SOT FDA Colloquia on Emerging Toxicological Science
Challenges in Food and Ingredient Safety

Developmental Neurotoxicity Testing: An Introduction to the State of the Science and Opportunities for Improvement

Charles V. Vorhees, PhD, Professor

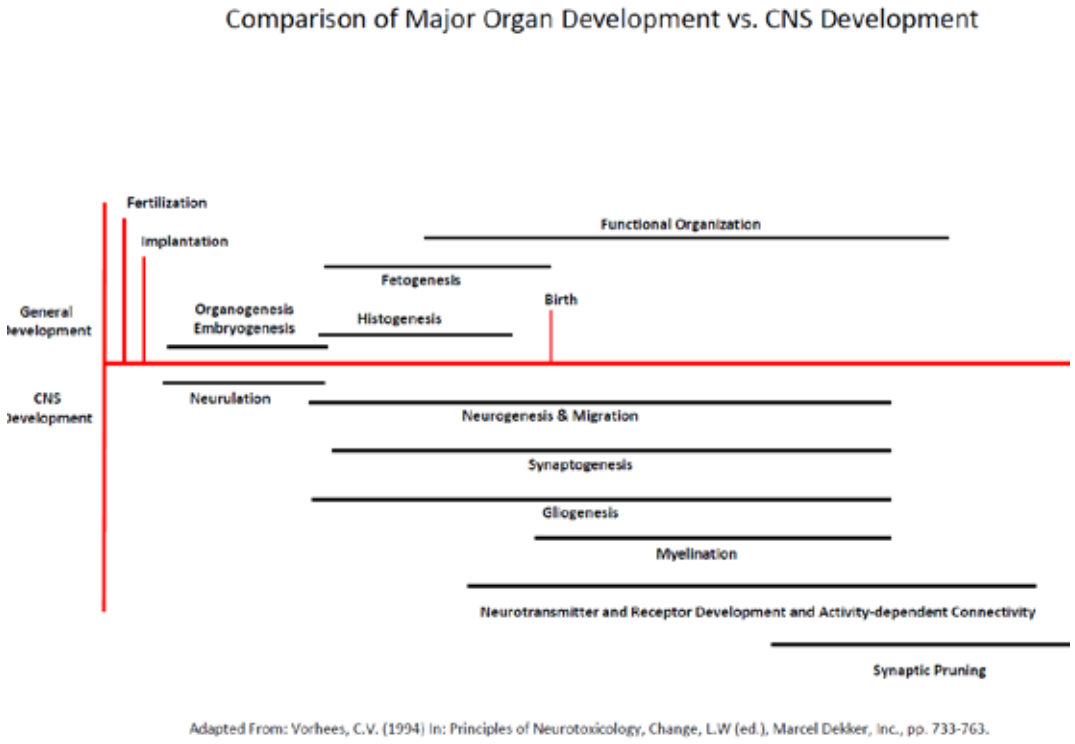
Div. of Pediatric Neurology, Cincinnati Children's Research Foundation and University of Cincinnati College of Medicine

