【S-6】

# Mechanisms of 5-azacytidine-induced damage and repair process in the fetal brain

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The fetal central nervous system (CNS) is sensitive to diverse environmental factors, such as alcohol, heavy metals, irradiation, mycotoxins, neurotransmitters, and DNA damage, because a large number of processes occur during an extended period of development. Fetal neural damage is an important issue affecting the completion of normal CNS development. As many concepts about the brain development have been recently revealed, it is necessary to compare the mechanism of developmental abnormalities induced by extrinsic factors with the normal brain development.

To clarify the mechanism of fetal CNS damage, we used one experimental model in which 5-azacytidine (5AzC), a DNA damaging and demethylating agent, was injected to the dams of rodents to damage the fetal brain. 5AzC induced cell death (apoptosis) and cell cycle arrest in the fetal brain, and it lead to microencephaly in the neonatal brain. We investigated the mechanism of apoptosis and cell cycle arrest in the neural progenitor cells in detail, and demonstrated that various cell cycle regulators were changed in response to DNA damage. p53, the guardian of genome, played a main role in these processes. Further, using DNA microarray analysis, the signal cascades of cell cycle regulation were clearly shown. Our results indicate that neural progenitor cells have the potential to repair the DNA damages via cell cyclearrest and to exclude highly affected cells through the apoptotic process. If the stimulus and subsequent DNA damage are high, brain development proceeds abnormally and results in malformation in the neonatal brain.

Although the mechanisms of fetal brain injury and features of brain malformation after birth have been well studied, the process between those stages is largely unknown. We hypothesized that the fetal CNS has the ability to repair itself post-injuring, and investigated the repair process after 5AzC-induced damage. Wefound that the damages were repaired by 60 h after the treatment and developmental processes continued. During the repair process, amoeboid microglial cells infiltrated in the brain tissue, some of which ingested apoptotic cells. The expressions of genes categorized to glial cells,

inflammation, extracellular matrix, glycolysis, and neurogenesis were upregulated in the DNA microarray analysis. We show here that the developing brain has a capacity to repair the damage induced by the extrinsic stresses, including changing the expression of numerous genes and the induction of microglia to aid the repair process.

# Mechanisms of 5-Azacytidine-induced damage and Repair Process in the Fetal Brain

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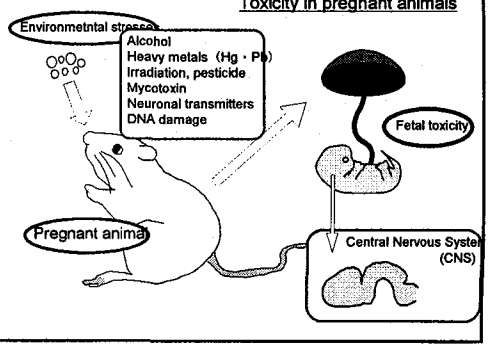
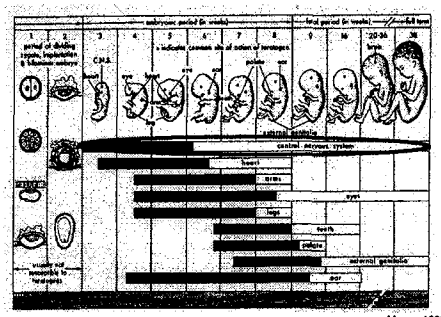
## Toxicity in pregnant animals

