第三屆環境醫學研討會
International Symposium of Environmental Medicine
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新時代新議題 **Current Environmental Health Issues**

発達神経毒性Developmental Neurotoxicity Perspectives and Problems in Risk Assessment

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名誉教授 Professor Emeritus, 東京大学　U. of Tokyo

Outline of Today's Topics

Why should we conduct behavioral studies *in vivo* that focus on the developing rodent brain?

- Developmental neurotoxicity (DNT) in children

- Behavioral test battery in rats and mice and DNT test guidelines

- Development of reliable behavioral methods for developmental neurotoxicity (DNT)

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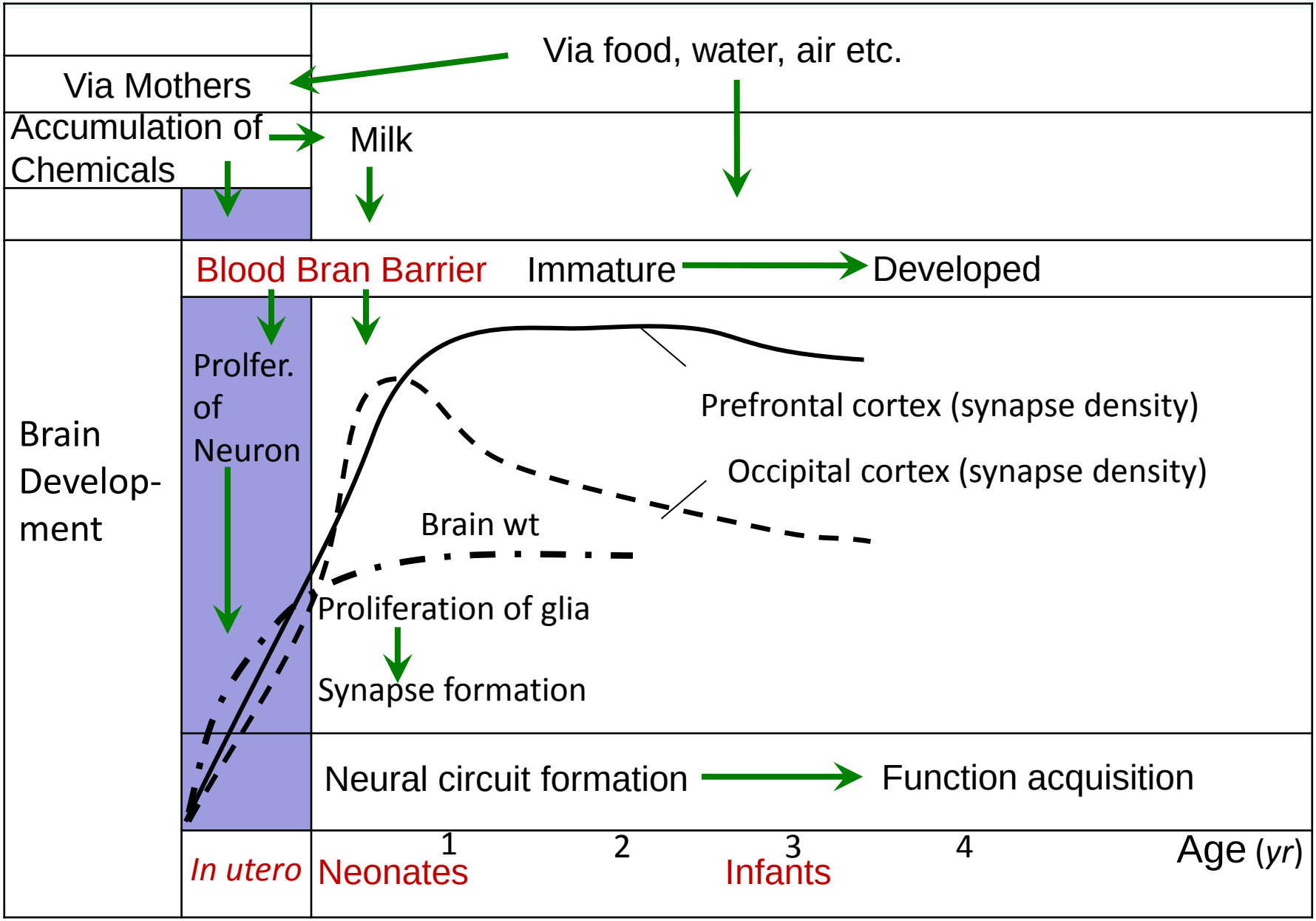
Brain Development and Intrusion of Environmental Chemicals to the Developing Brain



(Modified from Kuroda Y., *Kagaku*, 73:1234-1243, 2003))

Environmental Disease with “Clear-cut” Causality on Developmental Neurotoxicity

Minamata Disease
水俣病
（Methylmercury)

油症
（Mixture of PCB：Polychroinated-biphenyl; PCQ, Polychronated-quaterphenyl; PCDF, Polychrorinated-dibenzofuran）

Pictures were deleted because of the copyright

（Harada M.，1972)　　（http://www.kyudai-derm.org/part/yusho/index.html）

American Academy of Pediatrics
DEDICATED TO THE HEALTH OF ALL CHILDREN™

FROM THE AMERICAN ACADEMY OF PEDIATRICS

Organizational Principles to Guide and Define the Child Health Care System and/or Improve the Health of all Children

POLICY STATEMENT

Pesticide Exposure in Children

COUNCIL ON ENVIRONMENTAL HEALTH

KEY WORDS
pesticides, toxicity, children, pest control, integrated pest management

ABBREVIATIONS
EPA—Environmental Protection Agency
IPM—integrated pest management

abstract

FREE

This statement presents the position of the American Academy of Pediatrics on pesticides. Pesticides are a collective term for chemicals intended to kill unwanted insects, plants, molds, and rodents. Children encounter pesticides daily and have unique susceptibilities to their potential toxicity. Acute poisoning risks are clear, and understanding of chronic health implications from both acute and chronic exposure are emerging. Epidemiologic evidence demonstrates associations between early life exposure to pesticides and pediatric cancers, decreased cognitive function, and behavioral problems. Related animal toxicology studies provide supportive biological plausibility for these findings. Recognizing and reducing problematic exposures will require attention to current inadequacies in medical training, public health tracking, and regulatory action on pesticides. Ongoing research describing toxicologic vulnerabilities and exposure factors across the life span are needed to inform regulatory needs and appropriate interventions. Policies that promote integrated pest management, comprehensive pesticide labeling, and marketing practices that incorporate child health considerations will enhance safe use. *Pediatrics* 2012;130:e1757–e1763

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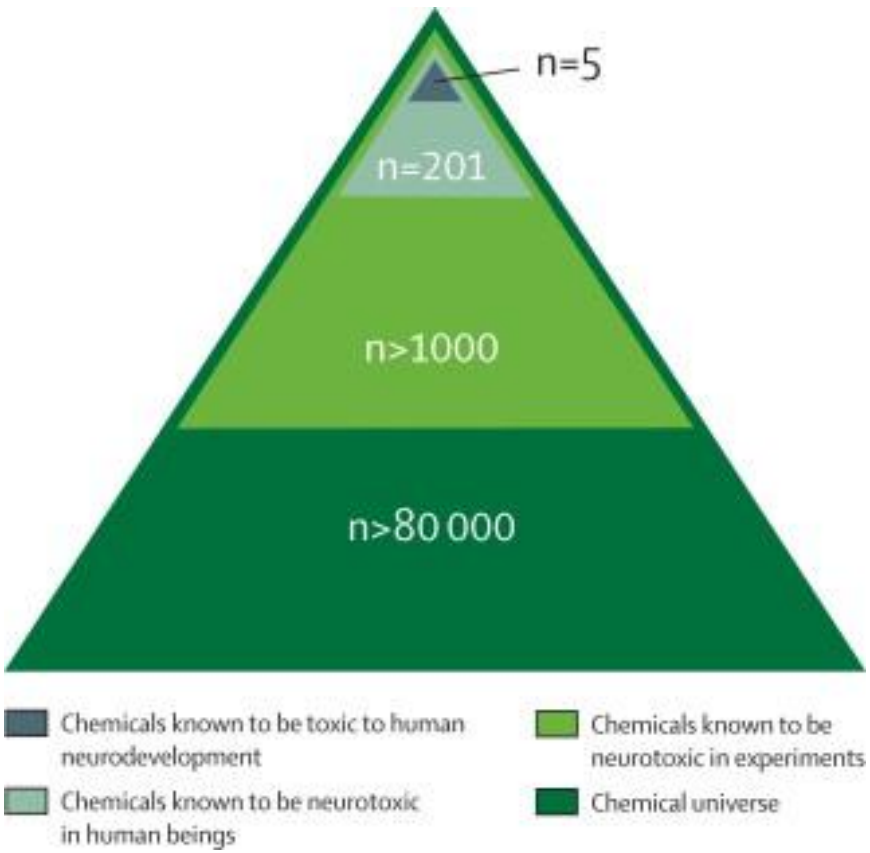
POLICY STATEMENT

Pesticide Exposure in Children

A serious concern on the epidemiologic evidence that demonstrates associations between early life exposure to pesticides and pediatric cancers, decreased cognitive function, and behavioral problems. It also proposes the regulatory actions and appropriate interventions for the protection of children's health.

Environmental Chemicals Are Potentially Harmful to the Developing Brain in Children



P Grandjean , PJ Landrigan,
The Lancet, 368: 2167 – 78, 2006

Only a small fraction of chemicals, such as Pb, methylmercury, PCBs, arsenic, and toluene, have been proven to exert developmental disorders in children.

The majority of chemicals do not have sufficient information on DNT in humans.

=== >>> In order not to overlook the potential neurotoxicity of chemicals, we need to establsh appropriate neurotoxicity test methodology.