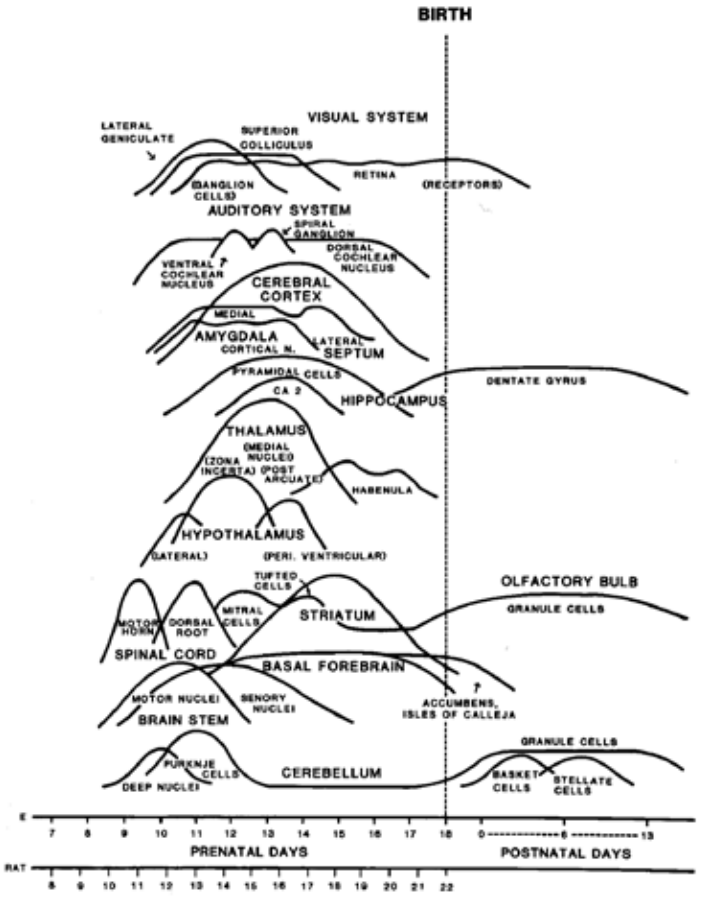
Email: charles.vorhees@cchmc.org

Conflict of Interest Statement

- I declare no conflicts of interest.

Brain Development

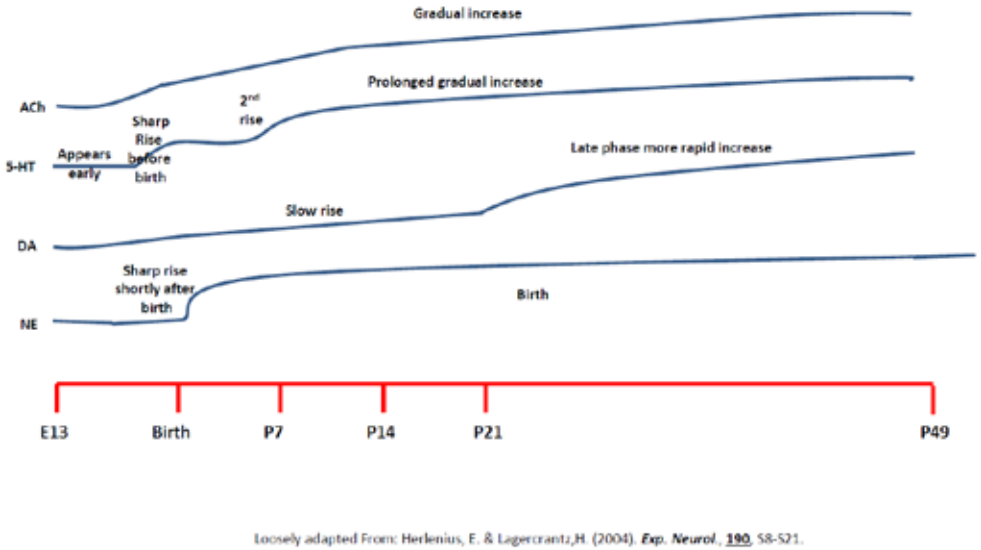




From: Rodier, P.M. (1980), ***Dev. Med. Child Neurol***., **22**, 525-545; modified by Vorhees, C.V. (1986) in Handbook of Behavioral Teratology, Riley, E.P. & Vorhees, C.V. (Eds.), Plenum Press, pp. 23-48.

SOT FDA Colloquia on Emerging Toxicological Science Challenges in Food and Ingredient Safety

Neurotransmitter ontogeny



Loosely adapted From: Herlenius, E. & Lagercrantz,H. (2004). *Exp. Neurol.*, 190, S8-S21.

Translatingtime.net

- Human gestation 270 d (38.5 wks)
- 1st trimester 0-13 wks
- 2nd trimester 13-26 wks
- 3rd trimester 26-38.5 wks
- rat gestation = 21 days
- PC = post-conception day

Neurogenesis

Human PC 8 wks translates to Rat E15
Human PC 13 wks translates to Rat **E19**
Human at PC 24 wks translates to Rat **P5**
Human PC 38 wks translates to Rat **P10-19 (no day of exact birth equivalency)**

This does not to account for:

- Apoptosis
- Synaptogenesis
- Synaptic pruning
- Myelinogenesis
- And other events in brain development

Complexity of brain development creates assessment challenges that DNT was not intended to solve

How has the Complexity of CNS Development been Translated into the Design of DNT Studies?

How has the Complexity of CNS Development been Translated into the Design of DNT Studies?

A: It hasn't!

How has the Complexity of CNS Development been Translated into the Design of DNT Studies?

A: It hasn't!

