Session 2

化学物質の内分泌かく乱作用に関する基礎的研究
in vivo/ in vitro試験系における試験研究の現状

EDC basic researchs on in vivo/in vitro screening/testing methods

国立医薬品食品衛生研究所・安全性生物試験研究センター・毒性部　菅野　純

Jun Kanno, MD, PhD

Division of Cellular and Molecular Toxicology,

Biological Safety Research Center,

National Institute of Health Sciences, Tokyo, Japan

このシンポジウム

- 環境省の行政指向性のシンポジウムの下での専門対象のセッション
- SPEED98開始以降、10年間の総括を踏まえて、今後の研究の方向性を示すこと
- 　菅野には、内分泌かく乱物質問題における
  - 低濃度問題
  - 逆U字現象
  - コントロールの問題
- 国際動向
  - 国際会議

this presentation handles

- ここでは、ヒトの身代わりとしての哺乳類実験動物について言及する
- Only about mammalian experimental species as surrgate of humans.

約10年前、内分泌かく乱問題が注目を集めた理由は、

ten years ago….

背景に

there were several background issues….

●DES daughter の事象があり、ホルモンにより早期暴露遅延影響（early exposure – late effect）が起こるが、その発癌機構が分からない」ということ (carcinogenesis mechanism unknown)

- ●弱いながらもエストロジェン活性を示す化学物質群があり（その他に抗アンドロジェン作用を示す化学物質群も指摘された）

  chemicals with weak estrogenic activity have identified.

- ●有害確定試験法とされていた、生殖毒性試験（1世代、2世代試験など）は陽性対照（DESやE_2）に対して感度が低く、多くの場合、人での薬用量において陰性結果であること

  pharmacological doses of E2 and DES turned out to be negative by regular one-gen, two-gen studies.

- ●当時やっとER β がクローニングされ、ERについての研究が、毒性学に応用できるほどには、意外なほど進んでいないことがわかった事

  ER beta had just been cloned, impressing toxicologists that we do not know much about ER system at all

- ●受容体系の用量作用関係はnon-monotonous（非単調関数）であるのが一般的であるのに対して、毒性学にはその様な概念が無いこと

  receptors react non-monotonously, whereas toxicology does not.

- ●ER発現が胎児で、生殖腺以前に中枢神経系を主体に見られること

  the earliest ER expression is found in brain of embryo. What for??

そして、

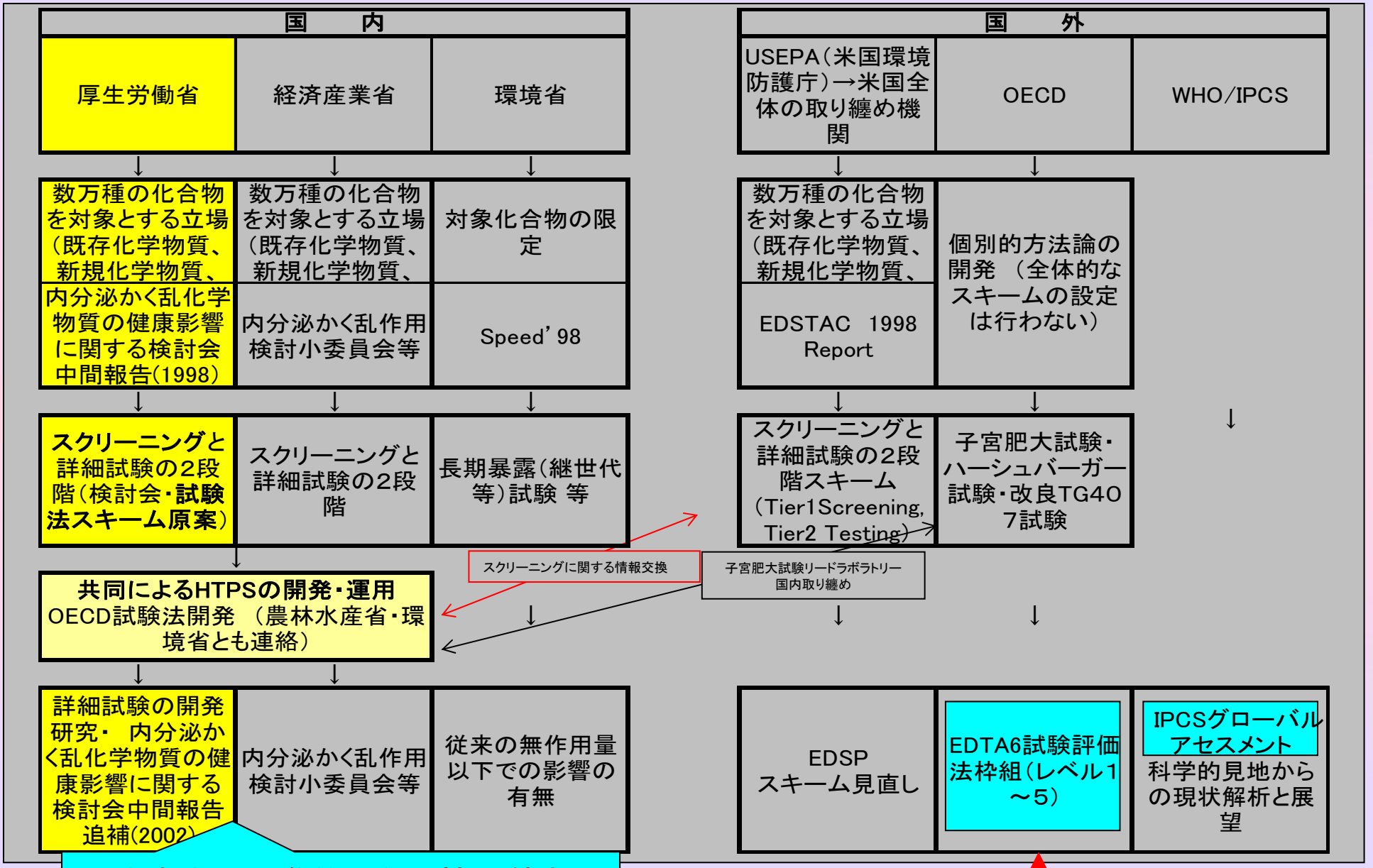
and

人に対する影響については、生物学的蓋然性の観点から懸念があり、人の身代わりとしての哺乳類実験動物での検討が必要と感じられるようになった。

biological plausibility made us to study this issue further.