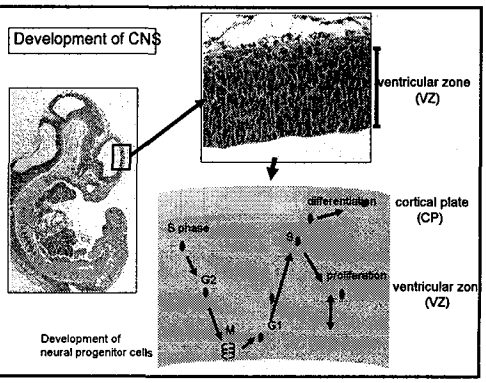



## Sensitive period of human fetal organs

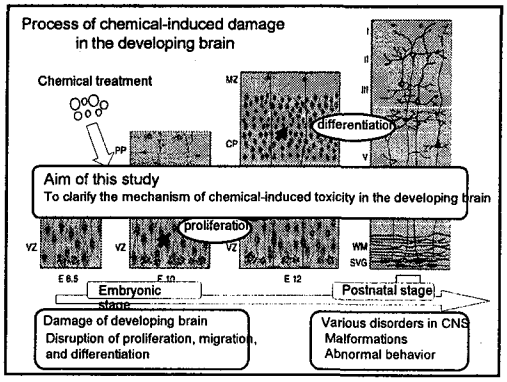



Moore, 1998

## Development of CNS

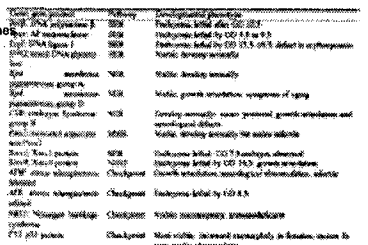



## Process of chemical-induced damage in the developing brain




## DNA damage affects brain development

Mutant mice of DNA repair genes

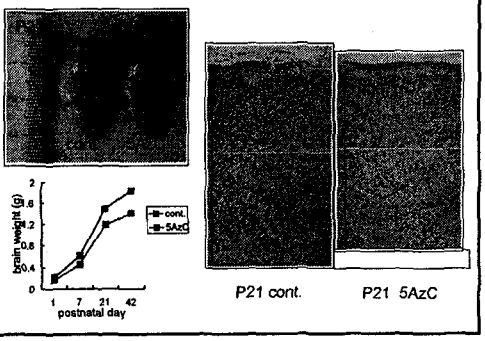


Vinson & Hales (2002)

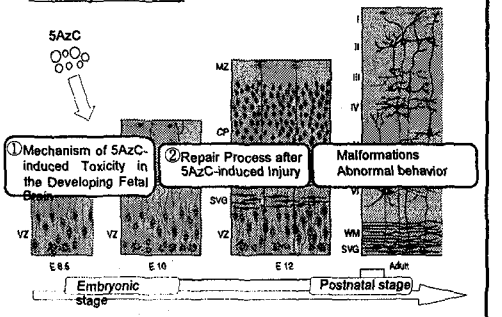
DNA damaging chemicals and stimuli

Irradiation, Ethylnitrosourea (ENU), Hydroxyurea (HU), Etoposide, Cyclophosphamide (CP), Arabinofuranosylcytosine (AraC), 5-Azacytidine (5AzC)

Microencephaly is induced by 5AzC-treatment

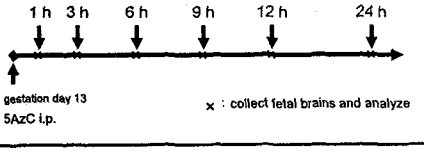



Summary of this study

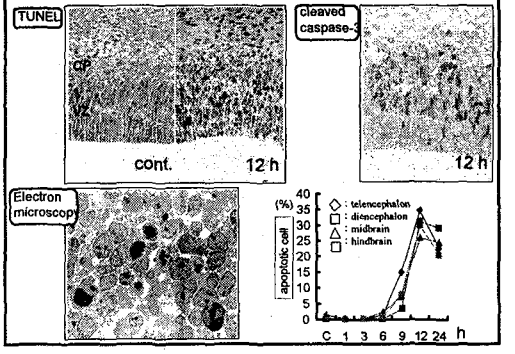



Materials and Methods

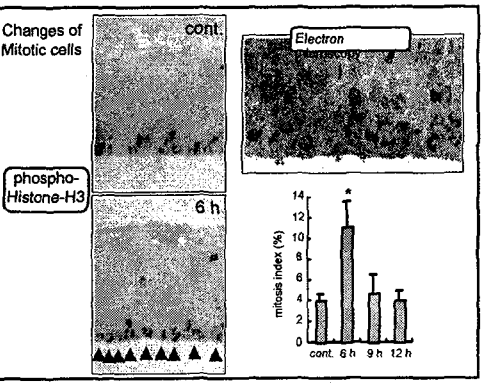
*chemical : 5-Azacytidine (5AzC; 10 mg/kg i.p.)*
animal : Wistar rat (gestation day 13)
analyses : histopathology (HE, electron microscopy, immunohistochemistry)
*BrdU labelling (FACS, immunohistochemistry)*
Cell cycle analysis (FACS)
DNA microarray (Gene Chip (Rat Expression Array 230A, AFFYMETRIX))
*RT-PCR、 Real-time PCR 、Western blotting*