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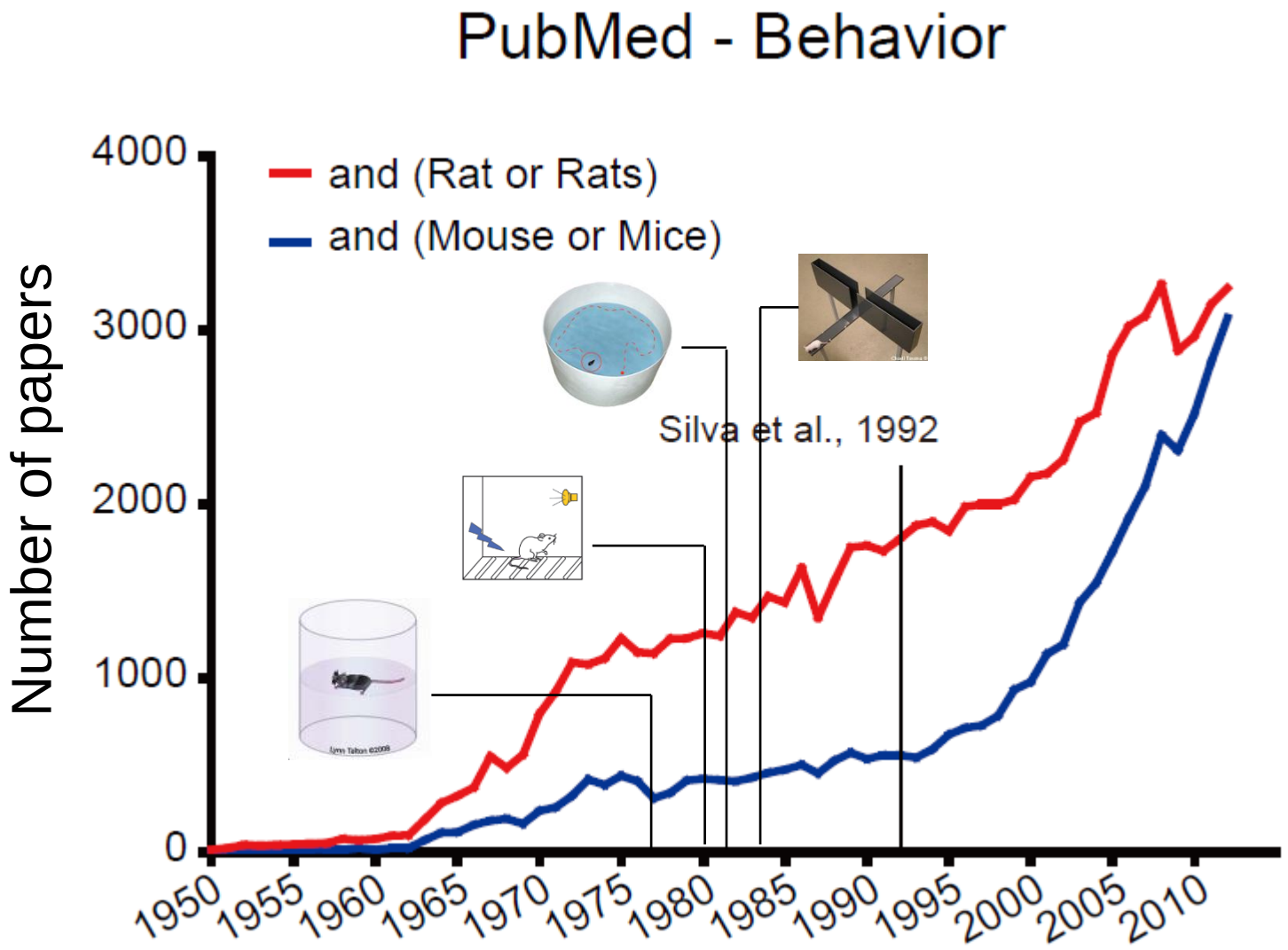
Trend of Number of Papers on Rodent Behavioral Tests in Biomedical Sciences



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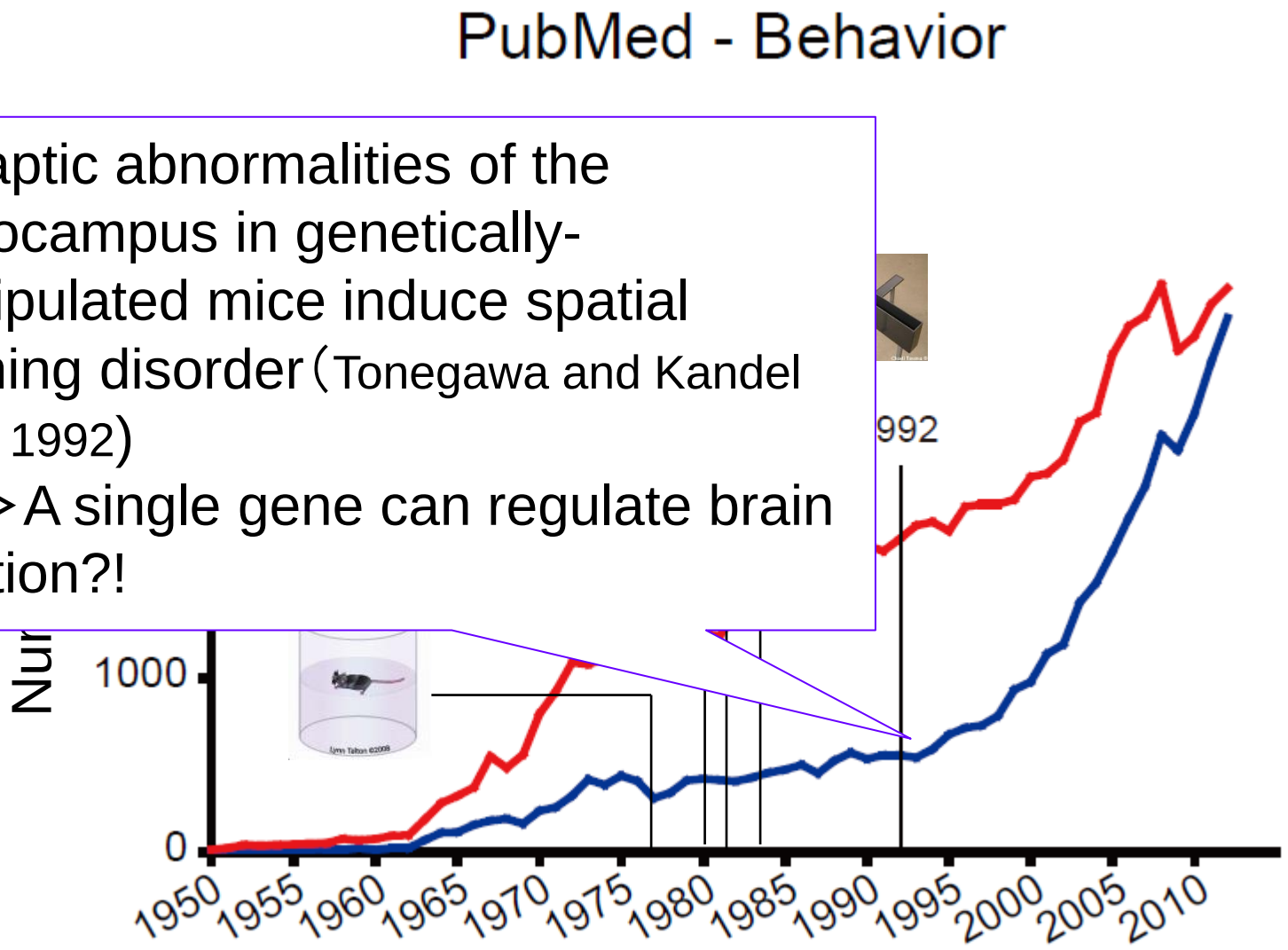
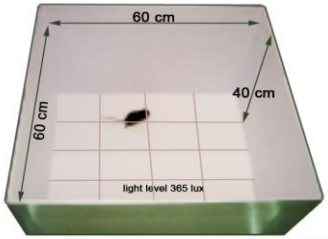


Synaptic abnormalities of the hippocampus in genetically-manipulated mice induce spatial learning disorder（Tonegawa and Kandel Labs, 1992）

・・・＞A single gene can regulate brain function?!

Behavioral Test Battery for the Rodent (Rats and Mice)

Basal Activity



Open-field

Motor

Rotarod

Sensory

Hot plate

Depression

Forced swim

Tail suspension

Anxiety

Light/Dark box

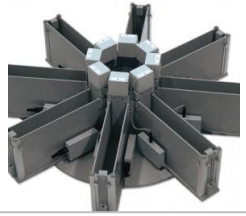
Elevated plus maze

Learning & Memory

Water maze

Object recognition

Y maze



Radial arm maze

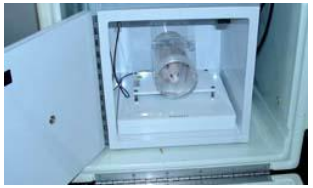
Operant conditioning

Sociality

Three-chamber

Resident-intruder

Startle Behavior



Acoustic startle response / Pre-pulse inhibition

An Example of Behavioral Test Battery

Order	Test Name	Tested Behavior and Response
1	General health check, Neurological screening	Physical characteristics, Neurological symptoms
2	Open field	Activity, anxiety-like behavior
3	Elevated plus maze	Activity, anxiety-like behavior
4	Hot plate	Pain perception
5	Rotarod	Motor learning, Coordination
6	Acoustic startle/Prepulse inhibition	Sensory motor gating, Startle response
7	Morris water maze	Working memory, Reference memory, Perseveration
8	Tail suspension	Depression-like behavior,
9	24-hour home-cage social interaction	Sociality, activity in a home-cage
10	Fear conditioning	Fear memory

(Translated and modified from: Takao, Koshimizu, Miyakawa, 2013 DOI：10.14931/bsd.4058)

