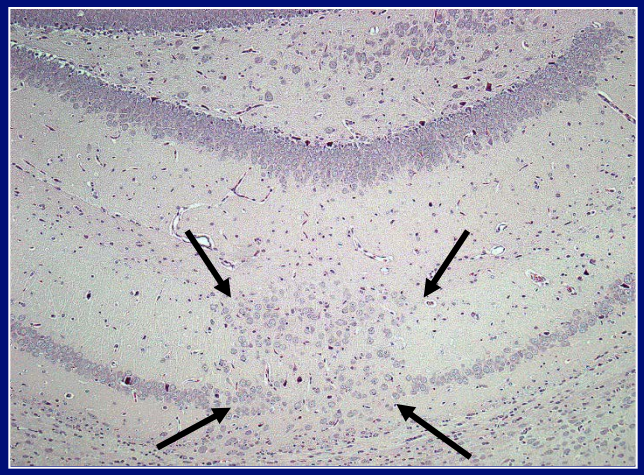
Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes are:

  (Neurohistologically)



Control. Hippocampal cross section, HE-stain

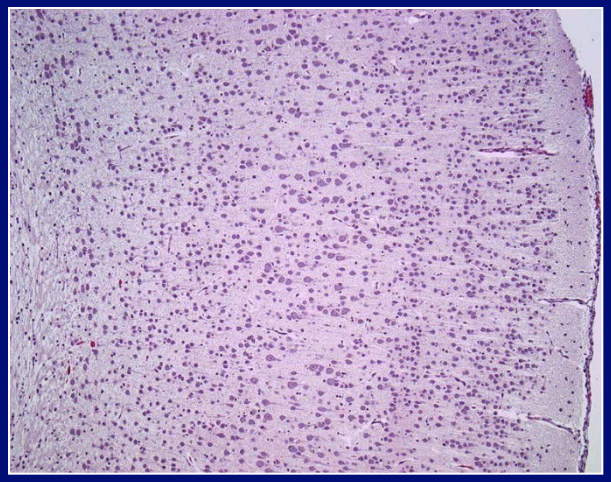


MAM-treated dams, PD 60 offspring, Note: Hippocampal neuronal heterotopia (CA1 layer) (arrows), HE-stain

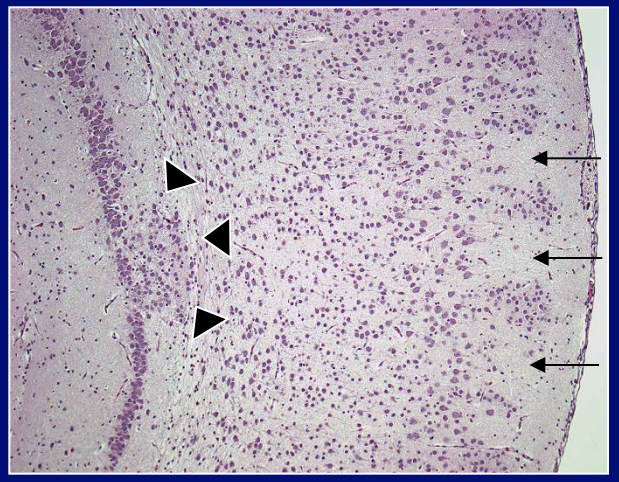
Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes are:

  (Neurohistologically)



Control. Cortex cerebri, HE-stain



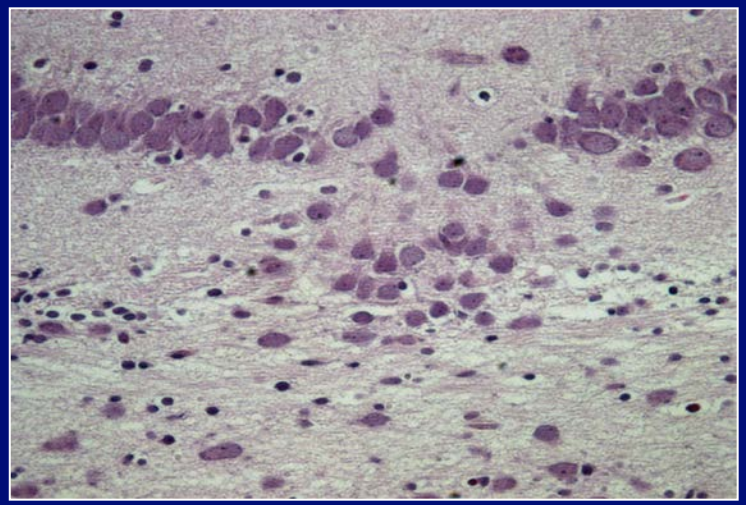
Cortex cerebri with distorted lamination (arrows), nodular heterotopia (arrowheads), HE-stain

Developmental neuropathology in DNT - studies

- Neuromorphological correlates for a perturbation of developing processes are:

  (Neurohistologically)

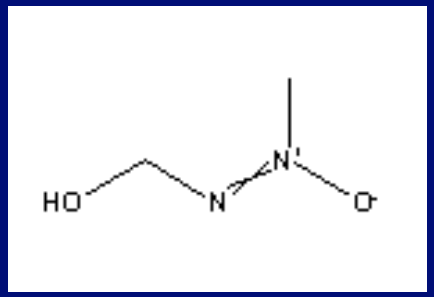
  - Clinical meaning of the „misplaced“ neuronal cell clusters:
    - Major heterotopiae may go along with altered transmitter and receptor properties
    - May be related to hyperexcitability and can display epileptiform activity
    - Cortical dysplasias may represent impaired synaptic plasticity that underlie learning disabilities



Positive control used: Methylazoxymethanol (MAM)

Cycas palm tree

Seeds of cycas spp. contain the glycoside cycasin (2- 4%). Cycasin is hydrolzyed by gut bacteria and hepatic metabolism to form methylazoxy methanol (MAM) :



Formula: $C_2H_6N_2O_2$

Cycasin has teratogenic, mutagenic, hepatotoxic, and carcinogenic properties.