(not enough data to pose immediate actions on weakly estrogenic chemicals)

内分泌かく乱化学物質試験法開発等の国内外の状況



Ministry of Environment

- SPEED'98
- 当時の科学的知見に基づき、専門家による検討会での意見を踏まえ、優先して調査研究を進めていく必要性の高い67物質（後に65物質）を取り上げ、毒性試験を実施した。

- 専門家による意見とは、内分泌かく乱化学物質問題の契機となった"Our Stolen Future"を記述したColbornらの論文等で取り上げられた63物質(群)とその類縁3物質及び巻貝のイボニシに生殖阻害を生じることが日本において明らかにされたトリフェニルスズ(TPT)の計67物質(群)を取り上げたものである。

Ministry of Environment

- SPEED'98 (67 selected chemicals)

- Result: Almost all results for mammalian targets turned out to be Negative.

  – No big surprise!
  – That is why no action ever taken under the name of EDC yet

OECD EDTA 6

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

Level 1 Sorting & prioritization based upon existing information	- physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability, - human & environmental exposure, e.g., production volume, release, use patterns - hazard, e.g., available toxicological data	
Level 2 *In vitro* assays providing mechanistic data	- ER, AR, TR receptor binding affinity - Transcriptional activation - Aromatase and steroidogenesis *in vitro* - Aryl hydrocarbon receptor recognition/binding - QSARs	-High Through Put Prescreens - Thyroid function - Fish hepatocyte VTG assay - Others (as appropriate)
Level 3 *In vivo* assays providing data about single endocrine mechanisms	- Uterotrophic assay (estrogenic related) - Hershberger assay (androgenic related) - Non -receptor mediated hormone function - Others (e.g. thyroid)	- Fish VTG (vitellogenin) assay (estrogenic related)
Level 4 *In vivo* assays providing data about multiple endocrine mechanisms	- enhanced OECD 407 (endpoints based on endocrine mechanisms) - male and female pubertal assays - adult intact male assay	- Fish gonadal histopathology assay - Frog metamorphosis assay
Level 5 *In vivo* assays providing adverse effects data from endocrine & other mechanisms for RA	- 1-generation assay (TG415 enhanced)[1] - 2-generation assay (TG416 enhanced)[1] - reproductive screening test (TG421 enhanced)[1] - combined 28 day/reproduction screening test (TG 422 enhanced)[1] 1 Potential enhancements will be considered by VMG mamm	- Partial and full life cycle assays in fish, birds, amphibians & invertebrates (developmental and reproduction)

受容体原性毒性Receptor Mediated Toxicity

内分泌かく乱化学物質問題は受容体原性毒性として
捉えることが出来る
Endocrine Disruptor Issue is Receptor Mediated Toxicity

- ホルモン活性化学物質Hormonally Active Chemicals (HACs)
  =ホルモン受容体を介した影響
  Hormone receptor-mediated effects

- 内分泌かく乱化学物質Endocrine Disrupting Chemicals (EDCs)
  = HACsで有害性を示すもの
  HACs that show adverse effects
  = 受容体原性毒性Receptor-Mediated Toxicity

Receptor Mediated Toxicity

- Examples:
  - TCDD : AhR KO* mice → virtually no toxic symptom.
    - *But, Wild type mice die of TCDD*
      - Symptoms are the results of gene expression through AhR
    - *Wild type mice die of their own gene products!*

  - Estrogens : ERKO mice → no DES effect.
    No uterotrophic response.
    - Analogy to TCDD/AhR: receptor mediated toxicity
    - Difference from TCDD/AhR
      = Intrinsic ligand (estradiol) is present for physiological function.
    - Difficulty for Toxicology: how to draw line between,,,
      Physiological responses <> adverse effects

Traditional Toxicity

Toxic Substance(●)

Target Sites

- Normal function
  - Proteins(Enzyme)
  - Membrane
  - Etc.

Receptor-Mediated Toxicity

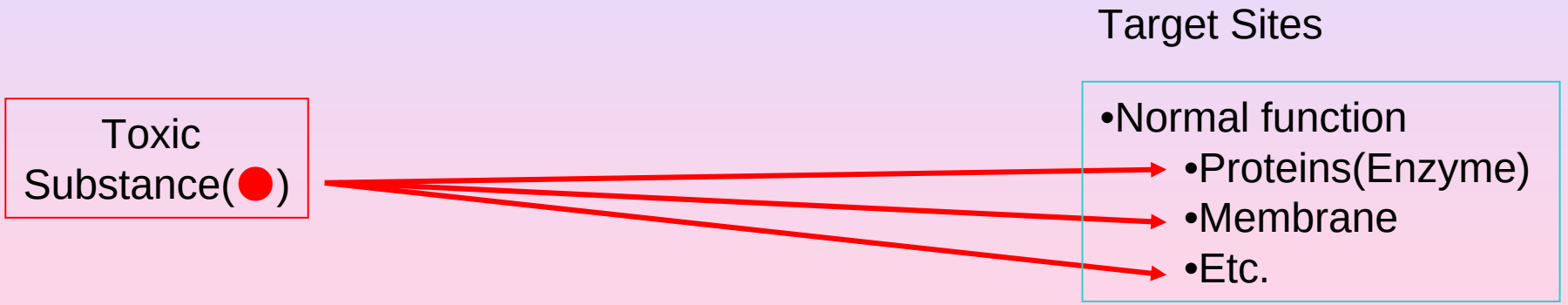
Toxic substance (■)