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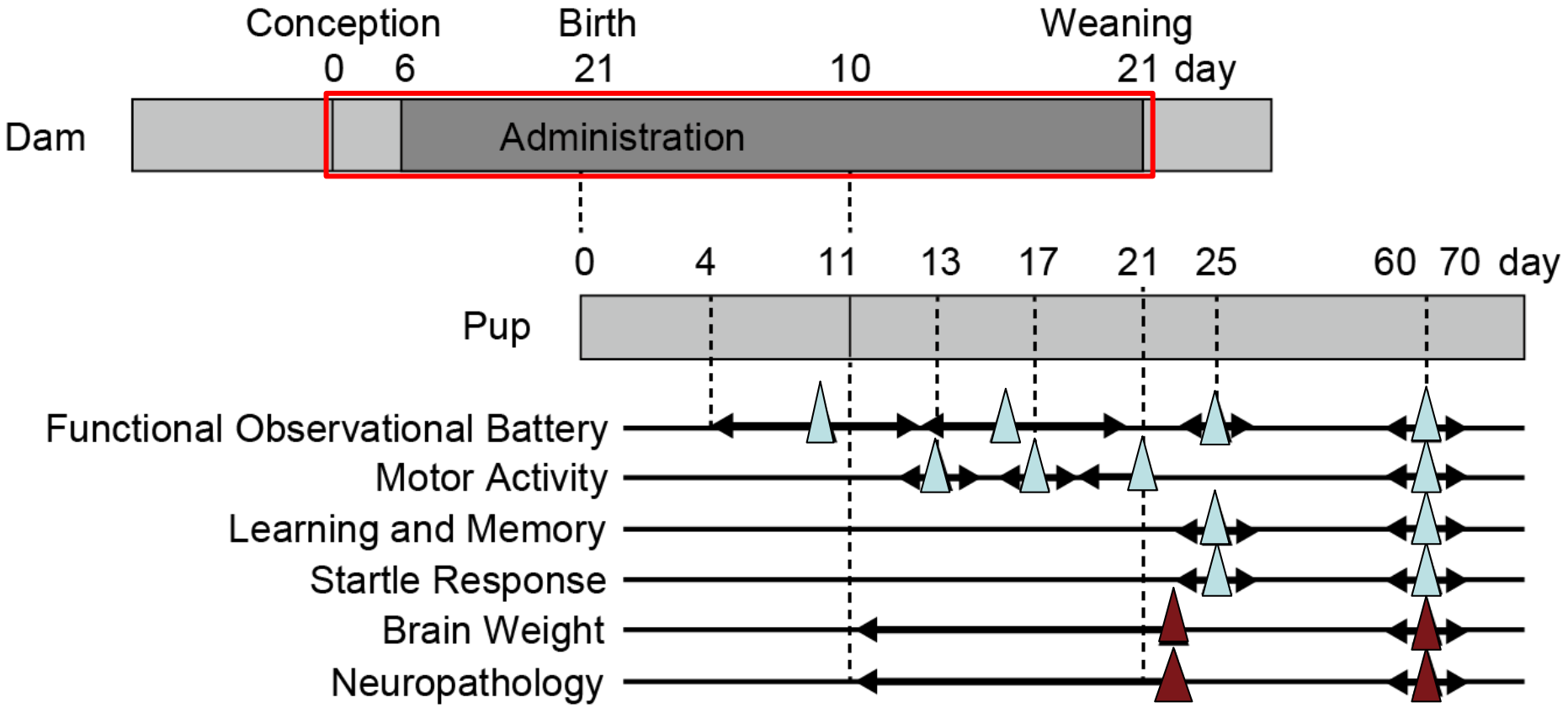
1. The order of tests: from “less stressful” to ”stressful”
2. At least one day apart between tests
3. Animals are handled one by one, which may be stressful to animals.

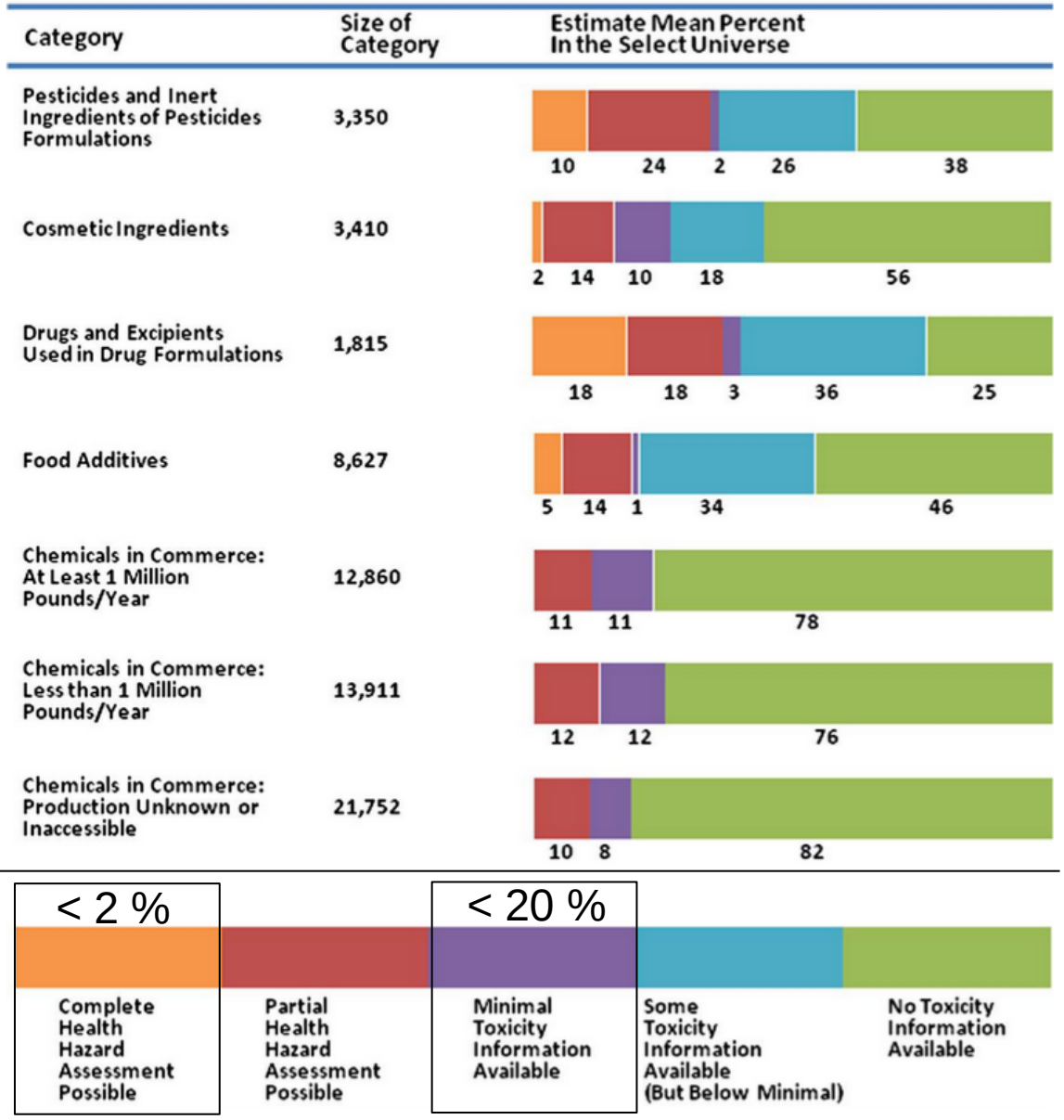
The OECD Guidelines for Testing Chemicals: TG426 Developmental Neurotoxicity (2007)



Number of Pups: 20 (animals) x 2 (sex) x 4 (doses) x 2 (time points) =320
Number of Dams: at least 20

Available online, <www.oecd-library.org>

Summary of the Data Available for conducting Health-Hazard Assessment of Chemicals



(Crofton K et al., Cong Anomal. 2012; Adapted and Modified

Estimated Period and Cost of guideline-based DNT tests for a chemical :

1.5 yr
US $100 million

High cost and nonmandatory requirement from the regulatory demand do not encourage to perform DNT tests
(Makris SL et al., Environ Health Perspect, 2009)

Prerequisite Toxicity Test Data at the Time of Application to Registration of New Pesticides (No prerequisite of DNT)

Acute toxicity

- Oral (rats, mice)
- Dermal (rats, mice)
- Inhalation (rats)
- Skin stimulation (rabbits, guinea pigs)
- Eye stimulation (rabbits, guinea pigs)
- Skin sensitivity (guinea pigs)
- (rats)
- Late-onset neurotoxicity (Chickens)

Subchronic/Chronic toxicity

- 21-day dermal (rats)
- 90-day inhalation (rats)
- 90-day neurotoxicity (rats)
- 28-day late-onset neurotoxicity (chickens)
- Subacute (90-day, rats, mice, dogs)
- Chronic (1-yr, rats, mice, dogs)
- Carcinogenicity (1.5-2-yr, rats, mice)
- Reproductive (rats)
- Developmental (Teratogenicity, rats, rabbits)
- Genotoxictiy (Mutagenicity)
- Other

Metabolism

- Fate in Animals
- Fate in plants

Pharmacology test

- Physiologic functions

Fate in the environment

- Soil
- Water

Persistency test

- Agroproducts
- Soil
- After harvest

Problems on the OCED DNT TG426

- TG426 is comprehensive but its implementation is extremely laborious.
- TG426 has not been updated since 2007, and seems to be obsolete (Beronius et al., 2012).
- DNT test data have not been utilized to derive safety standards (ADI / TDI) for various chemicals except for a few pesticides. Importantly, almost no toxicity information on pesticides is disclosed due to “proprietary right” of manufacturers.
- Uncertainties, unreliability as well as poor usability of TG426 in terms of methodology, evaluation, and regulation has been desrirbed (Beronius et al., 2012; Tsuji R and Crofton K, 2012)

“Do we really want to REACH* out to *in vitro*?” (1)

（Westerink R. H. S., NeuroToxicology, 39: 169–172, 2013)

*Registration, Evaluation, Authorisation and Restriction of Chemicals

- A use of vertebrates in animal experiments must be significantly reduced under the 3R’s policy; (Replace, Reduce and Refine the use of animals in science) in the “REACH” program enforced in EU in 2007

- Alternatives to in vivo animal tests for DNT have been proposed for screening of large numbers of chemicals, including pharmaceuticals and environmental chemicals (Crofton et al., 2011).