Positive control used: Methylazoxymethanol (MAM)

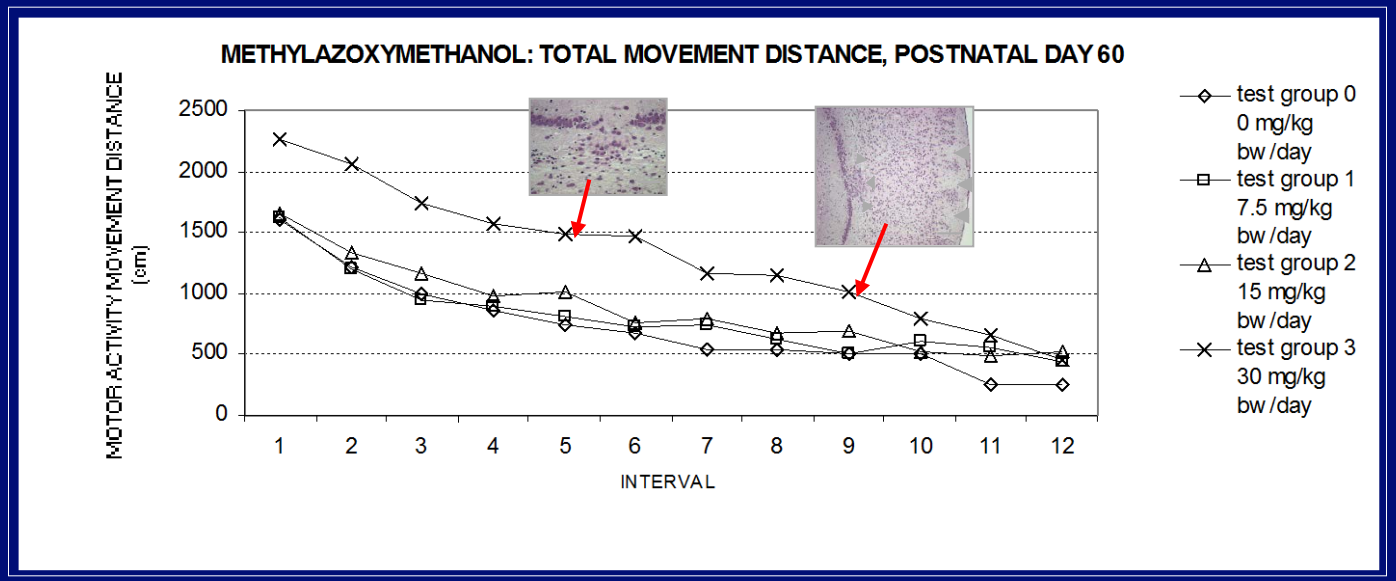
Food and medicinal use : cycad flour/leaves use is etiologically linked to a neurodegenerative disease found in an ethnically distinct native populations in the Western pacific region (e.g. Guam) - amyotrophic lateral sclerosis and parkinson-dementia complex (ALS/PDC).

Neurotoxicity: Cycasin and MAM pass the BBB (active carrier)

Toxic/lethal dose (dogs): 2 cycad seeds orally (gastroenterotoxic, hepatotoxic symptoms)

Antiproliferative action on dividing cells: MAM reacts with maternal and fetal nucleic acids by methylating guanine in the N^7 - position in both DNA and RNA

- MAM positive control:



Hyperactivity (motor activity testing) at PD 60 and the morphological correlate of neuronal heterotopiae after 1x 30 mg/kg MAM on GD 15

What are relevant considerations in the planning and conducting of developmental neuropathology in DNT-studies?

- Consider the *appropiate time for neuropathology*:

  PD 11? and/or PD 22? and approx. PD 60~70?

  Taking PD 11:

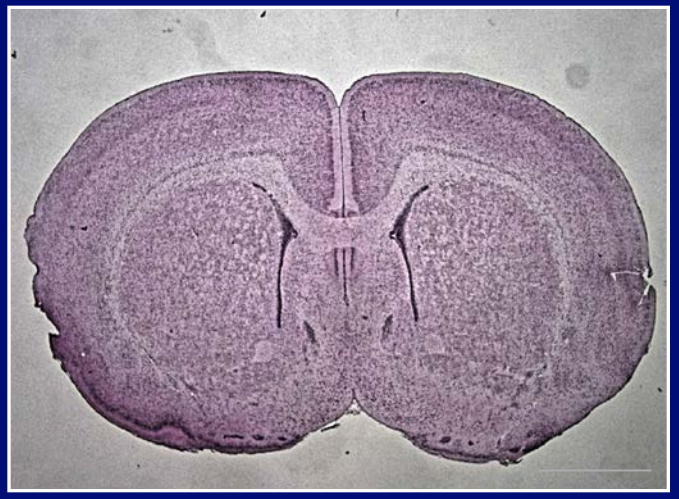
  - Transient structures (germ cell layers) can be examined
  - Some of the developing processes may be not sufficiently addressed (e.g. “rapid rate of myelination”)
  - More individual variation in size
  - More water - rich, friable nature of brain are disadvantages for handling/tissue processing and interpretation, much less relevant in PD 22 brains.

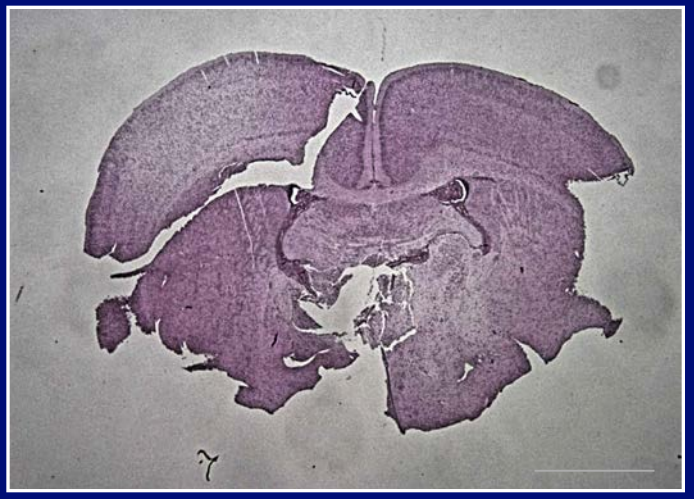
- Consider the *appropiate time for neuropathology*:

  PD 11? and/or PD 22? and approx. PD 60~70?

  Taking PD 11:

.



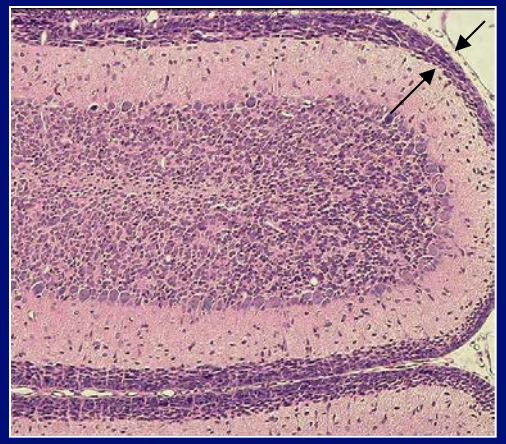


No analysis!

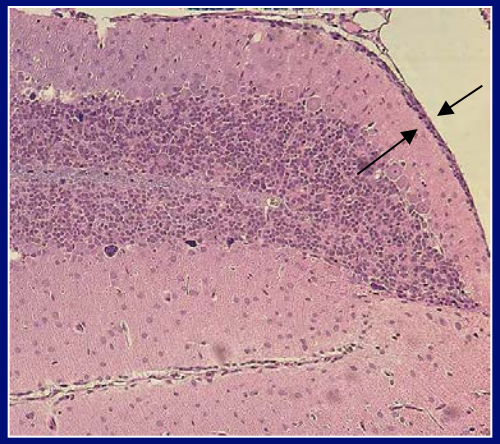
- Consider the *appropiate time for neuropathology*:

  PD 11? and/or PD 22? and approx. PD 60~70?