Receptor

Target Sites

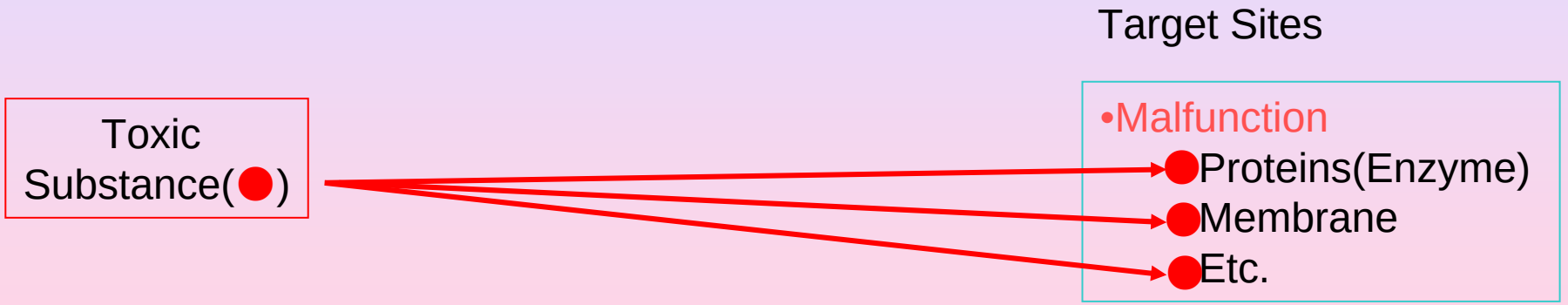
- Protein Expression
  - Normal Timing
  - Right Kind
  - Right Amount