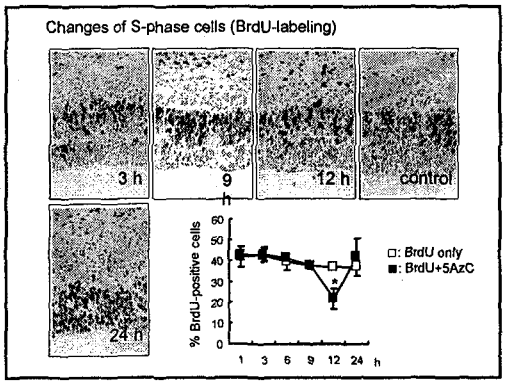

Induction of apoptosis by 5AzC-administration

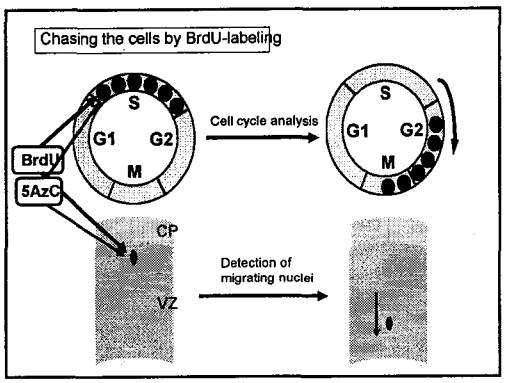


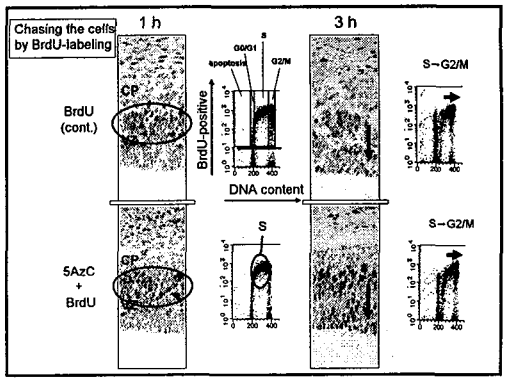

Changes of Mitotic cells

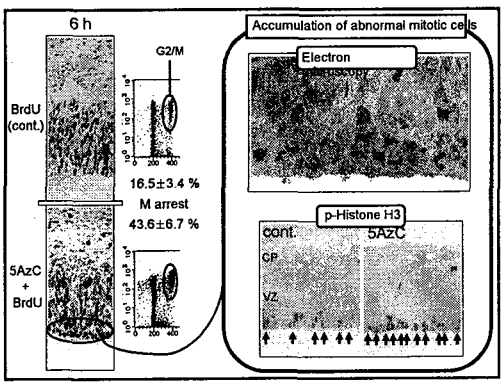


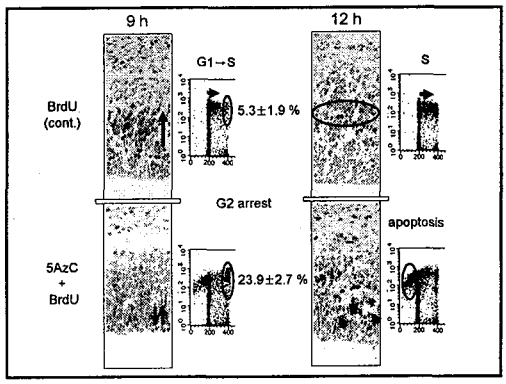

Changes of S-phase cells (BrdU-labeling)

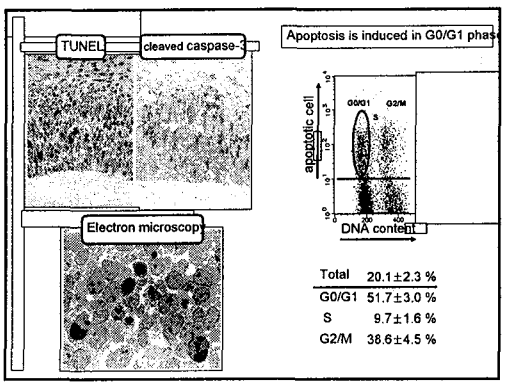


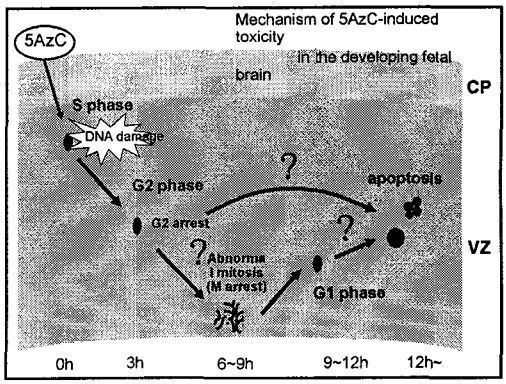

Chasing the cells by BrdU-labeling
S
G1
G2
M
BrdU
5AzC
Cell cycle analysis
CP
VZ
Detection of migrating nuclei