N.B. US EPA’s program: ToxCast™ uses "high-throughput screening assays" that determine the effects of chemicals on viable cells or purified proteins.

“Do we really want to REACH out to *in vitro*?” (2)

（Westerink R. H. S., NeuroToxicology, 39: 169–172, 2013)

Are *in vitro* tests appropriate to study higher brain function?

Pros. Neural cells and progenitors are useful for mechanistic studies

Cons. Cultured cells will NOT reflect neural function.

Annual memorial services for lab animals at research institutions in Japan





A hypothesis-driven approach using animal models is essential for DNT tests.

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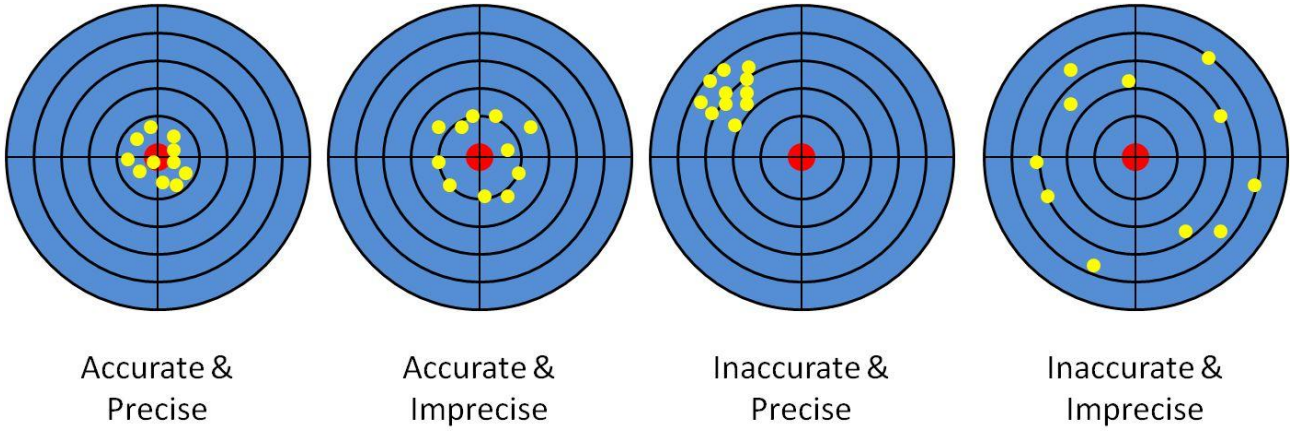
Usefulness of Behavioral Tests

- Reliability
  - Accuracy
  - Precision
  - Reproducibility
- Validity



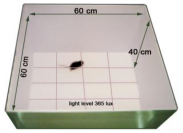
Behavioral test results should reflect the intention of a particular behavior, which is expected to be relevant to behavior in humans.

- Efficiency

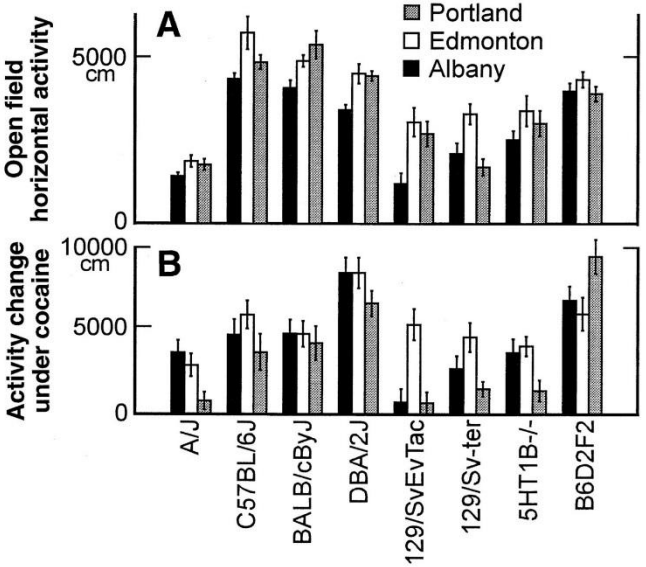
Because most behavioral tests are labor intensive, development of automated methods are needed.

A newly-developed method should be validated for its usefulness by many labs.

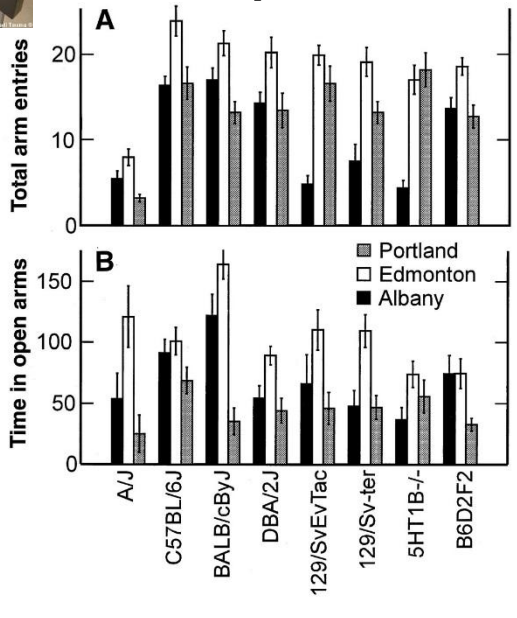
Significant Differences in Behavioral Test Results of Eight Mouse Strains Tested at Three Labs



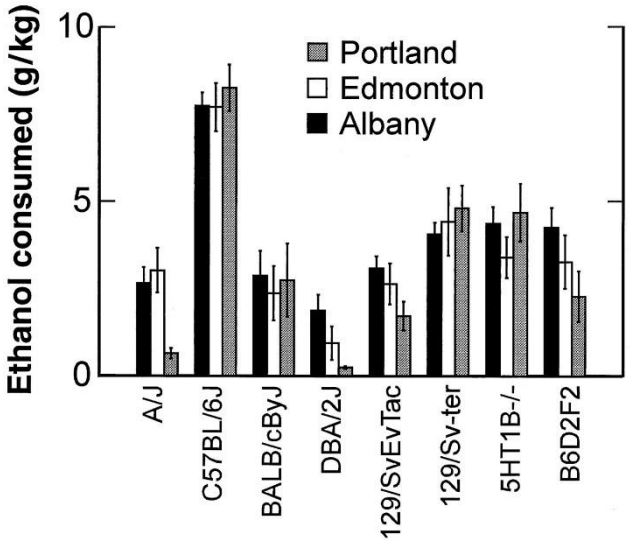
Open field



Elevated plus maze



Ethanol preference test



(Crabbe J C et al., Science, 1999)

The experimental conditions, including apparatus, test protocol, the start time and the day of a test were identical.

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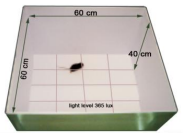
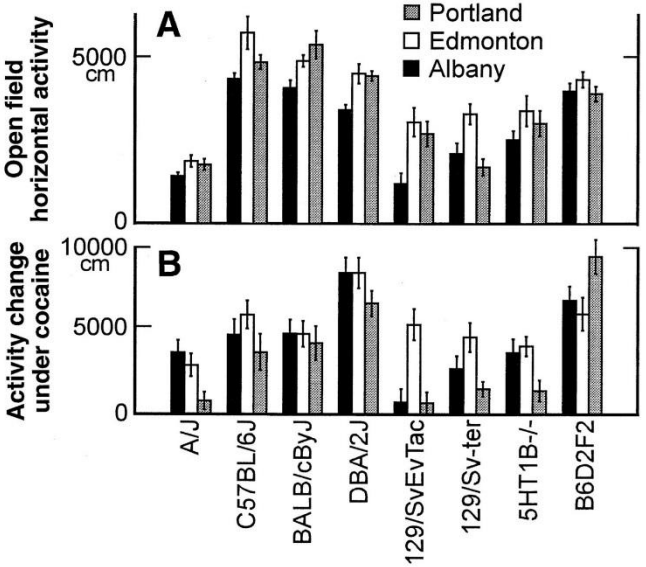
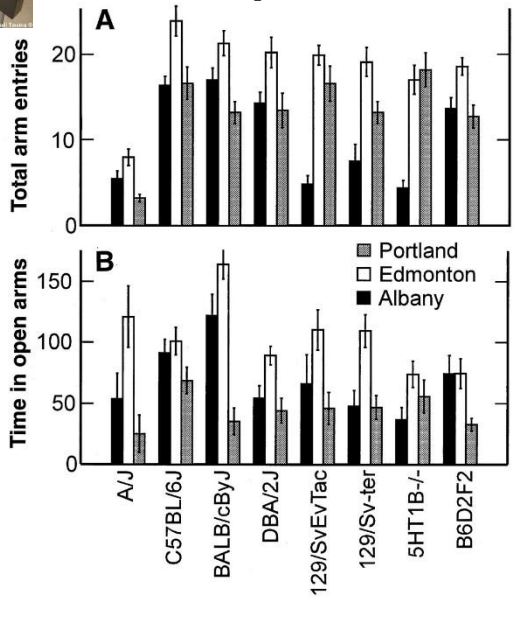