DNT studies have been defaults:

- Simplistic observations
- Movement (open-field)
- Acoustic startle response (ASR)
- Rudimentary learning
- Basic memory

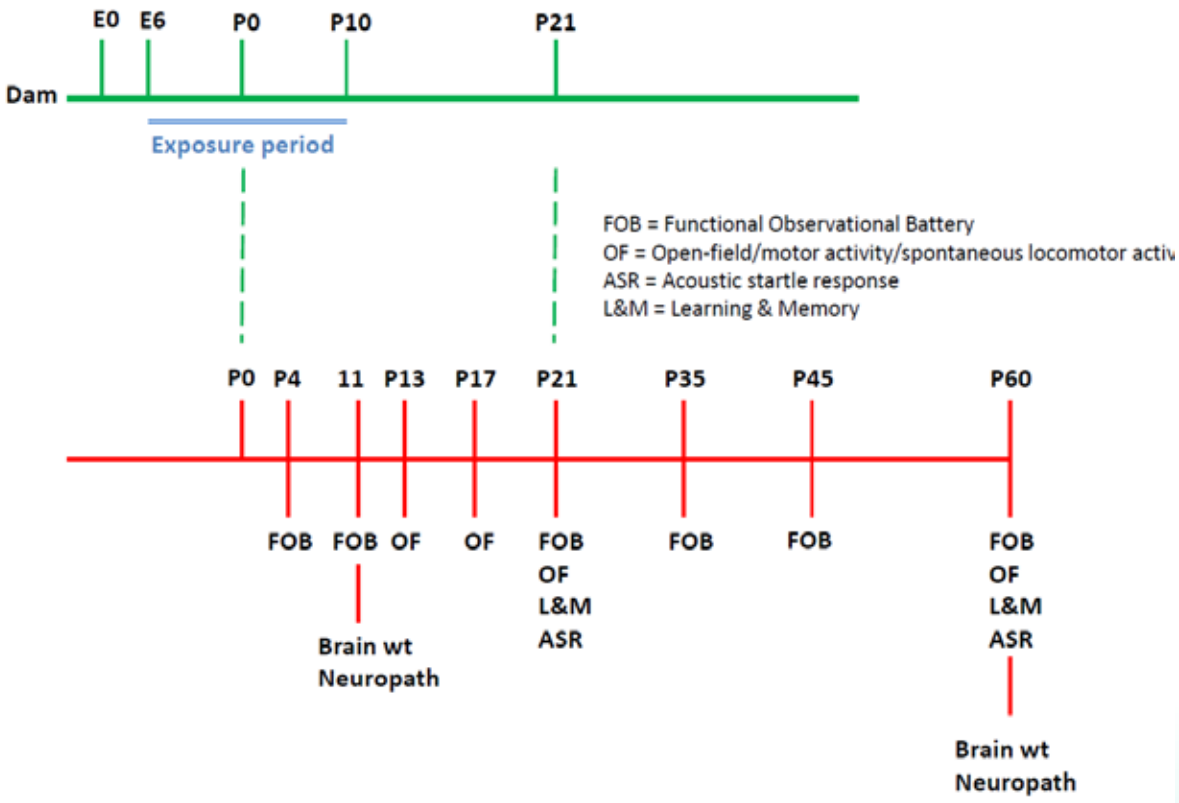
Why?

1. When written there were no methods to tackle CNS complexity; as you will hear later there are now ways to approach this.
2. Because guidelines were general (flexible) they resulted in least common denominators.

But it doesn't have to be this way!

Problem #1

- The design



EPA OPPTS 870.630 DNT Guideline

Adapted from: Tsuji & Crofton (2012). *Cong. Anom.*, 52, 122-128.

Time Points

- Why FOB at P4, 11, 21, 35, 45, 60? Who decided this? Based on what? There is no literature that supports these measures at all these ages.

Time Points

- Why FOB at P4, 11, 21, 35, 45, 60? Who decided this? Based on what? There is no literature that supports these measures at all these ages.
- Why OF at P13, 17, 21, 60? Rats don't walk well at P13. What neurotoxin has effects at P17 but not at P21 or vice versa?

Time Points

- Why FOB at P4, 11, 21, 35, 45, 60? Who decided this? Based on what? There is no literature that supports these measures at all these ages.
- Why OF at P13, 17, 21, 60? Rats don't walk well at P13. What neurotoxin has effects at P17 but not at P21 or vice versa?
- Why ASR at P21 and 60? Not unreasonable in this case but why P21; ASR is highly variable at this age.