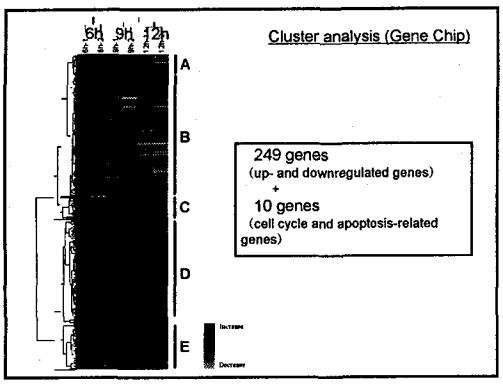

Chasing the cells by BrdU-labeling
1 h
3 h
BrdU (cont.)
5AzC + BrdU
CP
BrdU-positive
apoptosis
G0/G1
S
G2/M
DNA content
S→G2/M


6 h
BrdU (cont.)
5AzC + BrdU
G2/M
16.5±3.4 %
M arrest
43.6±6.7 %
Accumulation of abnormal mitotic cells
Electron microscopy
p-Histone H3
cont.
5AzC
CP
VZ

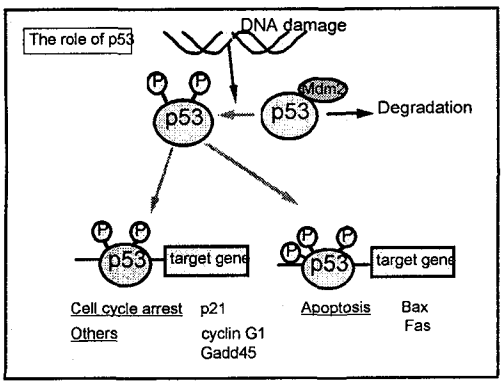

9 h
12 h
BrdU (cont.)
5AzC + BrdU
G1→S
5.3±1.9 %
S
G2 arrest
23.9±2.7 %
apoptosis

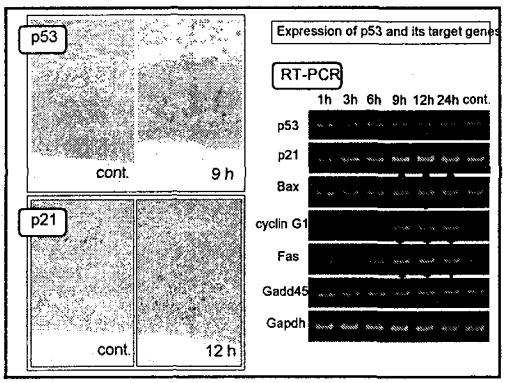

TUNEL
cleaved caspase-3
Electron microscopy
Apoptosis is induced in G0/G1 phase
apoptotic cell
G0/G1
S
G2/M
DNA content
Total 20.1±2.3 %
G0/G1 51.7±3.0 %
S 9.7±1.6 %
G2/M 38.6±4.5 %

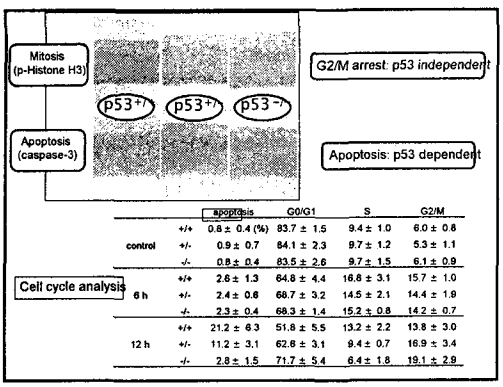

Mechanism of 5AzC-induced toxicity in the developing fetal brain
5AzC
S phase
DNA damage
G2 phase
G2 arrest
Abnormal mitosis (M arrest)
apoptosis
G1 phase
CP
VZ
0h
3h
6~9h
9~12h
12h~