Traditional Toxicity



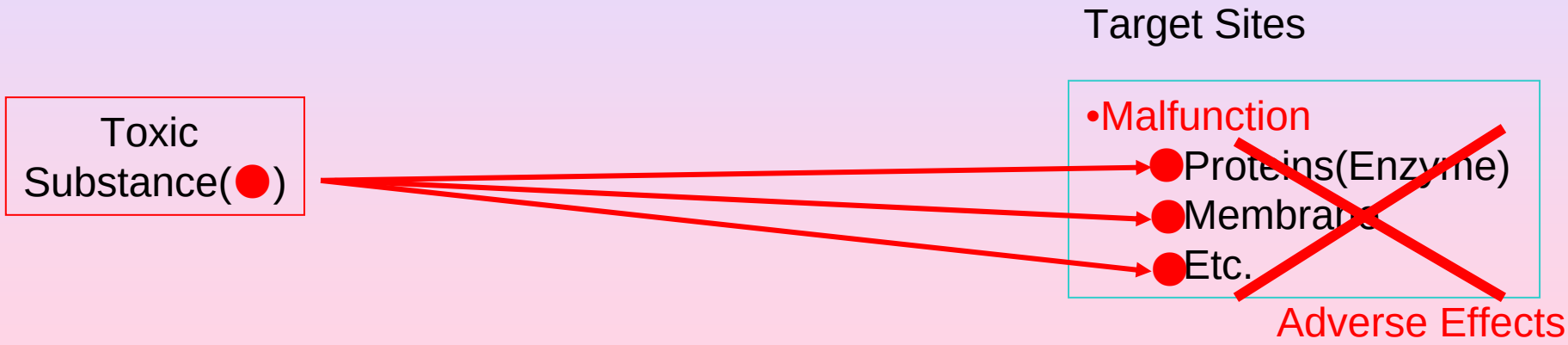
Receptor-Mediated Toxicity

Traditional Toxicity

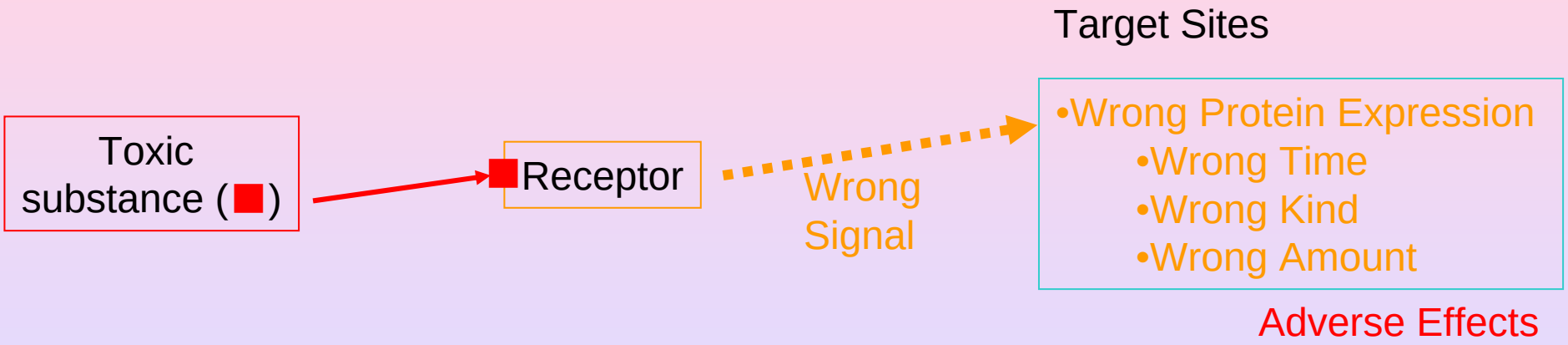


Receptor-Mediated Toxicity

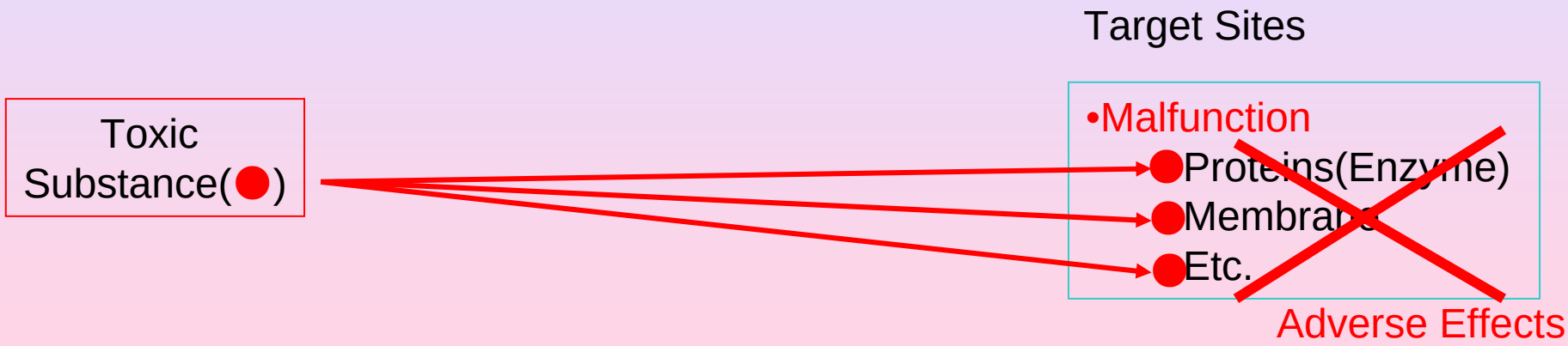
Traditional Toxicity



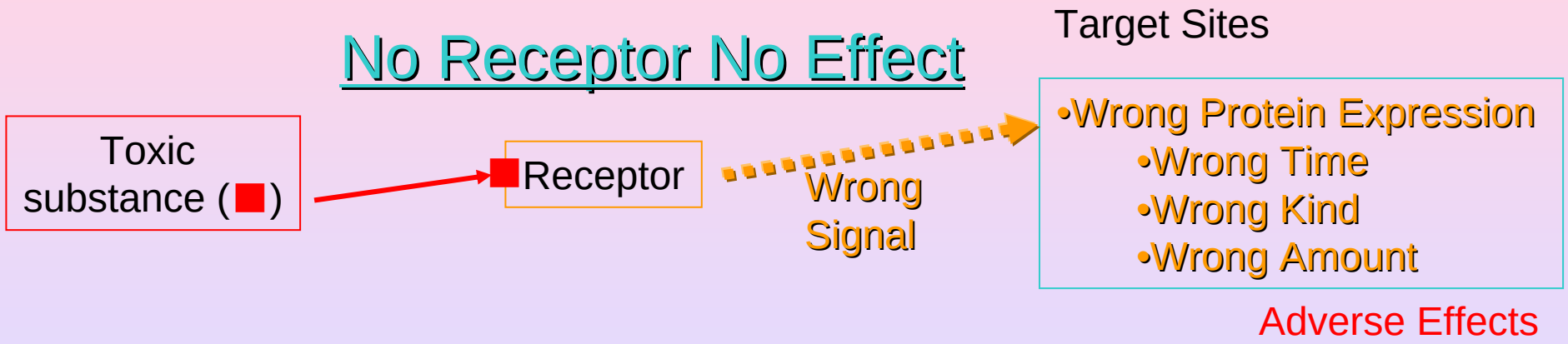
Receptor-Mediated Toxicity



Traditional Toxicity



Receptor-Mediated Toxicity



Receptor Mediated Toxicity

- Redundancy
  - Ligand binding characteristics
    - Common effect
    - Specific effects (SERM)
  - Receptor Subtypes (family, subunits)
- Signaling system – often works as Amplification system
  - Low dose effect
  - Dose Response Curve

    Non-monotonous: Inverted U-shaped, U-shaped
  - **Signal Cross-talk: additive / synergistic**
- Spatiotemporal Expression
  - Different effects in different sites at different stages of development, maturation, and aging

    The Fetal "Sensitive Window"