Time Points

- Why FOB at P4, 11, 21, 35, 45, 60? Who decided this? Based on what? There is no literature that supports these measures at all these ages.
- Why OF at P13, 17, 21, 60? Rats don't walk well at P13. What neurotoxin has effects at P17 but not at P21 or vice versa?
- Why ASR at P21 and 60? Not unreasonable in this case but why P21; ASR is highly variable at this age.
- Why L&M on P21 and 60? Rats cannot learning complex tasks at P21 so little information about cognitive function is possible at this age.

Problem #2

- The tests: Why these?
  - FOB = functional observational battery
  - ASR = acoustic startle response
  - OF = open-field; locomotor/motor activity
  - L&M = learning and memory
    - Passive avoidance (PA)
    - T or M-water maze

What is the FOB: 2 Examples

Idea: Quick, simple screen

Parameters	
Posture	Biting
Convulsions/tremors	Palpebral (eyelid) closure
Feces consistency	
Ease of removal from cage	Ease of handling animal in hand
Lacrimation/chromodacryorrhea	Salivation
Pilorection	Fur appearance
Papebral closure	Respiratory rate/character
Eye prominence	Mucous membrane/eye/skin color
Red/crusty deposits	Muscle tone
Mobility	Gait
Rearing	Arousal
Convulsions/tremors	Urination
Grooming	Defecation
Bizarre/stereotypic behavior	Gait score
Time to first step (s)	Backing
Approach response	Touch response
Startle response	Tail Pinch response
Pupil response	Eyeblink response
Forelimb response	Hindlimb response
Air-righting response	Olfactory response
Hindlimb extensor strength	Grip strength: hind and fore limb
Hindlimb foot splay	Rotorod performance
Body temperature	Body weight
Catalepsy	

FOB (also called the Irwin battery)

Autonomic
- Salivation
- Lacrimation
- Urination
- Defecation
- Respiration
- Pupil reflex
- Rectal temperature

Sensorimotor
- Touch response
- Palpebral reflex
- Startle (clicker)
- Pinna reflex
- Righting reflex

Neuromuscular
- Posture
- Gait
- Straub tail
- Body tone
- Ptosis
- Exopthalmos
- Grip strength
- Traction
- Twitches
- Convulsions

Behavioral
- Arousal
- Motor activity
- Vocalization
- Aggression
- Sniffing
- Grooming
- Scratching
- Rearing
- Stereotypy
- Bizarre Behavior
- Activity

What's in the FOB: 2 Examples

Parameters	
Posture	Biting
Convulsions/tremors	Palpebral (eyelid) closure
Feces consistency	
Ease of removal from cage	Ease of handling animal in hand
Lacrimation/chromodacryorrhea	Salivation
Piloerection	Fur appearance
Palpebral closure	Respiratory rate/character
Eye prominence	Mucous membrane/eye/skin color
Red/crusty deposits	Muscle tone
Mobility	Gait
Rearing	Arousal
Convulsions/tremors	Urination
Grooming	Defecation
Bizarre/stereotypic behavior	Gait score
Time to first step (s)	Backing
Approach response	Touch response
Startle response	Tail Pinch response
Pupil response	Eyeblink response
Forelimb response	Hindlimb response
Air-righting response	Olfactory response
Hindlimb extensor strength	Grip strength: hind and fore limb
Hindlimb foot splay	Rotorod performance
Body temperature	Body weight
Catalepsy	

FOB (also called the Irwin battery)

Autonomic
- Salivation
- Lacrimation
- Urination
- Defecation
- Respiration
- Pupil reflex
- Rectal temperature

Sensorimotor
- Touch response
- Palpebral reflex
- Startle (clicker)
- Pinna reflex
- Righting reflex

Neuromuscular
- Posture
- Gait
- Straub tail
- Body tone
- Ptosis
- Exopthalmos
- Grip strength
- Traction
- Twitches
- Convulsions

Behavioral
- Arousal
- Motor activity
- Vocalization
- Aggression
- Sniffing
- Grooming
- Scratching
- Rearing
- Stereotypy
- Bizarre Behavior
- Activity