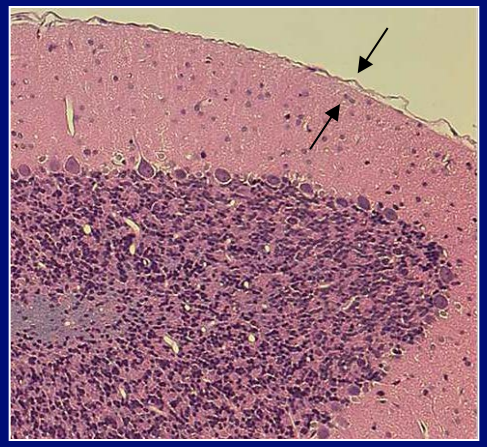
  Taking PD 11:



External germ cell matrix layer of the rat cerebellar cortex at PD 11 (arrows),
Kluever Barrera stain



External germ cell matrix layer almost completely disappeared at PD 22 (arrows),
Kluever Barrera stain



External germ cell matrix layer not longer present at PD 60 (arrows),
Kluever Barrera stain

- Consider the *appropiate time for neuropathology*:

  PD 11? and/or PD 22? and approx. PD 60~70?

  Taking PD 22:

  - Some changes in transient structures (e.g. external germinal matrix of the cerebellum) may be missed. Relevant?
  - Much better handling/tissue processing and interpretation
  - Less individual variation in size

  Taking PD 60~70:

  - Appropriate time for terminal sacrifice (- is the survival time long enough -) to show effects which become relevant only much later in life?

- Consider the *appropiate fixation procedure*:

Perfusion fixation or immersion fixation?

- Perfusion fixation must be employed for the PD 60~70 and PD 22 rats.
- Immersion fixation is sufficient for PD 11.
- Perfusion fixation employment for PD 22 and 11 brains remains controversial (disadvantage: artificial ventricle dilations).

- Consider how to achieve *homologous* brain sections*:

Advantage of “free-hand” sectioning:

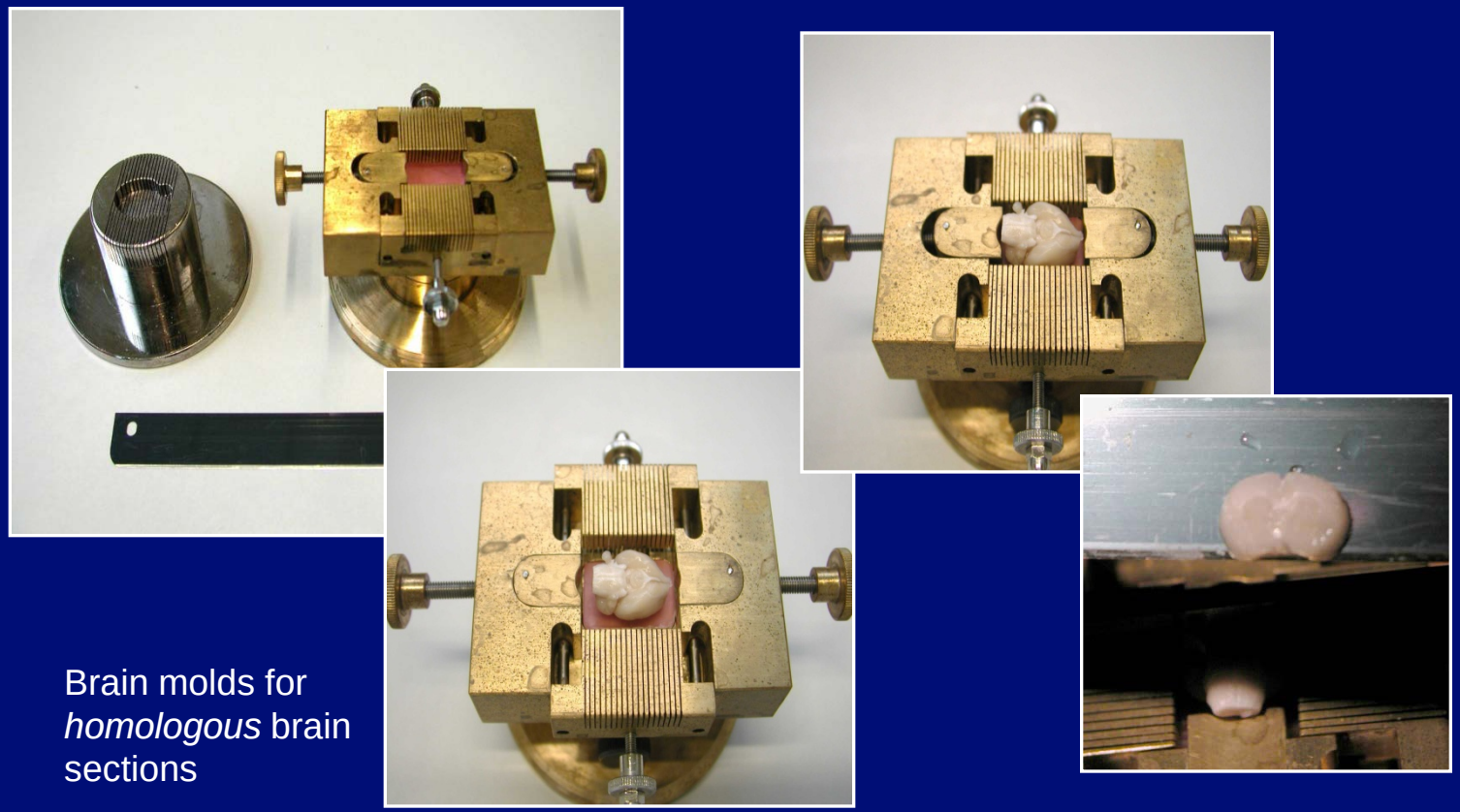
- Using neuroanatomical landmarks (optic chiasm, infundibulum, mammillary body) will produce more standardized sections, especially in juvenile brains, which change size rapidly
- Embedding in agar may help achieve parallel vertical cuts

Advantage of “brain matrix molds”:

- Correct vertical slices of even thickness

***homologous brain sections are mandatory for the morphometry component of the DNT - study**

- Consider how to achieve *homologous brain sections*:

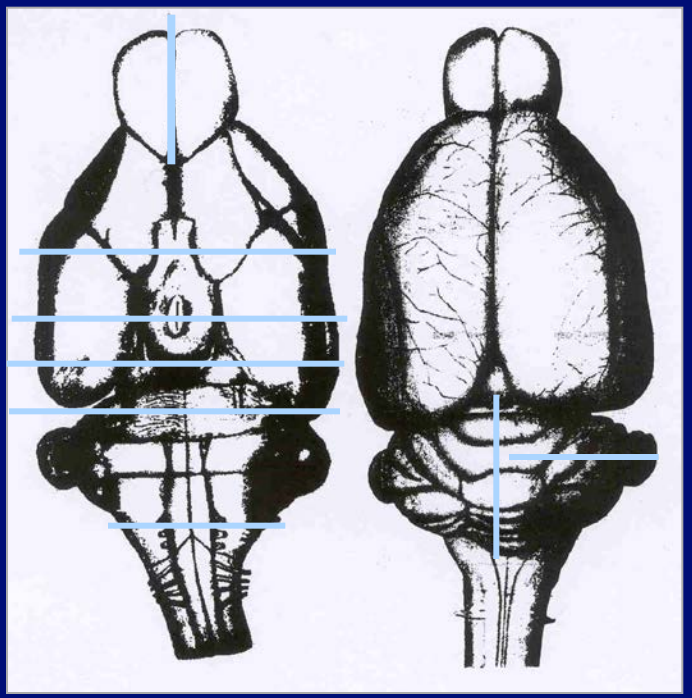


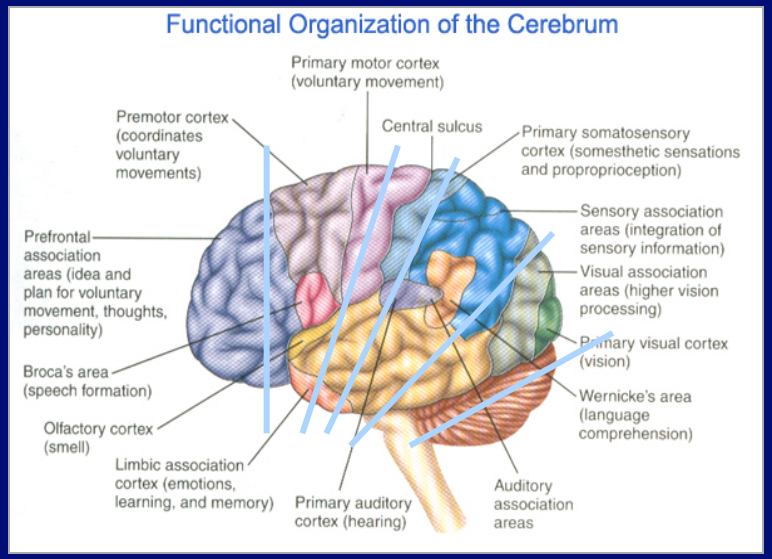
Brain molds for *homologous* brain sections

- Consider *representative sections from the* CNS *(brain)*:

ventral view dorsal view

Rat brain



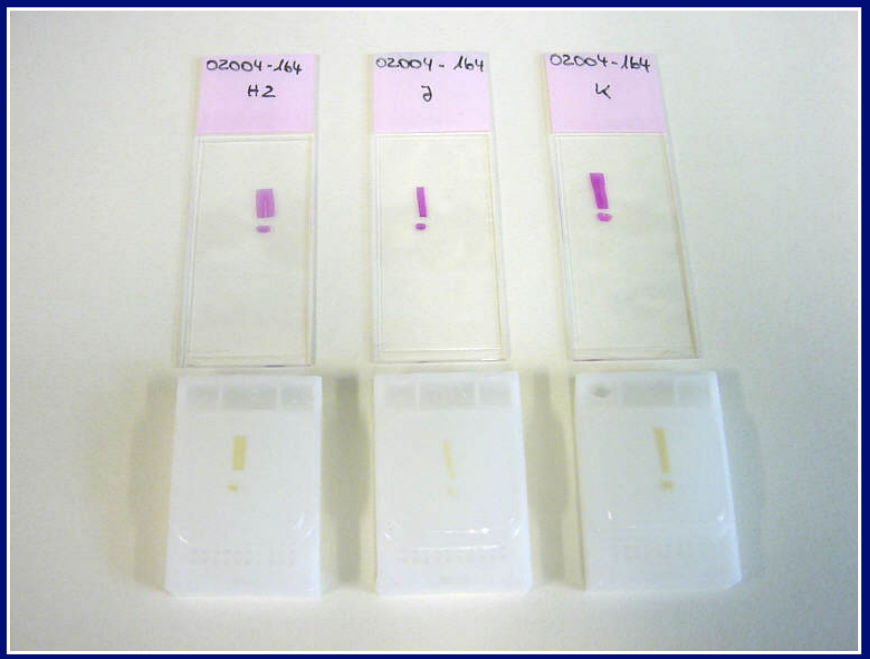


Brain levels to be taken

- Consider *representative sections from the* CNS *(brain)*:

Brain sections of one rat

- Consider *representative sections from the* CNS *(brain)*:



Representative sections from the spinal cord

- Consider *representative sections from the* PNS:

Sections from peripheral nerves and spinal ganglia

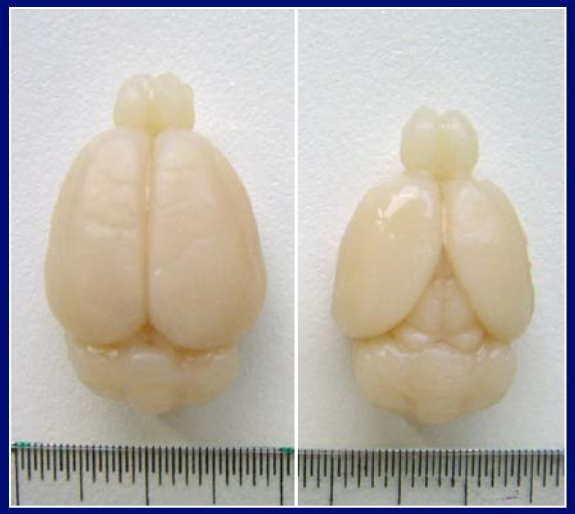
- Consider *personnel experience and training*:

  Knowledge of:

  - Neuroanatomical preparation skills
  - Histotechnical processing techniques
  - The normal neuroanatomy (level anterior or posterior to desired plane?)
  - Normal regressive processes vs. true degenerative processes
  - Functional - structural relations (major neurotransmitter circuits)

- Consider *gross lesions at necropsy* in DNT-studies:

  Careful necropsy adspection of all visible parts of the nervous system



Brain size estimation by adspection

- Consider *brain weight measurements* in DNT-studies:

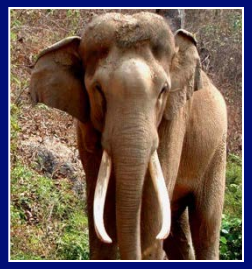
  Is brain weight a relevant parameter?

Absolute brain weight values		
Asian elephant	=	7,5 kg
Man	=	1,5 kg
Mouse	=	0,0004 kg

- Consider *brain weight measurements* in DNT-studies:

  Is brain weight a relevant parameter?

Relative brain weight values		
Mouse	=	3,2%
Man	=	2,1%
Asian elephant	=	0,15%



- Consider *brain weight measurements* in DNT-studies:

  Is brain weight a relevant parameter?