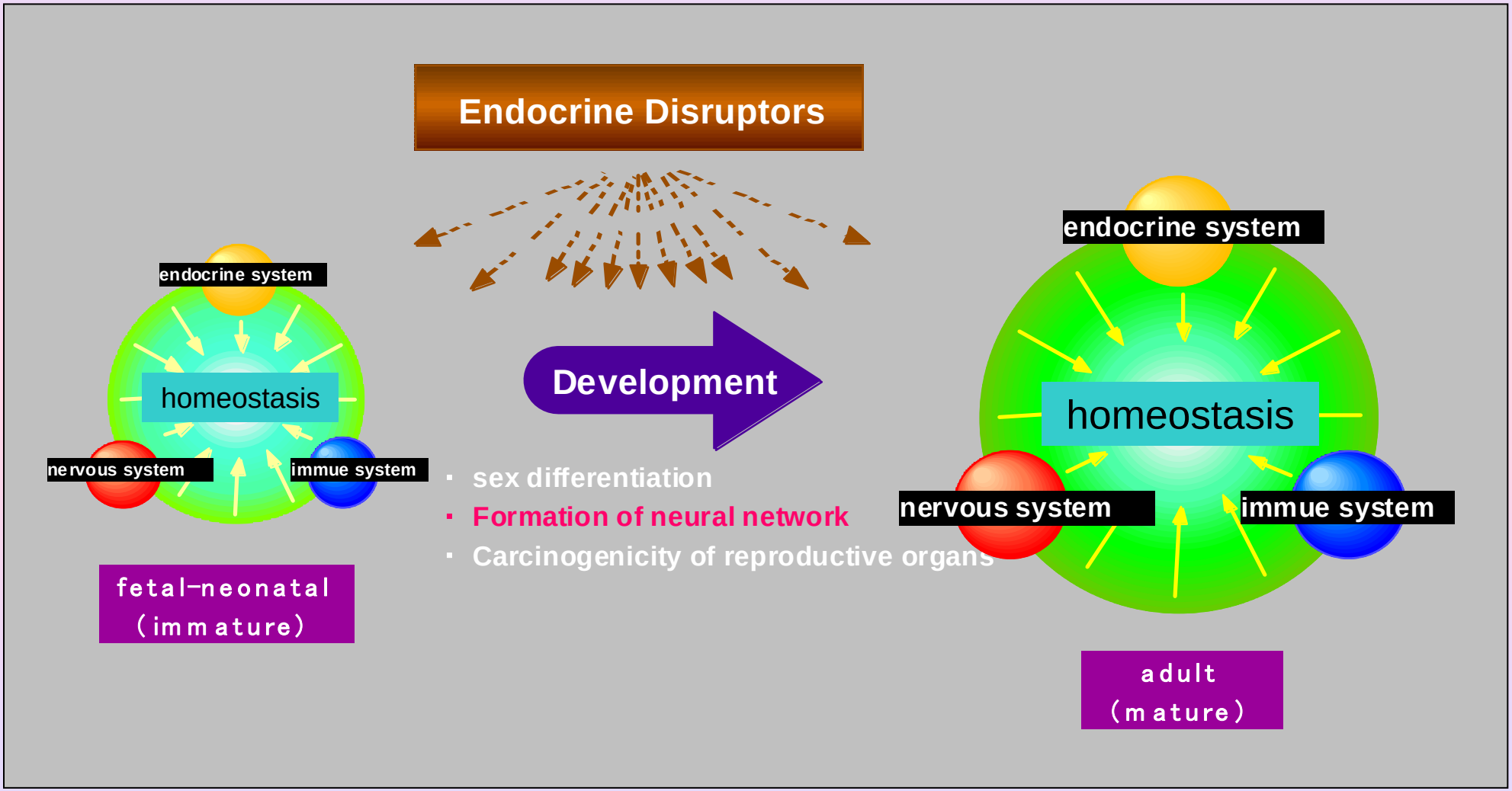
    << early exposure - late effect >>

A Paradigm-Shift in Toxicology

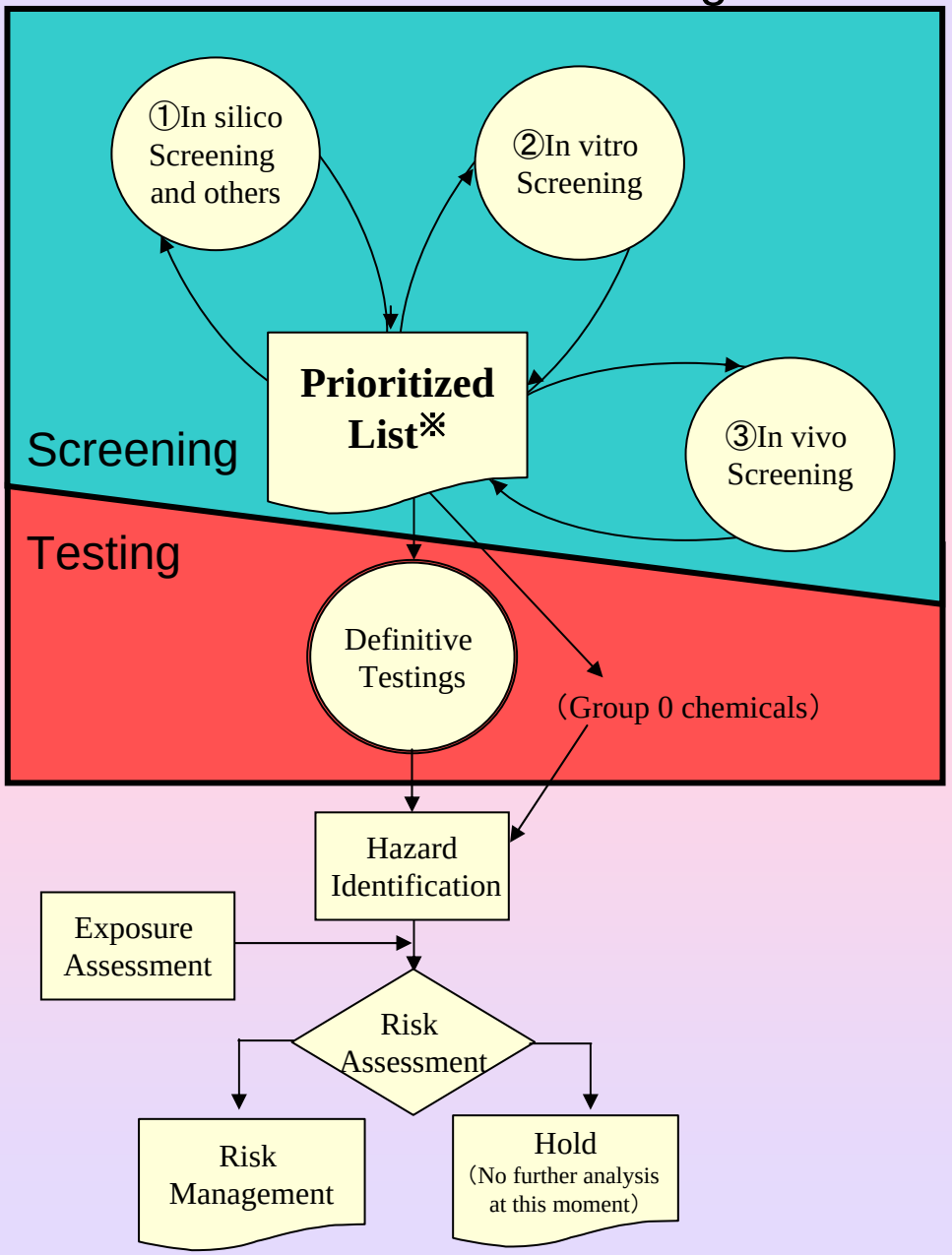
Traditional (Regular) Toxicology (using animals with strong homeostatic regulation)	Receptor-Mediated Toxicology	Dose-Range
Regular Toxicity (Membrane damage, Enzyme damage, etc.)		
NOEL of Trad Tox	AR system (antagonist) **H**	10^{-6} - 10^{-7} M
	ER system (agonist) **UT**	10^{-9} - 10^{-10} M

Reference : Oral contraceptive -- EE = ca. 0.5μg/kg/day
(P=1.0mg/tab EE=0.035mg/tab)

Sensitive time window and adverse effects
(Target is the immature homeostatic network)