| ession No. | Genes | Fold change 6h | 9h | 12h | Clust |
|---|---|---|---|---|---|
| **ll cycle** | | | | | |
| _022183.1 | *topoisomerase (DNA) II alpha (Top2a)* | | | 1.3 | E |
| 5341.1 | Cdc20 / p55CDC | | | 1.5 | D |
| 9589.1 | cyclin B (Ccnb1) | | | 1.4 | D |
| _012760.1 | lost on transformation 1 (Lot1) / pleiomorphic adenoma gene-like 1 (Plagl1) / Zac1 | -1.2 | | | D |
| _054008.1 | rgc32 protein (Rgc32) | | | -1.9 | A |
| _022683.1 | vasopressin-activated calcium-mobilizing receptor protein (VACM-1) / cullin-5 (Cul5) | | | -1.6 | A |
| _021662.1 | DNA polymerase delta, catalytic subunit (Pold1) | | | -1.5 | A |
| _017258.1 | B-cell translocation gene 1, anti-proliferative (Btg1) | | | -1.3 | A |
| **3-target genes** | | | | | |
| _012923.1 | cyclin G1 (Ccng1) | 1.9 | 3.0 | 3.2 | D |
| 4174.1 | p21 (Waf1 / Cip1) | | | 4.1 | E |
| 548539 | ESTs, Moderately similar to S15349 mdm2 protein - mouse (M.musculus) | | | 1.8 | D |
| _031621.1 | serum-inducible kinase (Snk) / Plk2 | | | 1.4 | D |
| _012568.1 | *insulin-like growth factor-binding protein (Igfbp3)* | | | 2.3 | 1.9 | D |
| **rowth factor** | | | | | |
| _022266.1 | connective tissue growth factor (Ctgf) | | | 1.6 | D |
| _019198.1 | fibroblast growth factor 17 (Fgf17) | | | 1.5 | D |
| _030859.1 | midkine (Mdk) | | | 1.3 | D |