	Parameters (PD 22)		Test groups (Males)				Test groups (Females)		
			1	2	3		1	2	3
Compound 1	TBW		-1%	+5%	-9%		-4%	-5%	-12%
	Brain (abs)		**-6%***	**-11%****	**-26%****		+2%	**-8%****	**-20%****
	Brain (rel)		+1%	**-10%***	**-21%****		0	**-11%***	**-17%****
Compound 2	TBW		-6%	**-17%****	**-17%****		**-7%***	**-26%****	**-23%****
	Brain (abs)		-2%	**-8%****	**-8%****		**-4%****	**-15%****	**-13%****
	Brain (rel)		+4%	**+8%****	**+7%***		+3%	**+12%***	**+8%****

- Consider *brain weight measurements* in DNT-studies:

  Conclusions

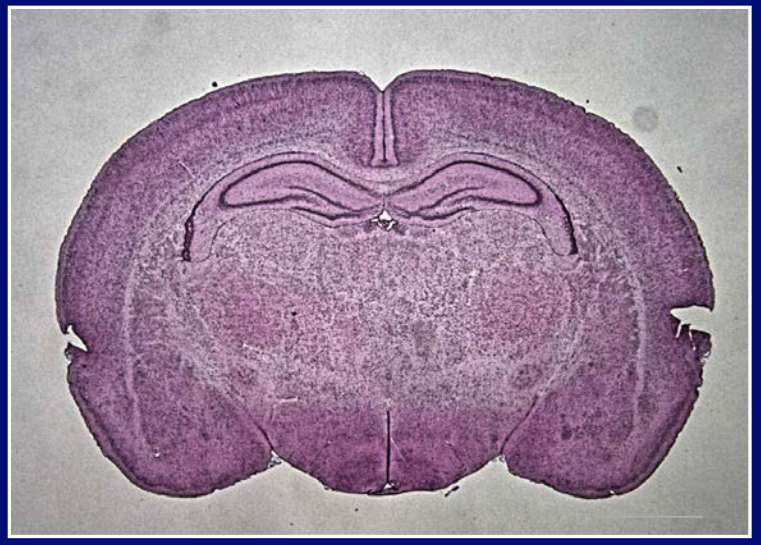
  - Significant decreases of relative brain weights (when related to body weight development) are a sensitive marker for a direct impact on brain development (e.g. after MAM treatment)
  - Significantly reduced body weight gain due to undernutrition may cause significant brain weight changes

- Consider *stage of development when performing light microscopy* in DNT-studies:

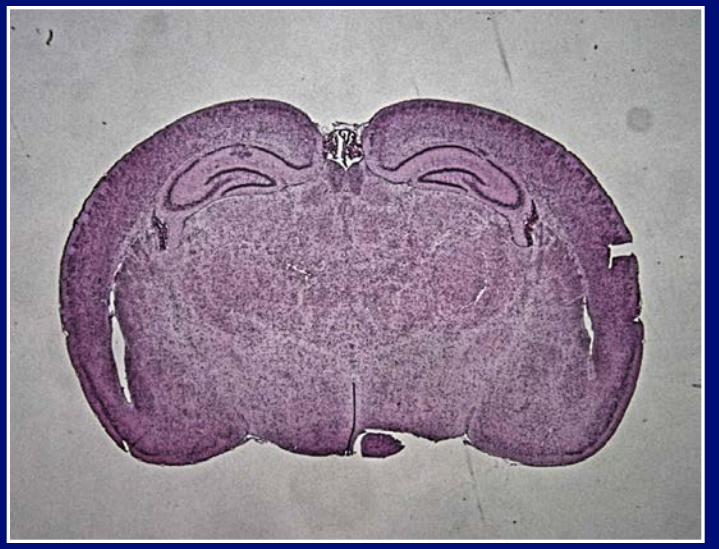
  - Brain from juvenile and young adults (central nervous system) - two developmental stages are examined (PD11/ 22 and PD 60/70)
  - Data from young adults (peripheral nervous system) - one developmental stage is examined (PD 60/70)

- Consider *stage of development when performing light microscopy* in DNT-studies:

Example 1:



Control rat brain, PD 60 offspring, HE-stain



MAM-treated dams, comparable findings in PD 11, 22, and 60 offspring, HE-stain

- Consider *stage of development when performing light microscopy* in DNT-studies:

Conclusions

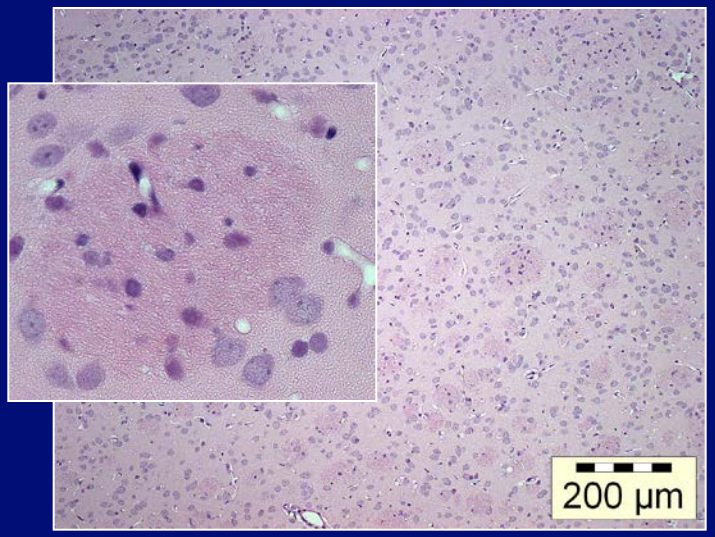
Example 1: Create a check list - Evidence for neurodevelopmental impact?

-	Brain weight decrease	+	
-	Specific lesions detected	+	stand-alone criteria
-	Persistent (PD 11, 22, and 60)	+	
-	Transient (PD 22 only)	-	
-	Neuroclinical correlates	+	

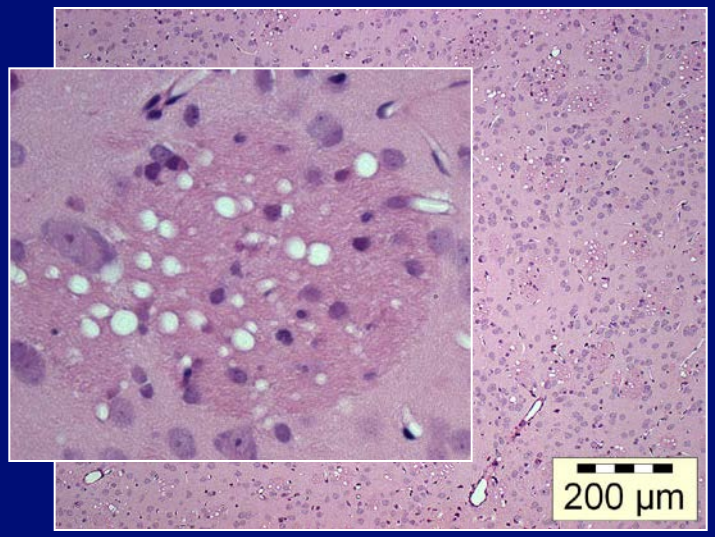
Yes - Specific indicators present, and the consideration of all parameters gives clear evidence of a neurodevelopmental insult

- Consider *stage of development when performing light microscopy* in DNT-studies:

Example 2:



Control rat brain, PD 22 offspring: corpus striatum, HE-stain



Treated dams, findings in PD 22 pups after direct pup dosing only, HE-stain

- Consider *stage of development when performing light microscopy* in DNT-studies:

Conclusions

Example 2: Create a check list - Evidence for neurodevelopmental impact?