MHLW Scheme for ED screening and testing



Developing priority list DB

Compound		Reporter gene assay				In silico		SPR assay		Uterotropic Assay
CAS	Name	ERaEC50(pM	ERaEffect	ERbEC50(pM	ERbEffect	ERa-Score	ERb-Score	SPRERaE	SPRERaL	Uterotropic Assay
000057-63-6	Estradiol,ethynyl	6	P	10	P	0.995	0.854	H	H	++
000050-28-2	Estradiol,17b					0.928	1.225	H	H	++
000057-91-0	Estradiol,17a					1.183	0.805	H	H	++
026538-44-3	a-Zearalanol (2,4-Di					-0.071	-0.098	H	H	
000084-19-5	3,4-Bis(4-acetoxyph	53.7	P	50.7	P	0.3329				
000056-53-1	Diethylstilbestrol	82	P	27.77682	P	0.719	0.759	H	H	++
000053-16-7	Estrone	831	P	15.22533	P	0.534	0.542	H	L	
000531-95-3	Equol	37000	P	3548.825	P					
001478-61-1	4,4'-(HEXAFLUORO	80200	P	10264.73	P				N	
001478-61-1	4,4'-(HEXAFLUORO	80200	P	10264.73	P				N	
000479-13-0	Coumestrol	104000	P	9445.462	P					
007507-01-9	3,4-Bis(4-Hydroxypl	139000	P	2467.847	P	-0.158	0.348	H	H	
000446-72-0	Genistein	144000	P	695.7919	P	-0.315	0.291	L	N	
000068-22-4	Norethrindrone	171000	P	226459.5	P	-0.449	0.268	L	L	
000077-40-7	Bisphenol B	196000	P	62940.04	P	-0.122	-0.117			
059517-19-0	3,3'-Dimethyl-4,4'-E	231000	P	66935.05	P	-0.425	-0.244			
000486-66-8	Diadzein(4',7-Dihydr	268000	P	24117.57	P	-0.209	-0.135	L	L	
000131-55-5	2,2',4,4'-Tetrahydrox	328000	P	506320.7	P	-0.303	-0.22	H	H	
052222-87-4	6-BENZOYL-2-NAF	342000	P	659548.2	P	-0.134	0.006	K	L	
000341-58-2	2,2'-Bis(Trifluorome	346000	P	880156.1	P	-0.91	-0.441	H	L	
000140-66-9	4-Octylphenol(tert)	391000	P	41614.38	P	-0.33	-0.069	K	N	
000521-18-6	Stanolone	475000	P	20463.19	P	0.49	0.601	L	L	
. .										
. .										