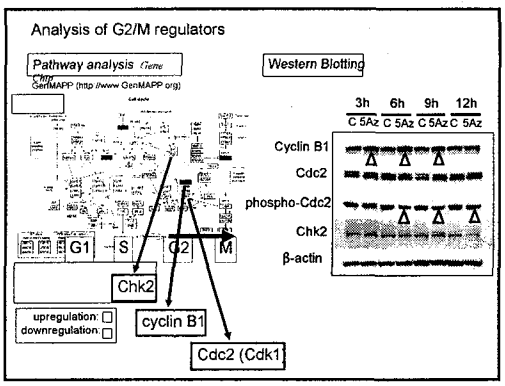


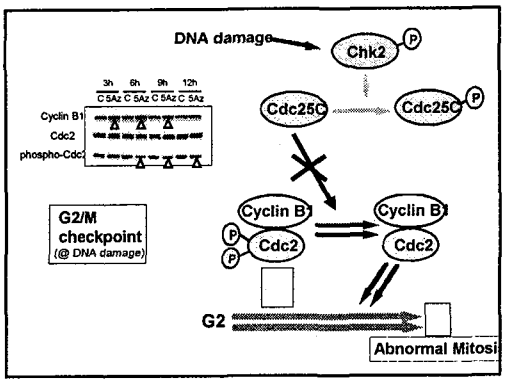



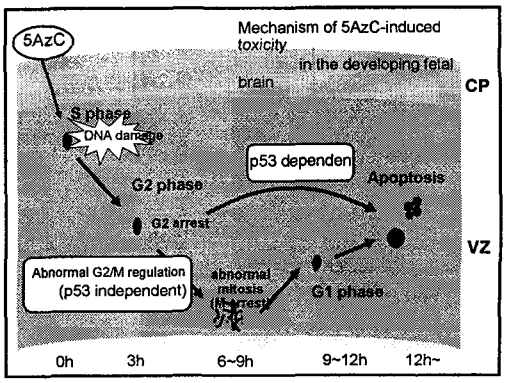



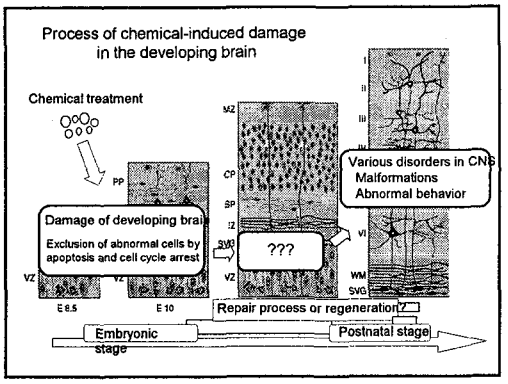

Cell cycle analysis

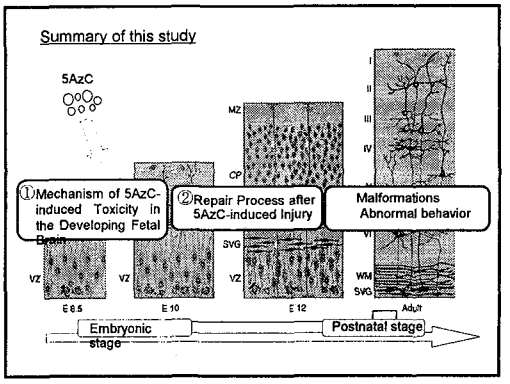
| | | apoptosis | G0/G1 | S | G2/M |
|---|---|---|---|---|---|
| control | +/+ | 0.8 ± 0.4 (%) | 83.7 ± 1.5 | 9.4 ± 1.0 | 6.0 ± 0.8 |
| | +/- | 0.9 ± 0.7 | 84.1 ± 2.3 | 9.7 ± 1.2 | 5.3 ± 1.1 |
| | -/- | 0.8 ± 0.4 | 83.5 ± 2.6 | 9.7 ± 1.5 | 6.1 ± 0.9 |
| 6 h | +/+ | 2.6 ± 1.3 | 64.8 ± 4.4 | 16.8 ± 3.1 | 15.7 ± 1.0 |
| | +/- | 2.4 ± 0.6 | 68.7 ± 3.2 | 14.5 ± 2.1 | 14.4 ± 1.9 |
| | -/- | 2.3 ± 0.4 | 68.3 ± 1.4 | 15.2 ± 0.8 | 14.2 ± 0.7 |
| 12 h | +/+ | 21.2 ± 6.3 | 51.8 ± 5.5 | 13.2 ± 2.2 | 13.8 ± 3.0 |
| | +/- | 11.2 ± 3.1 | 62.6 ± 3.1 | 9.4 ± 0.7 | 16.9 ± 3.4 |
| | -/- | 2.8 ± 1.5 | 71.7 ± 5.4 | 6.4 ± 1.8 | 19.1 ± 2.9 |





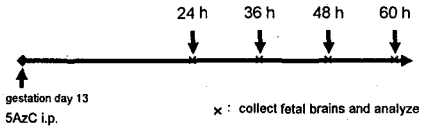



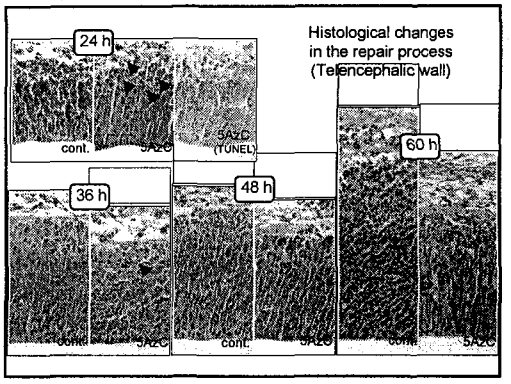



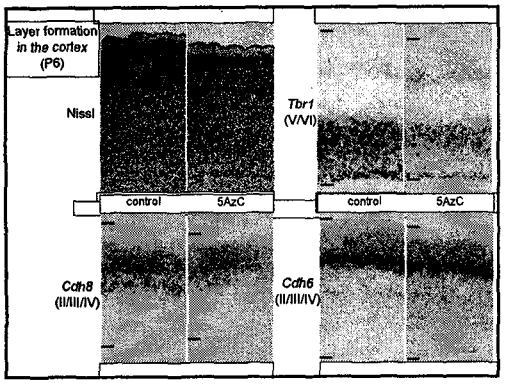



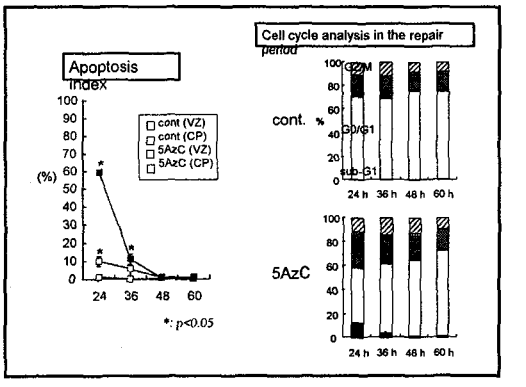

## Materials and Methods

*chemical : 5-Azacytidine (5AzC; 10 mg/kg i.p.)*
animal : Wistar rat (gestation day 13)
analyses : Histopathology (HE, immune-electron microscopy, immunohistochemistry)
Cell cycle analysis (FACS)
DNA microarray (Gene Chip (Rat Expression Array 230A, AFFYMETRIX))
RT-PCR. Real-time PCR