Open field



Elevated plus maze

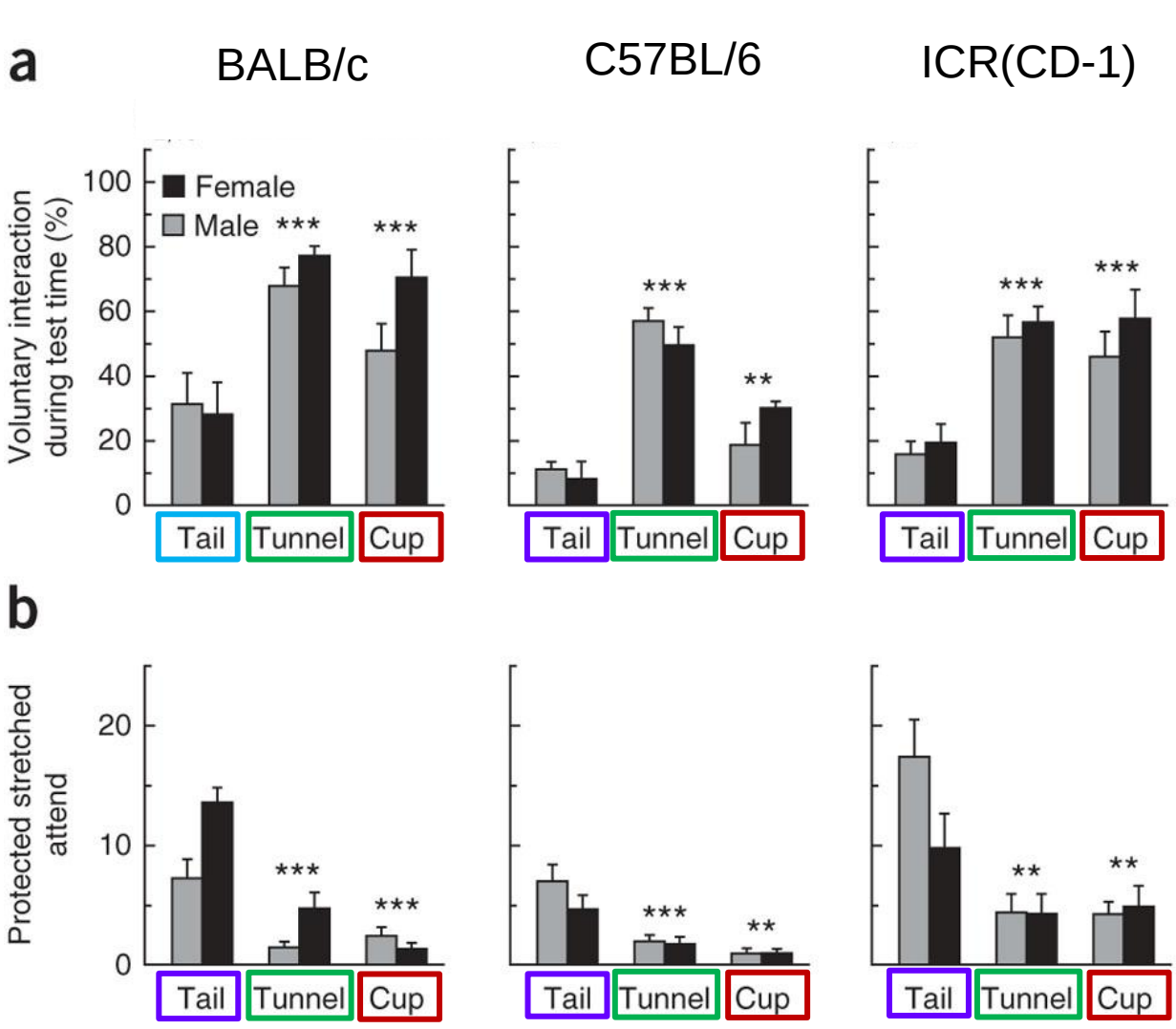


An experimenter's effect ?!

(Crabbe J C et al., Science, 1999)

A researcher at one of the three lab was allergy to rats, and wore a heavy-duty mask, which, according to the author, might be stressful to rats to examine anxiety-like behavior by elevated plus maze and open field test.

Handling Methods (Tail, Tunnel, and Cup) Affect Anxiety-like Behavior



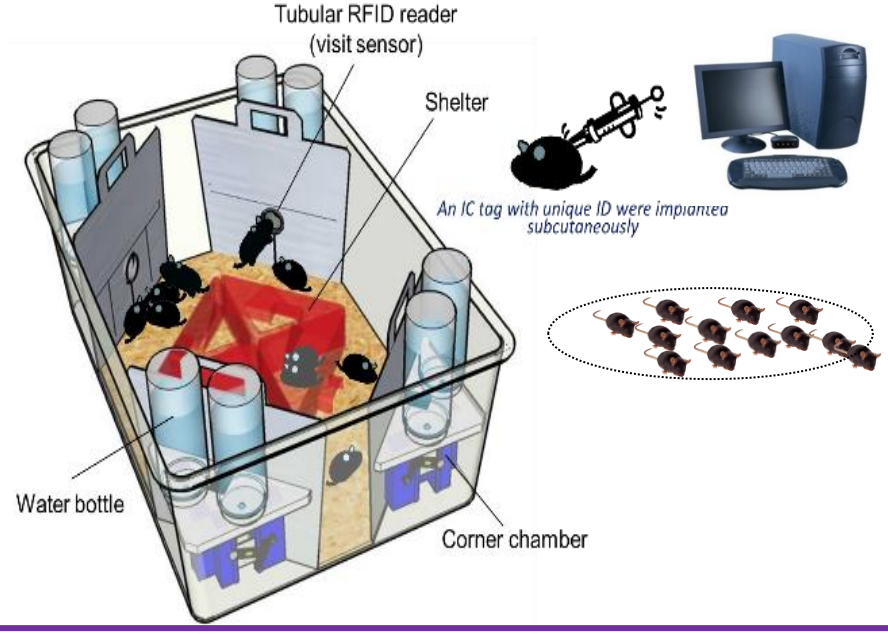
Tail suspension

Tunnel-like container (without a direct handling)

Cup (on the palm)

Hurst J L & West R S, Nature Methods 7: 825–826 (2010)

Characteristics of Automated Behavior Assay System, IntelliCage



Endo et al., Behav. Brain Res., 2011

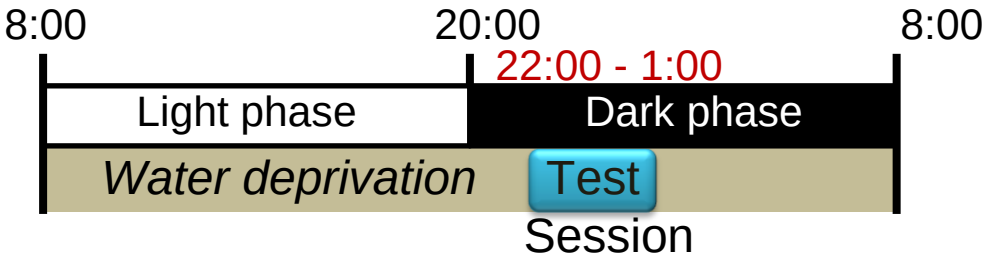
Four operant chambers at each corner

1. Less stressful home-cage testing apparatus, because experimenters do not have to touch animals by hands during behavioral tests even at the time of cleaning of the apparatus.

2. The apparatus is automatically controlled to each mouse in which an IC chip is implanted.

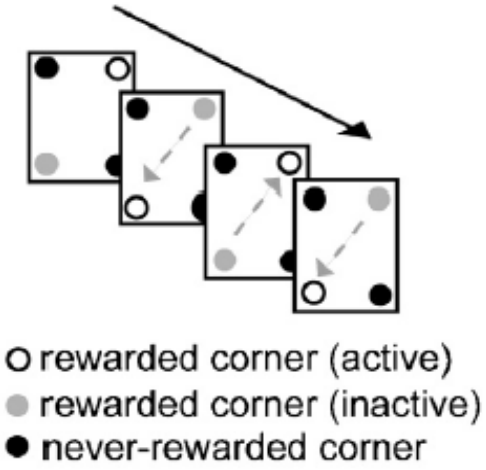
3. A maximum of 16 mice can be kept in a cage which enables to study sociality

Automated Behavior Assay System, IntelliCage

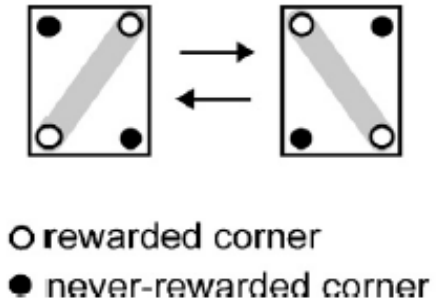


Endo et al., Behav. Brain Res., 2011

1. Behavioral sequencing task



2. Serial reversal learning



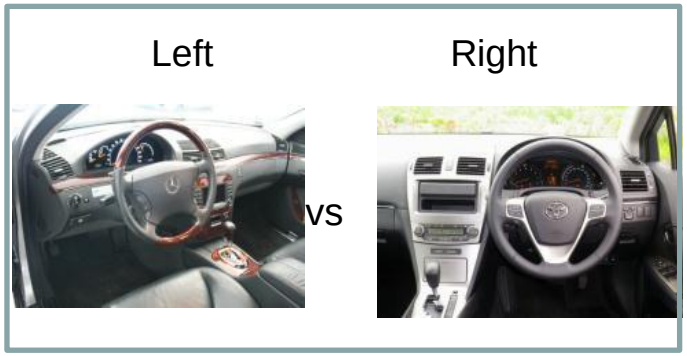
Mice can drink water by visiting one of the two rewarded corners, and must learn to alternately visit the other corner to drink water, if they drink water continuously.

The assignment of rewarded and never-rewarded corners was reversed serially.