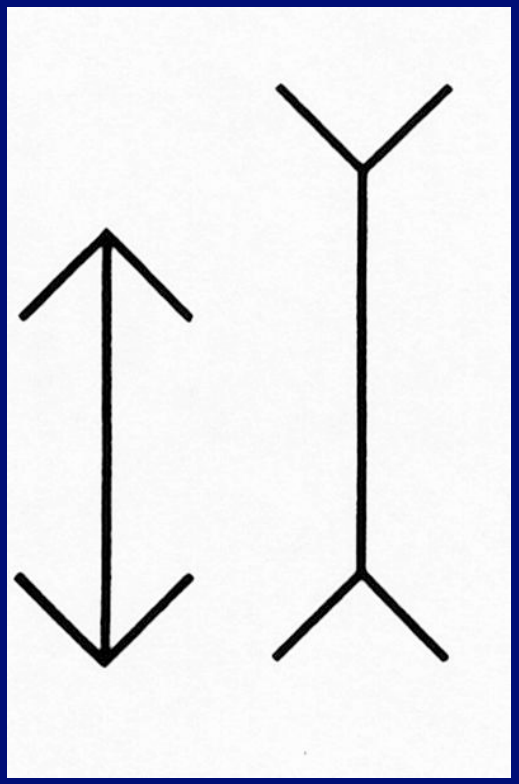
-	Brain weight decrease	-
-	Specific lesions detected	-
-	Persistent (PD 11, 22, and 60)	-
-	Transient (PD 22 only)	+
-	Neuroclinical correlates	-

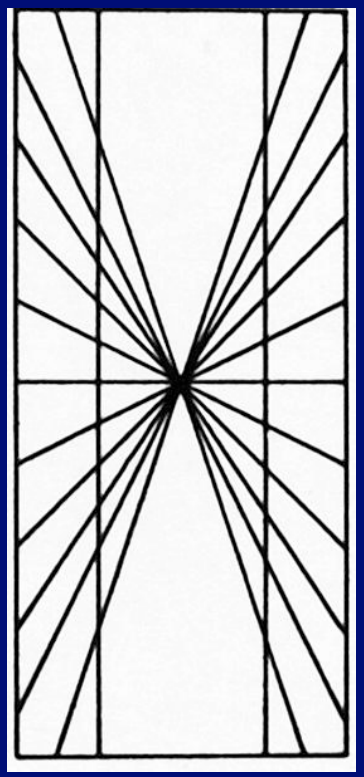
No - Transient character only after direct pup dosing on Day 22, and no indications for further endpoints involved

- Background for the need of an appropiate animal model to test developmental neurotoxicity impacts
- Developmental neuropathology in DNT-studies
- **The quantitative brain analysis in DNT-studies**

- Consider *the morphometry approach* in DNT-studies:

How sure are we about a quantitative change?

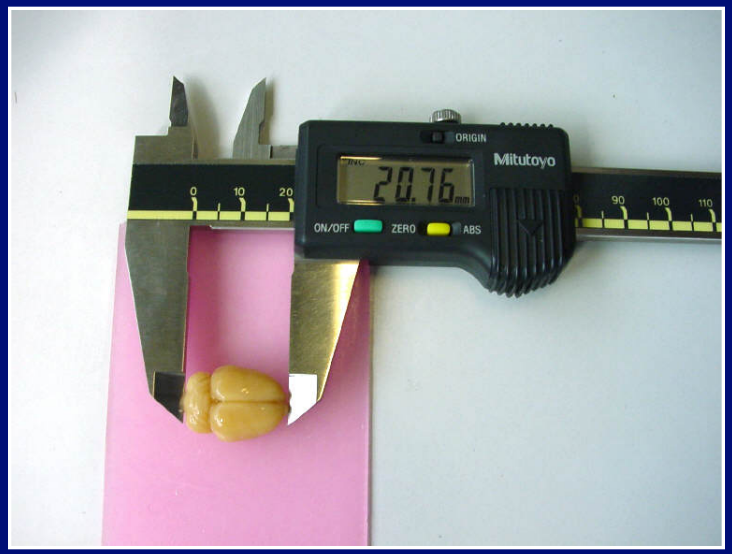




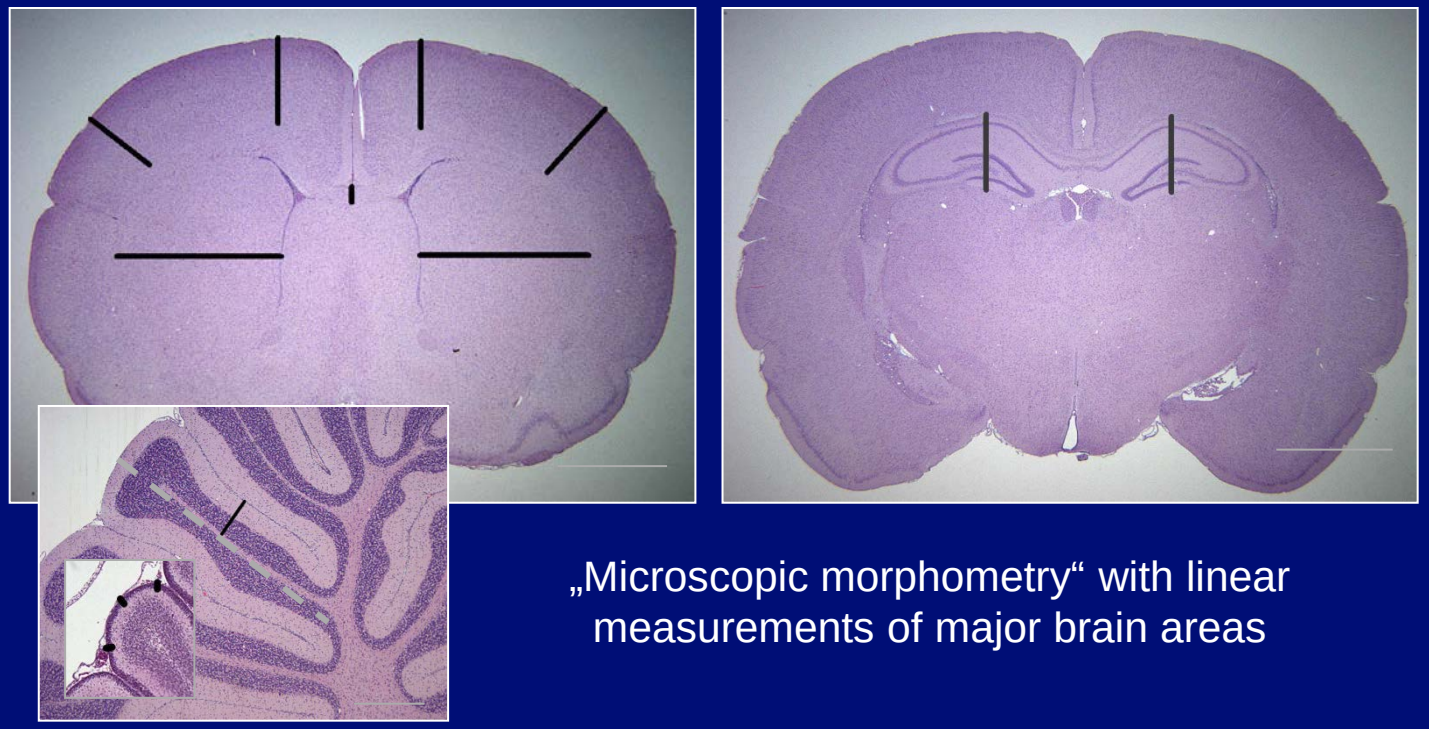
- Consider *the morphometry approach* in DNT-studies:

  - Some toxicants may interfere with DNA synthesis or by inhibiting or delaying neuronal migration during neurodevelopment.
  - A variety of neuroanatomic regions may be only reduced in size while other biomarkers of effect are missing (no GFAP - response, no astrogliosis).
  - Changes in size may be the primary and only microscopic lesion present.
    - EPA guideline calls for a “simple” morphometric analysis
    - As primary morphometric method, although its inherent weakness, linear measurements are used in a first - tier approach as they are simple to conduct and most cost - effective.

- Consider *the morphometry approach* in DNT-studies:
  - Lengths and width measurements of cerebrum and cerebellum during necropsy (e.g. by using Vernier calipers)



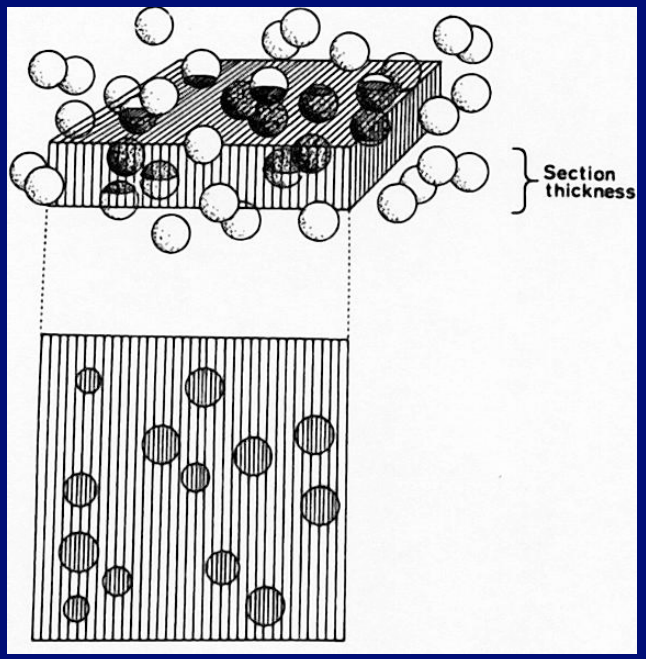
- Consider *the morphometry approach* in DNT-studies:
  - Linear measurements



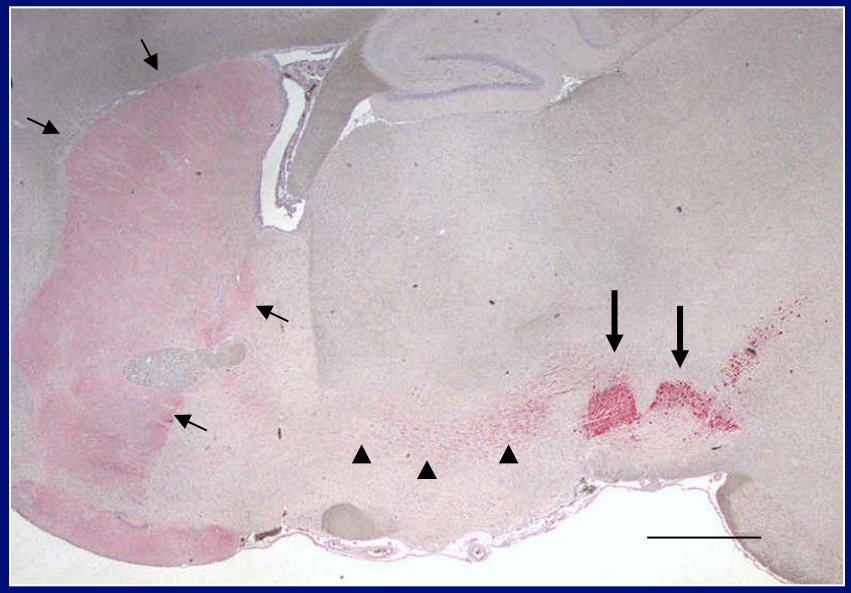
„Microscopic morphometry“ with linear measurements of major brain areas

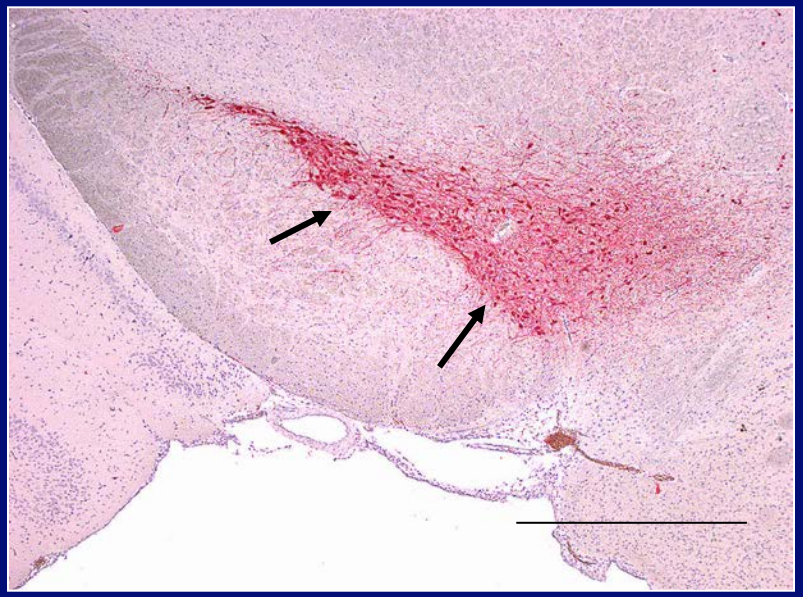
- Consider *the morphometry approach* in DNT-studies:
  - Linear measurements – pitfalls and recommendations
  - Lack of sensitivity because of inherent variability of tissue sections (oblique sections, section artefacts) - cut as accurately as possible in a right angle to the longitudinal axis!
  - Variations in the level of the anatomic location - look at well - defined neuroanatomical landmarks during dissection - highly homologous section are needed
  - Measure at least thickness of major brain layers (neocortex, hippocampus, cerebellum)