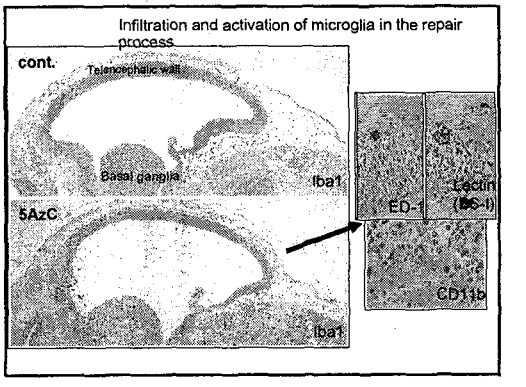



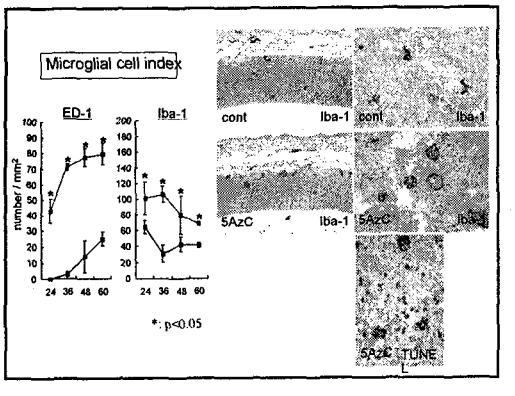

## Responses to injuries (adult)

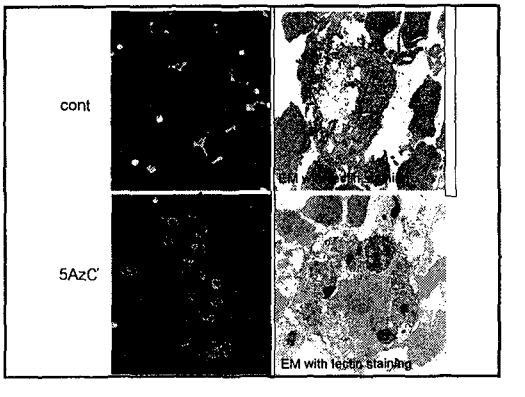
Injury
Microglia and macrophage — *Inflammatory Cytokines*
Oligodendrocyte precursors
*(and Meningeal cells)*
Astrocyte — *Inhibitory Molecules for*
*Regeneration*
Scar formation

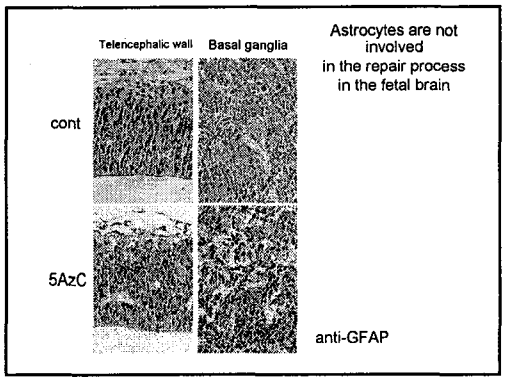
## Responses to injuries (embryo)

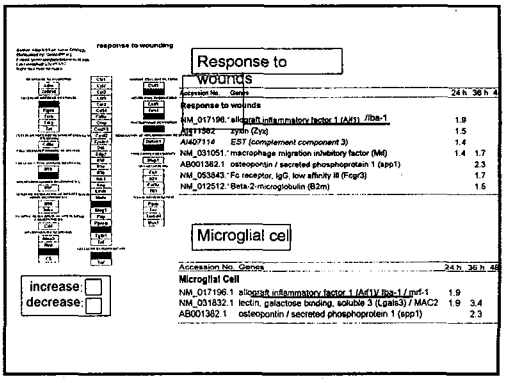
Injury
?
?
Regeneration — ?