Mice must realign their behavioral sequence

High Reproducibility of Behavioral Flexibility Test in Three Laboratories

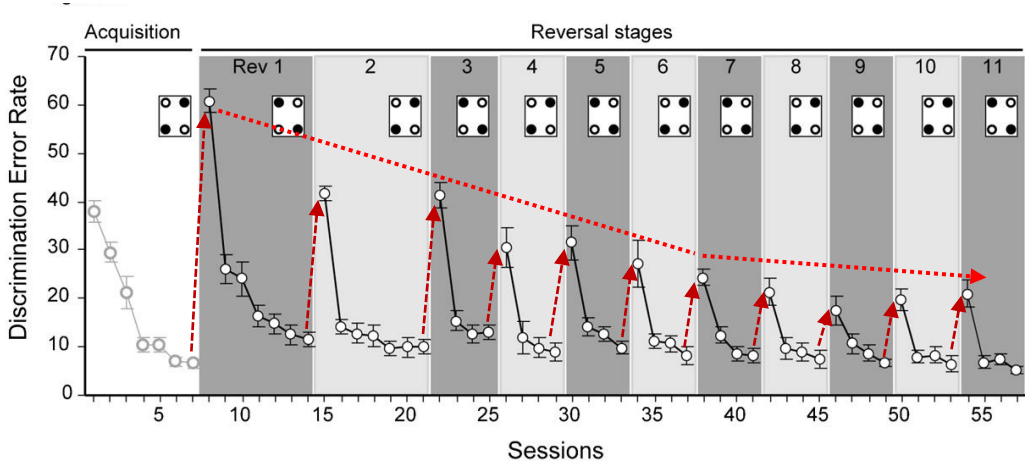
Behavioral Flexibility Determined by Serial Reversal Learning



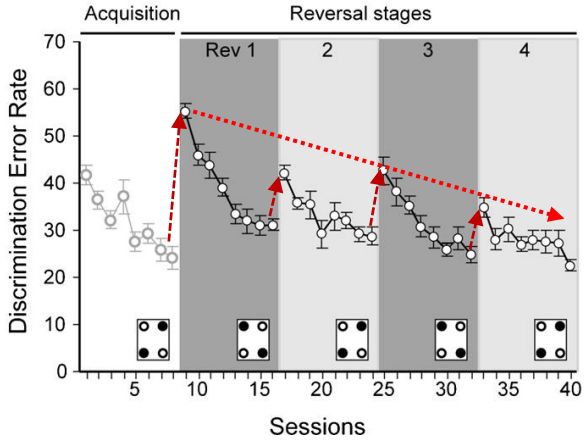
How flexibly one can adjust to a new situation by changing an already-established rule.

Very small variations for each point, and high reproducibility in three independent labs.