- Consider *extended morphometry approach* in DNT-studies:
  - Stereology morphometry
    - consider as a second – tier approach on otherwise identified potential target areas in the brain
    - optical dissector stereology may be more sensitive for detecting certain types of cell loss (e.g. TMT) as it is an unbiased stereologic determination of neuron numbers
    - considerable technical effort is needed



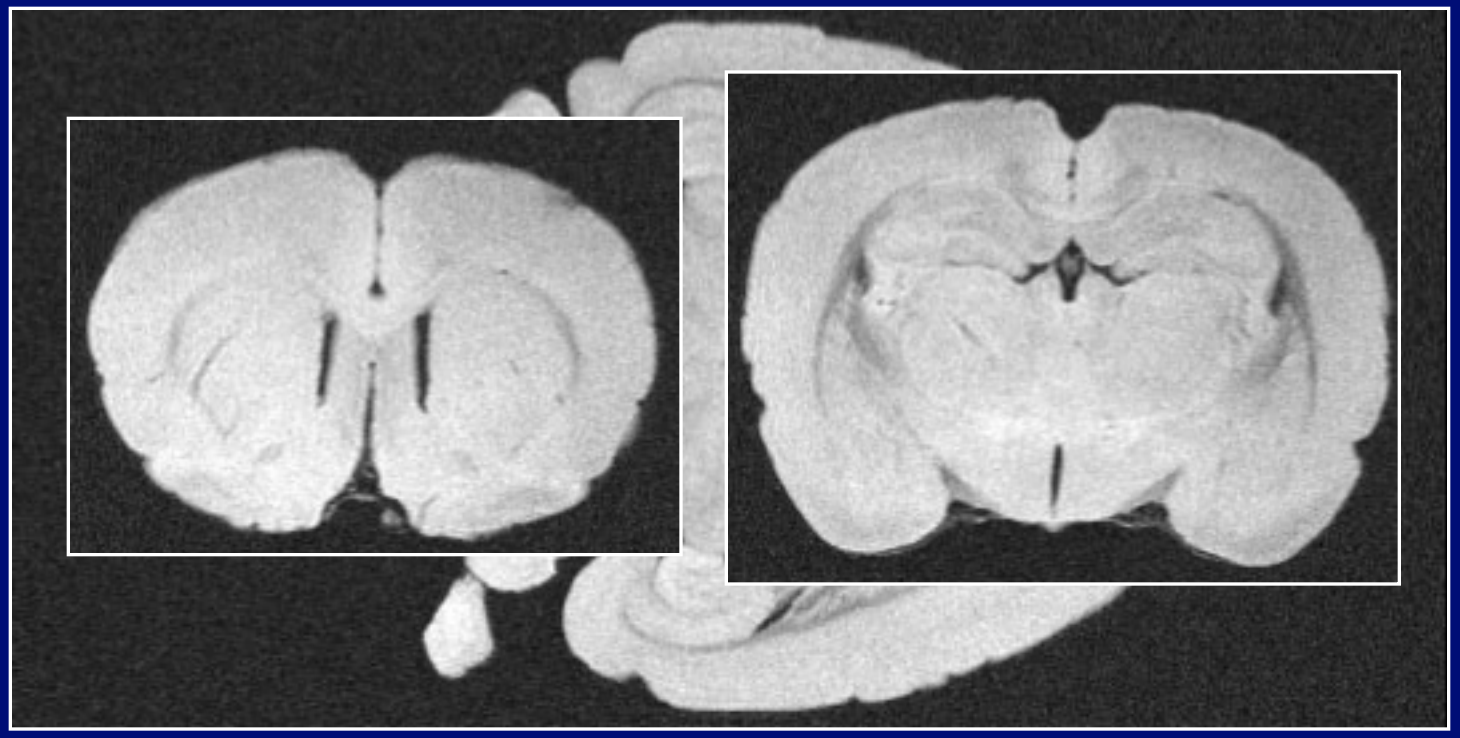
- Consider *extended morphometry approach* in DNT-studies:
  - Stereology morphometry





Longitudinal and cross (substantia niagra pars compacta) section: TH+ immunohistochemistry does specifically mark dopaminergic neurons and their fiber networking

- Consider *new morphometry approach* in DNT-studies:
  - Magnetic resonance imaging (MRI)



Summary:

- Neuropathology in DNT - studies contributes to detect, to characterize and to visualize impacts on the structure of the developing nervous system.
- The relevance of brain morphometry data has to be evaluated under the auspices of further neuromorphological or neurochemical or neurobehavioural deviations from normal development.
- A tiered approach is appropiate.
- The current DNT - study exactly follows this approach, although critical issues remain unresolved.
- Critical issues are the appropiate time of sacrifice, fixation procedure and the kind of morphometry.
- New techniques (e.g. MRI) may find there way to routine analysis in future

Selected references:

1.Spencer PS, Kisby GE, Palmer VS, and Obendorf P (2000): Cycasin, methylazoxymethanol and related compounds. In: Experimental and Clinical Neurotoxicology (eds. PS Spencer and HH Schaumburg). Oxford University Press, New York Oxford, pp. 436-447

2.Kaufmann W (2000): Developmental Neurotoxicity. In: The Laboratory Rat (ed. G. Krinke). Academic Press, London, pp. 227-250

3.Bolon, B, Garman, RH, Jensen, KF, Krinke, G, and Stuart, B (2006): A 'Best Practices' Approach to Neuropathologic Assessment in Developmental Neurotoxicity Testing—for Today, *Toxicol Pathol, 34,* 296-313

4.Kaufmann W, Gröters S (2006): Developmental neuropathology in DNT-studies – a sensitive tool for the detection and characterization of developmental neurotoxicants, *Reproduct Toxicol, 22/2,* 196-213

5.Kaufmann W (2011): The developing central nervous system. In: Fundamental Neuropathology for Pathologists and Toxicologists: Principles and Techniques (eds. B Bolon and M Butt). John Wiley & Sons, pp. 339-363

6.Garman RH, Li AA, Kaufmann W, Auer RN, and Bolon B (2016): Recommended Methods for Brain Processing and Quantitative Analysis in Rodent Developmental Neurotoxicity Studies. *Toxicol Pathol, 44,* 14-42