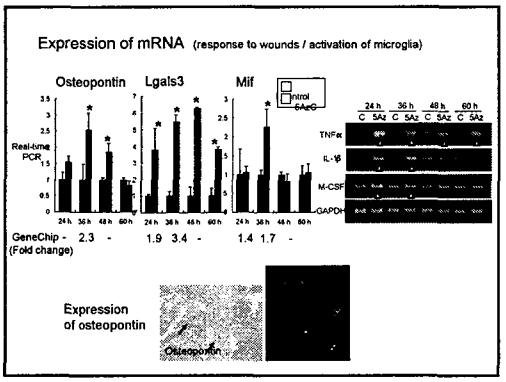


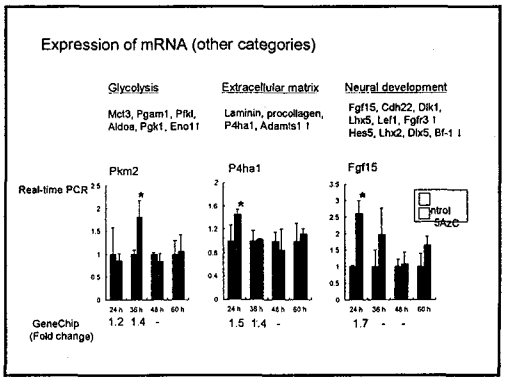



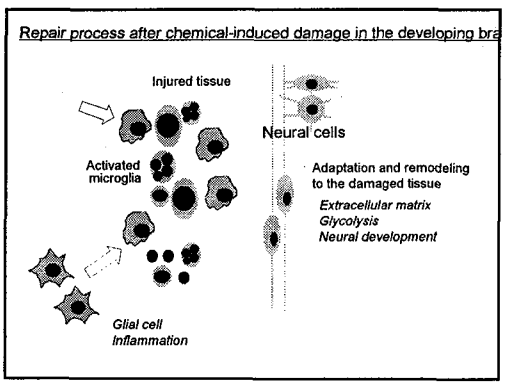



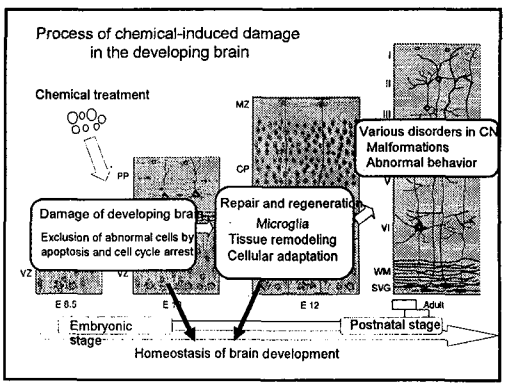

Telencephalic wall
Basal ganglia
cont
5AzC
Astrocytes are not involved in the repair process in the fetal brain
anti-GFAP


Response to wounds
response to wounding
Microglial cell
increase
decrease


Expression of mRNA (response to wounds / activation of microglia)
Osteopontin
Lgals3
Mif
Real-time PCR
GeneChip - (Fold change)
2.3 - 1.9 3.4 - 1.4 1.7 -
TNFα
IL-1β
M-CSF
GAPDH
Expression of osteopontin
Osteopontin


Expression of mRNA (other categories)
Glycolysis
Mct3, Pgam1, Pfkl, Aldoa, Pgk1, Eno1↑
Extracellular matrix
Laminin, procollagen, P4ha1, Adamts1 ↑
Neural development
Fgf15, Cdh22, Dlk1, Lhx5, Lef1, Fgfr3 ↑
Hes5, Lhx2, Dlx5, Bf-1 ↓
Pkm2
P4ha1
Fgf15
Real-time PCR
GeneChip (Fold change)
1.2 1.4 - 1.5 1.4 - 1.7 - -


Repair process after chemical-induced damage in the developing bra
Injured tissue
Activated microglia
Neural cells
Adaptation and remodeling to the damaged tissue
Extracellular matrix
Glycolysis
Neural development
Glial cell
Inflammation


Process of chemical-induced damage in the developing brain
Chemical treatment
MZ
CP
PP
VZ
SVG
WM
Various disorders in CN
Malformations
Abnormal behavior
Damage of developing bra
Exclusion of abnormal cells by apoptosis and cell cycle arrest
Repair and regeneratio
Microglia
Tissue remodeling
Cellular adaptation
E 8.5
E 12
Adult
Embryonic stage
Postnatal stage
Homeostasis of brain development

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