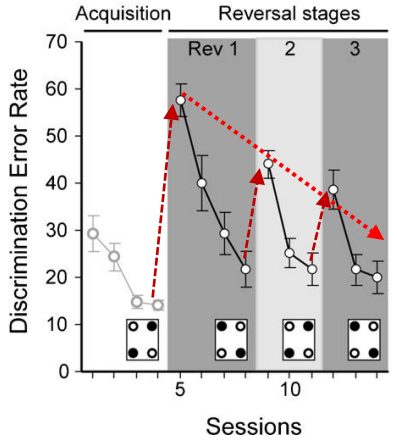
C57BL/6 @ U Tokyo



C57BL/6 @ U Zurich

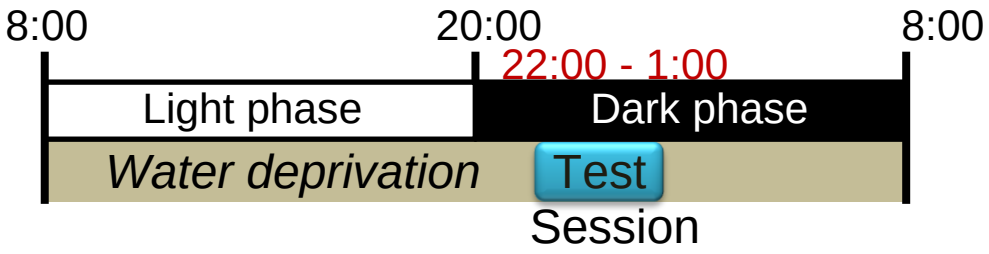


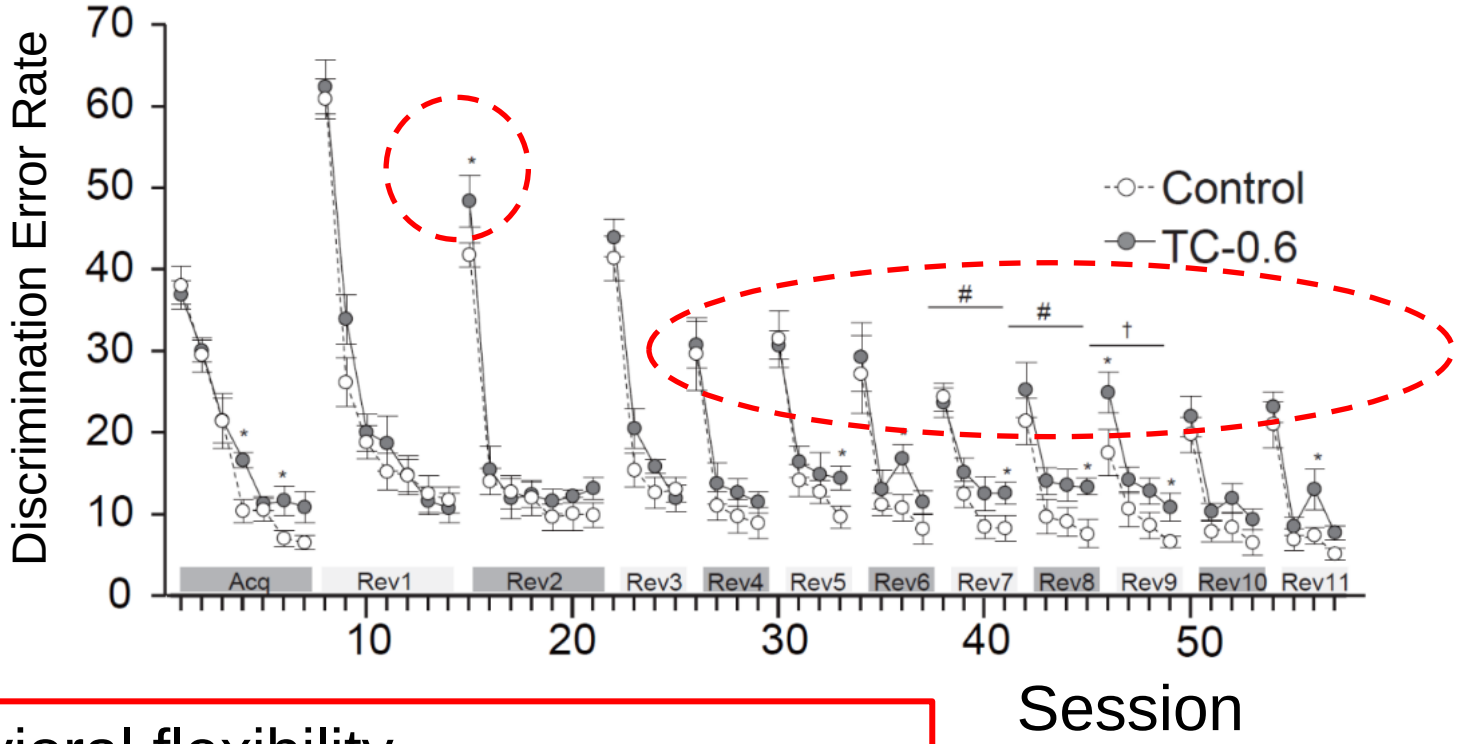
C57BL6 @Jichi Med U



Endo et al., Behav Brain Res, 2012

Detection of Impaired Behavioral Flexibility as a DNT Index



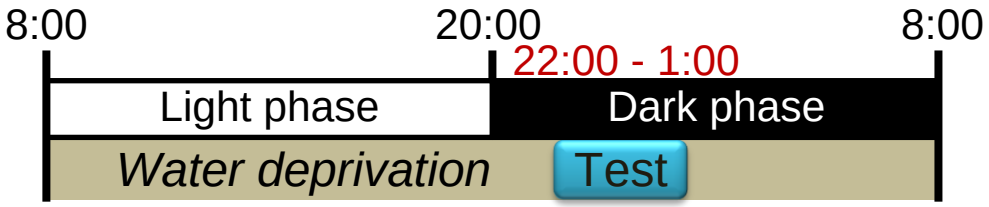


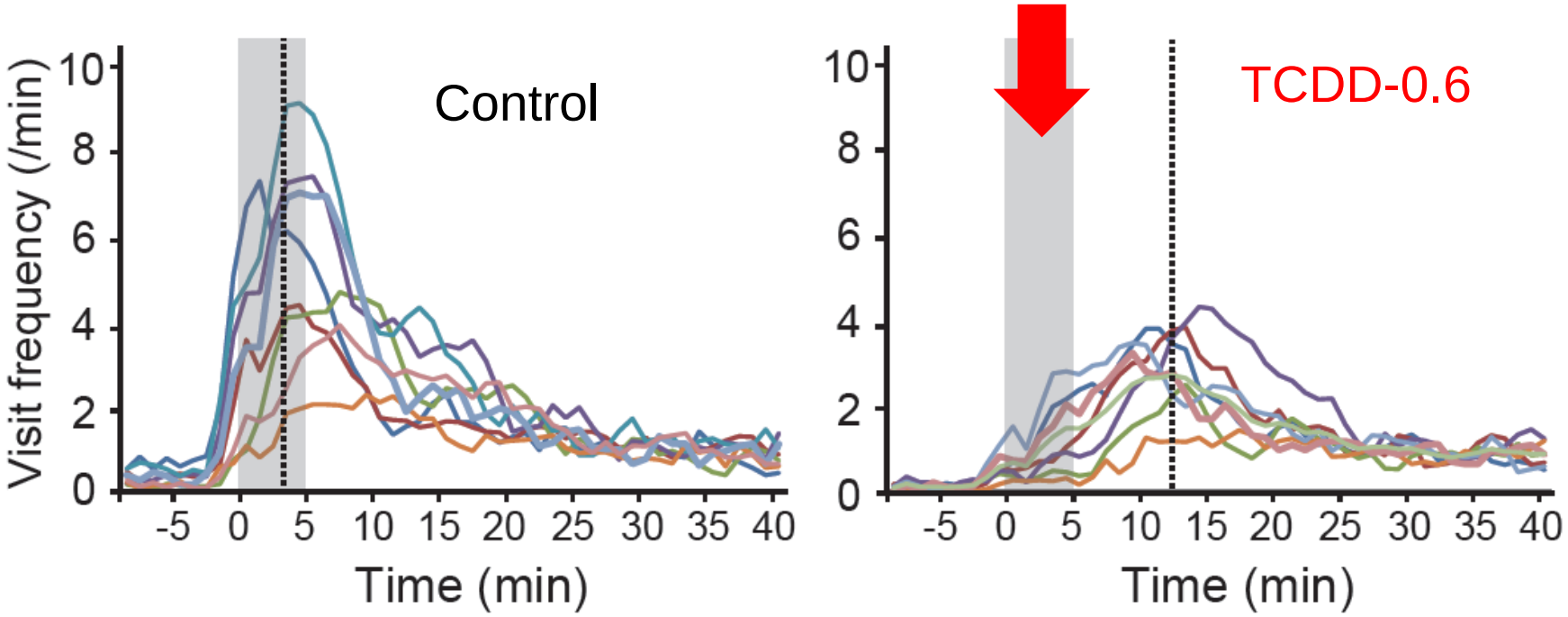
Behavioral flexibility

- With very small variations
- Throughout 57 days

Endo *et al. PLOS ONE*, 2012

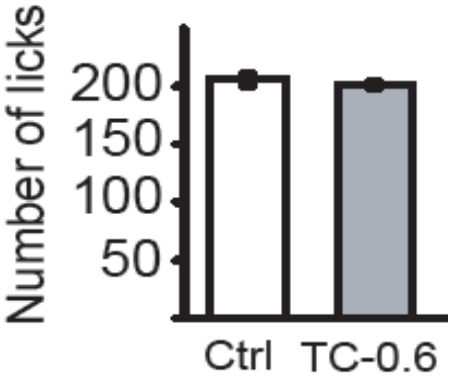
Detection of Social Dominance/Submissiveness as a DNT Index Under a Competitive Condition

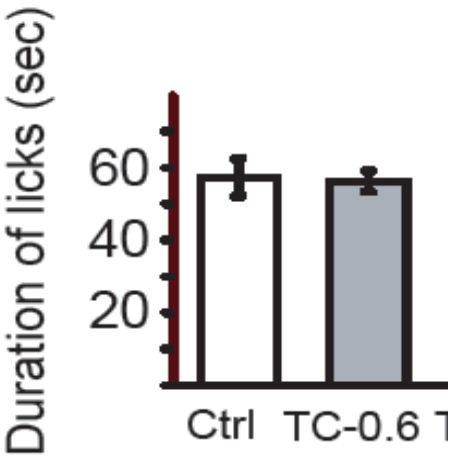


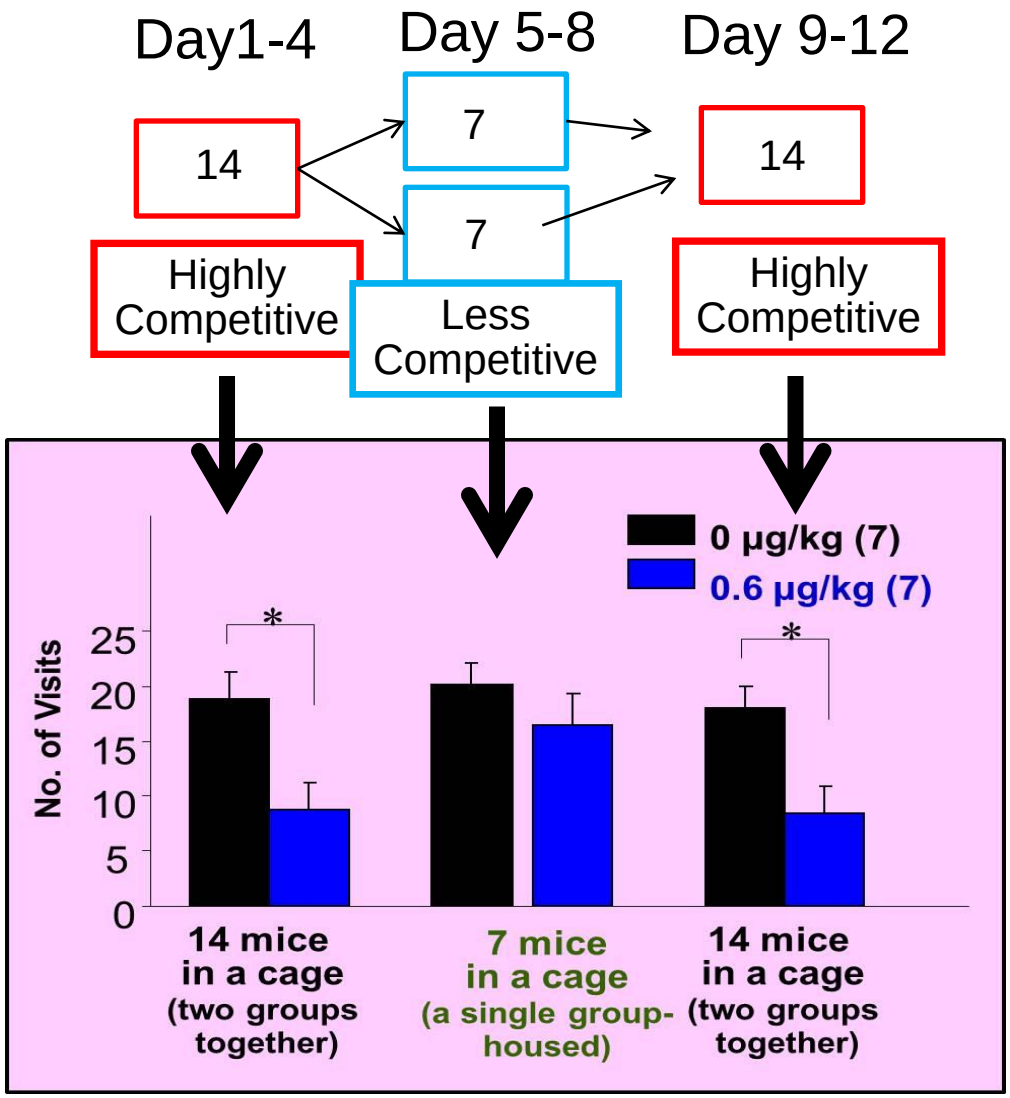


Endo *et al. PLoS ONE*, 2012

Social Submissiveness of TCDD-Exposed Mice Was Dependent on a Competitive Condition







Endo *et al. PLoS ONE*, 2012

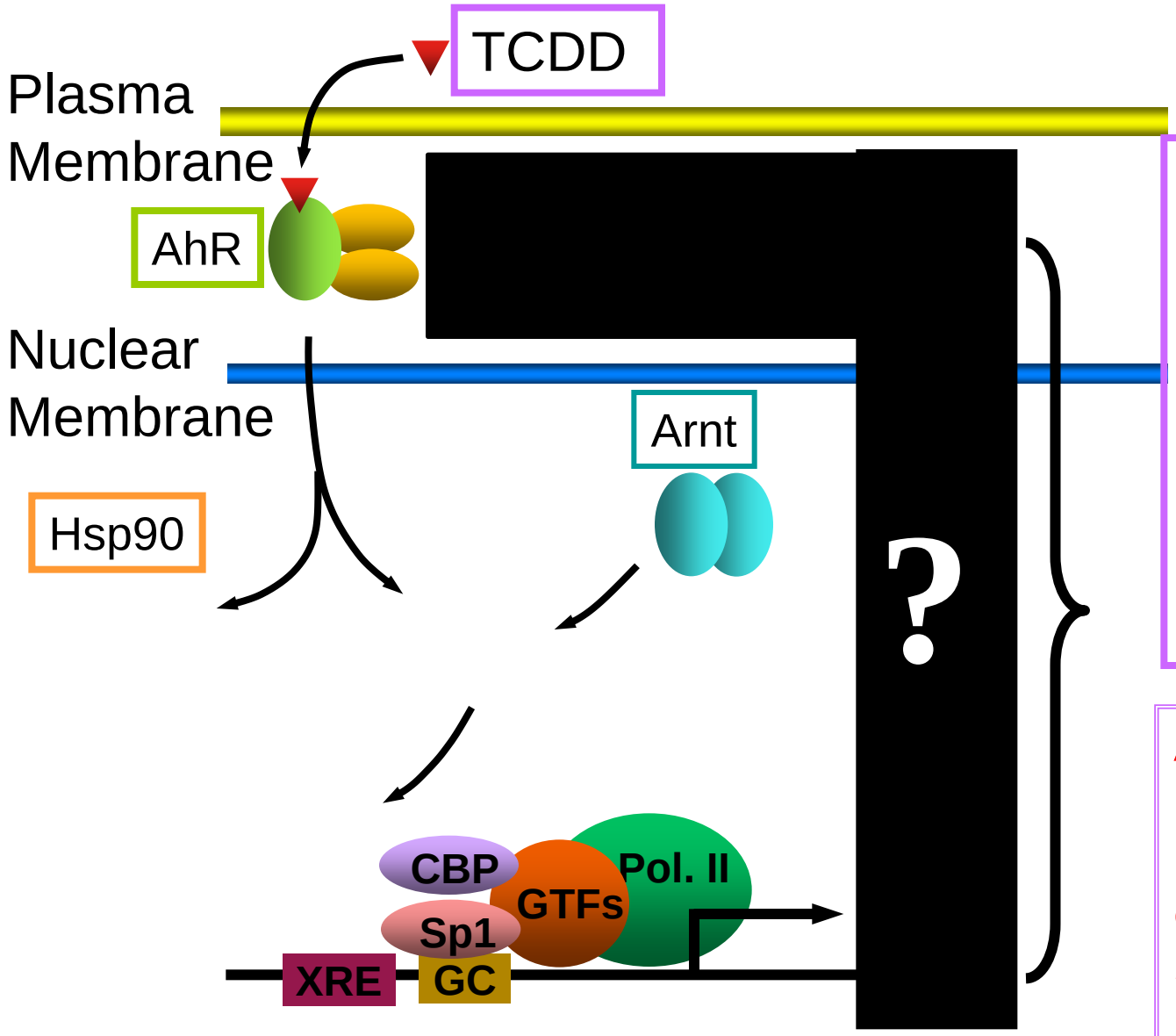
Questions to be Addressed for DNT

Once behavioral test results show developmental neurotoxicity in the tested animals, some scientifically important questions will arise.