Reporter assay data

***In silico* screening score data**

SPR assay data

Uterotropic data

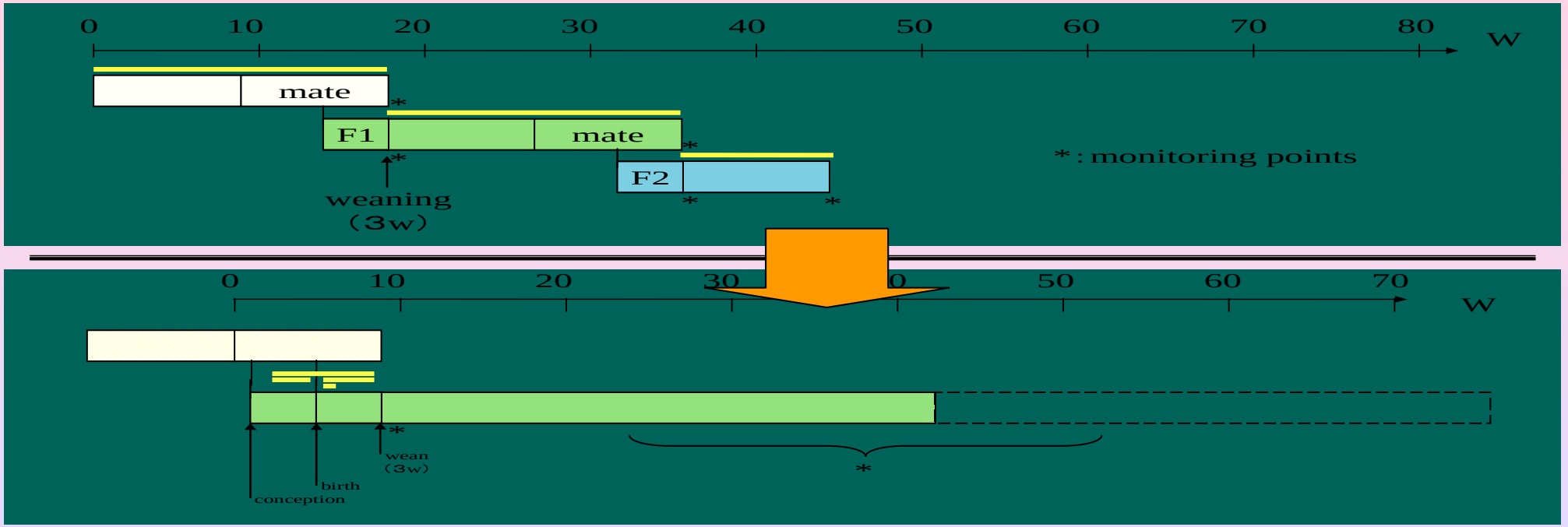
Other data

EC50 to be posted

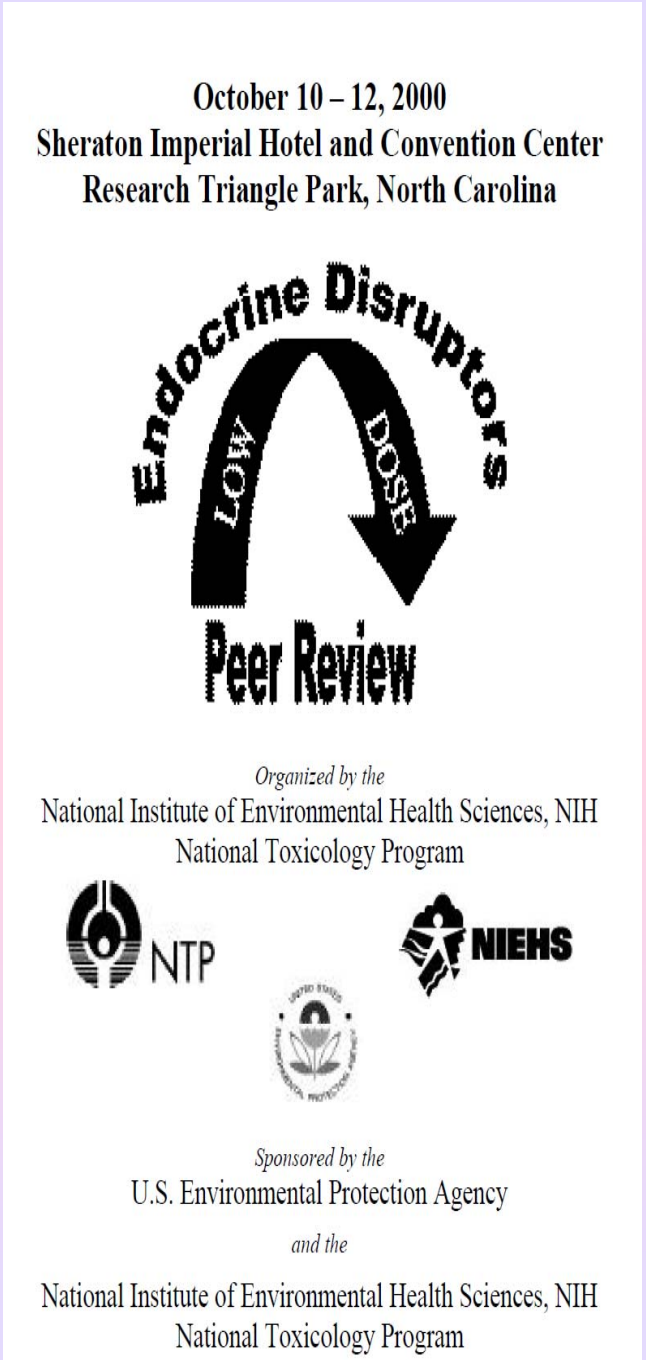
Rearrangement of a priority list by a combination of accumulated data

Definitive testing end points:

- Multi-generation study endpoints are not sensitive to known estrogens such as DES and 17beta-estradiol
- “Low-dose effects” = Early Exposure - Late Effect
  - non-reprotox endocrine endpoints
  - Neurological endpoints, Immunological endpoints
  - may look like ageing-related phenotypes



低用量問題

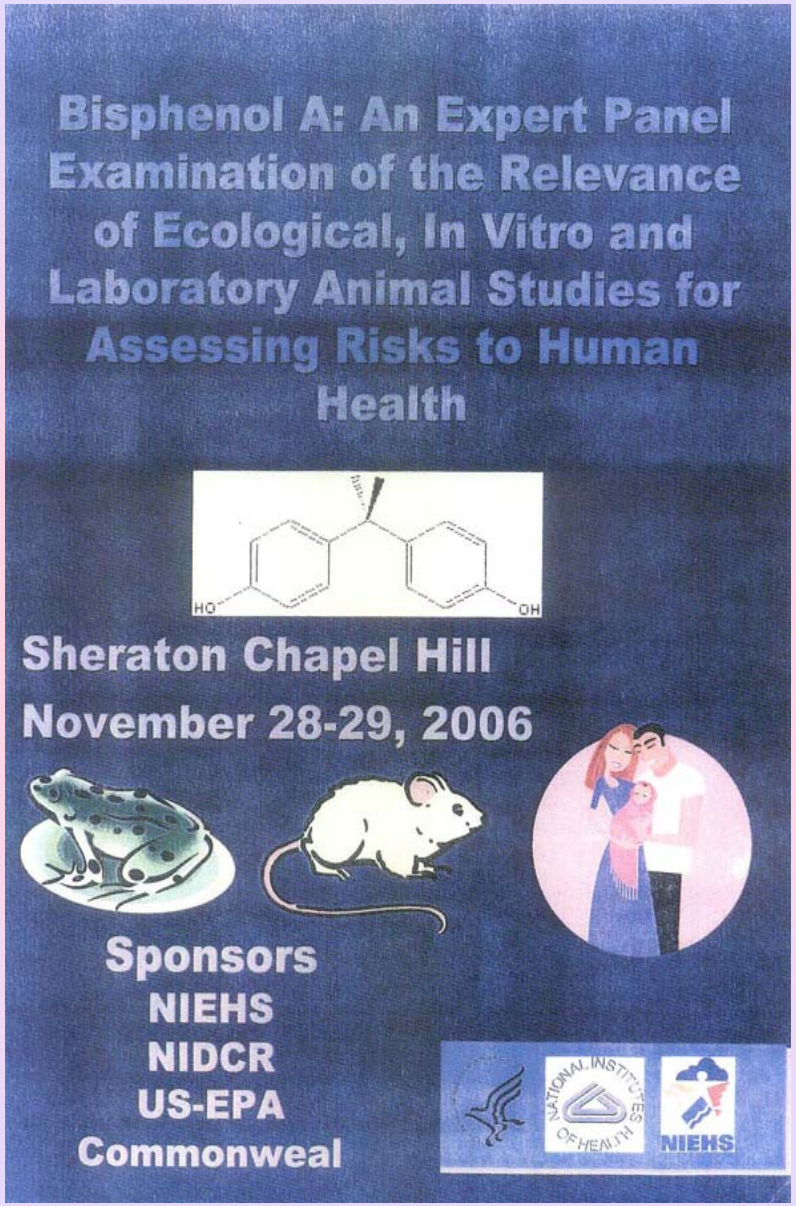


左記の会合をはじめ、Dr. John AshbyらとProf. Fred vom Saalらの間で様々な論議が行われたが、その中には陰性対照と陽性対照の問題があった。
argument about postive/ negative control