1. What is the mechanism?
2. When does DNT occur?
3. How long does DNT last?
4. Do abnormal phenotypes change with time?

However, in the viewpoint of risk assessment, it is extremely important to note DNT phenotypes induced by low-dose chemical exposure are likely to be overlooked by histological staining or biochemical analysis of the whole brain, as described in OECD TG426.

Aryl Hydrocarbon Receptor (AhR) Mediated Toxicity



Toxicity

Cancer

Immune suppression

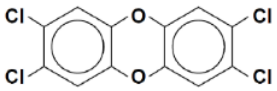
Cleft palate
Hydronephrosis

Wasting syndrome/Death

Abnormality in higher brain function (Learning/memory, emotion, and Sociality)

Importance of Hypothesis-Driven Approach:
Micro-Morphologic Analysis Reveals Abnormality in Dendritic Development in AhR Signal-Activated Mice

TCDD exposure



Kimura et al., Neurotox Teratol, 2016

Activation of AhR signals

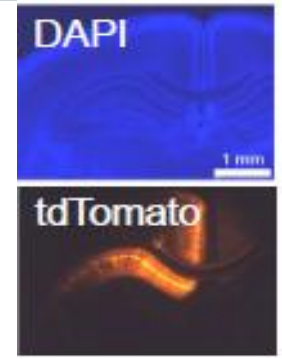
Abnormal dendritic growth

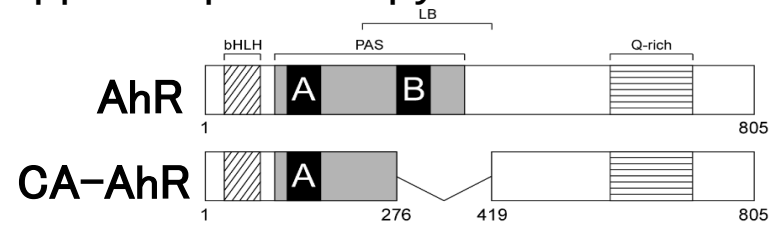
Abnormalities in Cognitive Function and Sociality

Hypothesis:
There are molecular or morphologic disruptions which cannot be detected by methods recommended for use by toxicity test guidelines.

Constitutive Activation of AhR Signaling Disrupted Dendritic Growth in Mice

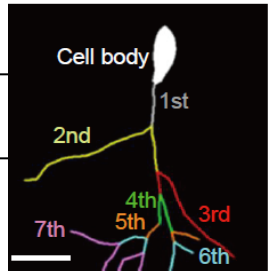
Expression of CA-AhR in hippocampal CA1 pyramidal neurons

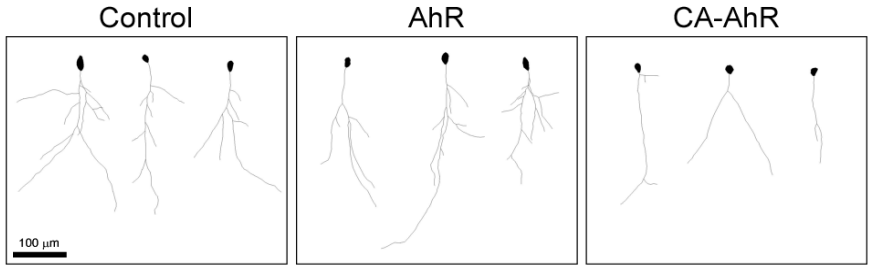


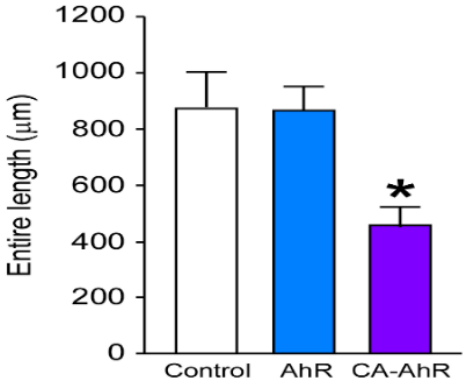


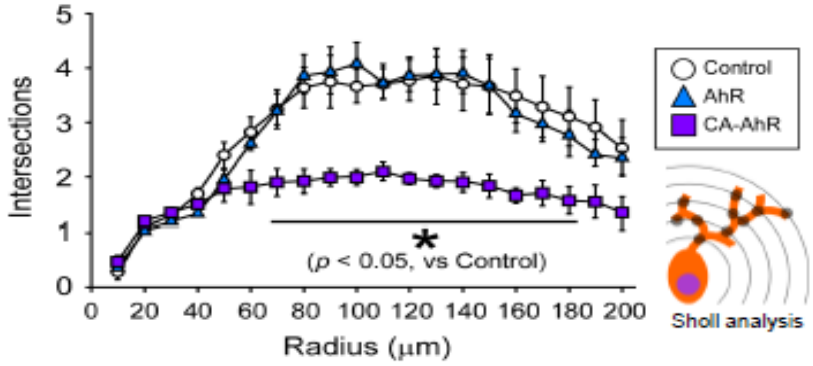
Constitutive Activation of AhR Signals

Abnormal Dendritic Growth









Kimura et al., Neurotox Teratol, 2016

Importance of Hypothesis-Driven Approach:
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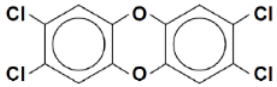


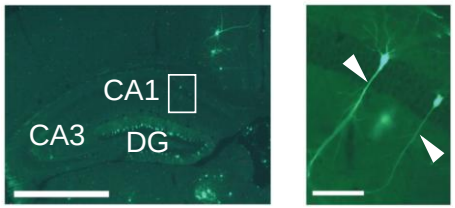
Kimura et al., Neurotox Teratol, 2016

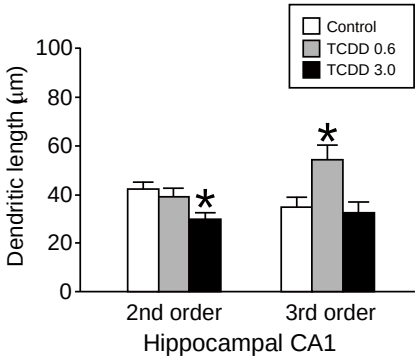
Activation of AhR signals

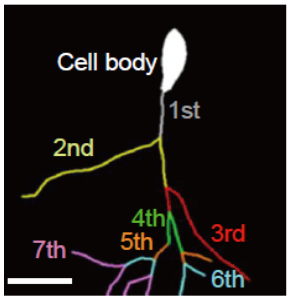
Abnormal dendritic growth

An example that may be overlooked by TG426-based histology

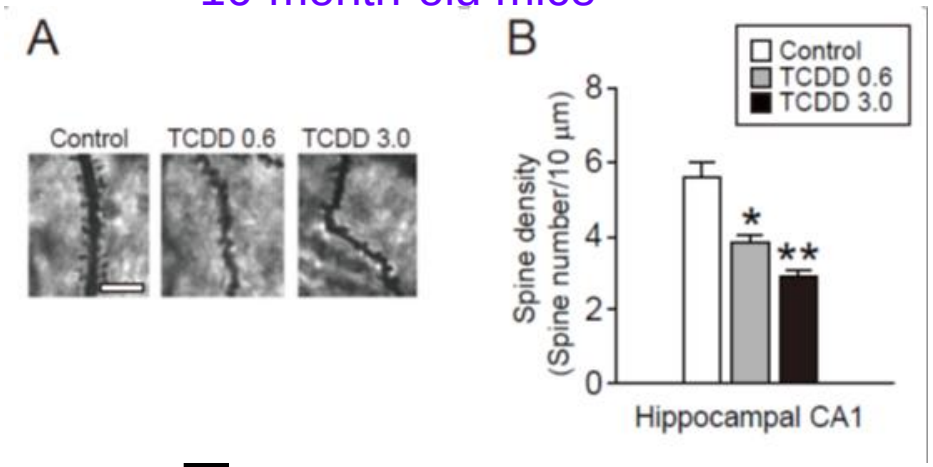
14-day-old mice







16-month-old mice



Abnormalities in Cognitive Function and Sociality

Take-Home Messages

- Extremely important not to ‘overlook’ or ‘neglect’ the possible risk of environmental chemical exposure on the developing brain

- Implementation of hypothesis-driven tests may be useful to make the DNT test guidelines more concise and realistic for actual use.

- Importance of *in vivo* behavioral tests to study cognitive and behavioral toxicity should not be compromised by a recent move towards *in vitro* tests.

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