この問題は:
●従来型の生殖毒性試験では経口避妊薬に含まれるエストロジェン量が陰性結果となることが明確に示されたため、薬用量相当のDESはもとより、それよりも弱いエストロジェン活性を示す物質の影響は、従来型毒性試験では取り扱えないことが示された。
→もともと、陽性対照を置くという概念が無い試験法である
pharmacuetical doses of E2/DES were negative
// No concept of positive control in multi-gen studies

●シグナルを扱うことから動物の扱いに最新の注意を払う必要がある等が指摘された。
very precise control on animal handling is important for handling studies on signal toxicity

の2点に集約される問題点を提起している。



2006年10月にNIEHSのJerry Heindelらが主催するワークショップが行われた。結論としては、所謂低用量現象が哺乳類実験系でも観測されることなどが取り上げられた。

human exposure to BPA is within the range that is predicted to be biologically active in over 95% of people sampled. The wide range of adverse effects of low doses of BPA in laboratory animals exposed both during development and in adulthood is a great cause for concern with regard to the potential for similar adverse effects in humans.

Reproductive Toxicology, in press, 2007

Chapel Hill Bisphenol A Expert Panel Consensus Statement: Integration of Mechanisms, Effects in Animals and Potential to Impact Human Health at Current Levels of Exposure

Frederick S. vom Saal[1†], Benson T. Akingbemi[2], Scott M. Belcher[3], Linda S. Birnbaum[4], D. Andrew Crain[5], Marcus Eriksen[6], Louis J. Guillette[7], Russ Hauser[8], Jerrold J. Heindel[9], Shuk-Mei Ho[10], Taisen Iguchi[11], Susan Jobling[12], Jun Kanno[13], Ruth A. Keri[14], Karen E. Knudsen[15], Gerald A. LeBlanc[16], Michele Marcus[17], John A. McLachlan[18], J. Peterson Myers[19], Angel Nadal[20], Retha R. Newbold[21], Nicolas Olea[22], Gail S. Prins[23], Catherine A. Richter[24], Beverly S. Rubin[25], Carlos Sonnenschein[26], Ana M. Soto[27], Chris E. Talsness[28], John G. Vandenbergh[29], Laura N. Vandenberg[30], Debby R. Walser-Kuntz[31], Cheryl S. Watson[32], Wade V. Welshons[33], Yelena Wetherill[34] and R. Thomas Zoeller[35]

National Toxicology Program
U.S. Department of Health and Human Services

NTP

Center for the Evaluation of Risks to Human Reproduction

NTP-CERHR EXPERT PANEL REPORT on the REPRODUCTIVE and DEVELOPMENTAL TOXICITY of BISPHENOL A

November 26, 2007

Selection by design (size), litter effect handling, statistics, route of exposure

Strengths/Weaknesses: This study utilized a good choice of methods to examine functional disruptions in sexually dimorphic behaviors. Weaknesses include a lack of clarity about the nature of disruption of sexually dimorphic behavior patterns that was indicated in the authors' conclusions, the somewhat small sample size, the use of a single dose level, which was not confirmed, and the lack of clarity of the statistical methods regarding litter.

Utility (Adequacy) for CERHR Process: This paper is inadequate for the evaluation process due to statistical methodology.

Strengths/Weaknesses: A strength is the examination of effects on uterine indices in female offspring. A slight weakness is the use of only 6 females per group; however, the panel noted that the results appeared to be consistent across animals and across endpoints, especially in the 50 mg/kg bw/day treatment group.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate and of high utility for the evaluation process.

Strengths/Weaknesses: Endpoints are a strength, but inadequate methodological detail (i.e., sample size or adequate control for litter effects) precludes any informed judgment of study quality.

Utility (Adequacy) for CERHR Evaluation Process: This study is inadequate for the evaluation process based on insufficient methodological details.

Strengths/Weaknesses: The strengths of this paper are the care taken to control for extraneous estrogenic exposure, the delivery of BPA at 2 doses, both low, delivery from GD 1 to PND 16, the reasonable sample sizes, and the inclusion as outcome measurements of behavior, anatomy, and an index of neurochemical effects in the brain. Significant weaknesses include the use of sc osmotic pumps, uncertainty about sample size and whether litter effects were adequately controlled for.

Utility (Adequacy) for CERHR Evaluation Process: This is inadequate for the evaluation process due to the combination of route of administration and statistical concerns.

There are sufficient data to suggest that developmental exposure to Bisphenol A causes neural and behavioral alterations related to sexual dimorphism in rats and mice (ca. 2.5 mg/kg/d, gestation and lactation in rats, (*326*); LOEL 0.00002 mg/kg/d, fetal mice, (*406*); 0.0002 mg/kg/d, fetal mice, (*404*), 0.04 mg/kg/d, weaning to puberty, rats, (*370*); 0.1 mg/kg/d, GD3 – PND 20, rats, (*361*); 0.2 mg/kg/d, GD3 – PND20, mice, (*435*); 0.01 mg/kg/d, GD11-18, mice, (*413*), although other studies report no change in a related measure, the size of the sexually dimorphic nucleus of the pre-optic area (SDN-POA) [300 µg/kg/d, rats (*372*); NOEL of 320 mg/kg/d, rats, (*342*)].

1. For pregnant women and fetuses, the Expert Panel has different levels of concern for the different developmental endpoints that may be susceptible to bisphenol A disruption, as follows:
    - For neural and behavioral effects, the Expert Panel has some concern
2. For infants and children, the Expert Panel has the following levels of concern for biological processes that might be altered by Bisphenol A, as follows:
    - some concern for neural and behavioral effects
    - minimal concern for the effect of accelerated puberty

Summary

- EDC issue is Receptor-Mediated Toxicity
  (Wrong Signal)
- Target is Neuro-Immuno-Endocrine Network or Homeostatic system
  (common/shared signal molecules)
- Modification of NIE network at embryonic, perinatal and/or infantile stages results in Irreversible changes in the later stages of life.
- Practical arguments on how to deal with the low dose issue will begin shortly with real low dose data.

end