What's in the FOB: 2 Examples

Parameters	
Posture	Biting
Convulsions/tremors	Palpebral (eyelid) closure
Feces consistency	
Ease of removal from cage	Ease of handling animal in hand
Lacrimation/chromodacryorrhea	Salivation
Piloerection	Fur appearance
Papebral closure	Respiratory rate/character
Eye prominence	Mucous membrane/eye/skin color
Red/crusty deposits	Muscle tone
Mobility	Gait
Rearing	Arousal
Convulsions/tremors	Urination
Grooming	Defecation
Bizarre/stereotypic behavior	Gait score
Time to first step (s)	Backing
Approach response	Touch response
Startle response	Tail Pinch response
Pupil response	Eyeblink response
Forelimb response	Hindlimb response
Air-righting response	Olfactory response
Hindlimb extensor strength	Grip strength: hind and fore limb
Hindlimb foot splay	Rotorod performance
Body temperature	Body weight
Catalepsy	

FOB (also called the Irwin battery)

Autonomic
Salivation
Lacrimation
Urination
Defecation
Respiration
Pupil reflex
Rectal temperature

Sensorimotor
Touch response
Palpebral reflex
Startle (clicker)
Pinna reflex
Righting reflex

Neuromuscular
Posture
Gait
Straub tail
Body tone
Ptosis
Exopthalmos
Grip strength
Traction
Twitches
Convulsions

Behavioral
Arousal
Motor activity
Vocalization
Aggression
Sniffing
Grooming
Scratching
Rearing
Stereotypy
Bizarre Behavior
Activity

31 non-concordant items

Quiz #1

- Based on what you just saw: What does the FOB measure?
- What central processes underlie the tests?

None, apparently

What Kind of Data do the FOB Provide?

- Example

FOB Data Example

Table 4
Summary of FOB findings for Type I pyrethroids.

	Test substance													
	Pyrethrins		Resmethrin			S-Bioallethrin			Permethrin		Bifenthrin		Tefluthrin	
	4[a]		4[a]			2[a]			4[a]		8[a]		8[a]	
	400[b]	800[b]	350[b]	500[b]	750[b]	150[b]	200[b]	300[b]	200[b]	250[b]	40[b]	55[b]	10[b]	15[b]
Home cage observations														
Sitting, head held low				1		1 (4)	2 (4)				1			
Flattened, limbs may be extended		1		1	1			1				1		
Rearing				2					1	1	1	2	1	
Splayed hindlimbs				1	1			2		1	1 (1)	2 (1)	1 (1)	2 (1)
Clonic convulsions (repetitive movement of mouth/jaw)			1 (1)		3 (1)		2			1	1	3		
Clonic convulsions (back twitches)	1		3	5	5	2	1				3	5		2
Clonic convulsions (head/body twitches) myoclonus		1	1	4	6		3	6	2	2	1	2	1	1
Clonic convulsions (irregular jerking, limbs)		1		3	1		1	2	2	4	3	2		2
Clonic convulsions (whole body)		1		3	2		1	4	1	5	1	4		
Slight tremors	1	2	3	6	7	3	4	2	4	8	6	6	2	3
Moderately coarse tremors			1	1	2		1	1		1		3		
Markedly coarse tremors								2						
Extremely coarse tremors								1				1		
Biting of self										1 (1)				
Handling observations														
Salivation		1 (1)		1 (1)			2	1		1 (1)	4 (1)	2 (1)		
Ventral staining	1	1			1				1	1	3	5	1	1
Ventral wetness											1	2		
Abdominogenital wetness												1		
Slightly soiled fur											2			1
Red deposits—nose		2 (1)	1 (1)	3 (1)	3 (1)	1	2	3			6 (1)	9 (1)		
Pale mucous membranes												1		
Pale skin												2		
Pulsating eyes												4		1
Exophthalmus													1	
Moderately difficult to remove from cage	1					1			1					1
High difficulty in handling											1			
Open field observations														
Ataxia, excessive sway, rocks, lurches				1			1	4	1		1	3	1	1
Slightly impaired mobility				1			2	2			1	1	1	1
Moderately impaired mobility								2				1		
Walking on tiptoes	1 (1)	3 (1)	2 (1)			1	1	1		1	8	2	1	
Body drags, body sways, abdomen contacts surface										1		2		
Hindlimbs splayed or dragging			1					1				2	1	2
Hunched body	1	1	3	1	1	2	3	2	1	1	5	4	2	1
Gait impairment—slight							3		1	1				
Gait impairment—considerable								3						
Gait impairment—severe, cannot walk without falling								1						
Clonic convulsions (back twitches)		1		3	1			2				2		1
Clonic convulsions (head/body twitches)		2 (1)	3 (1)	9 (1)	5 (1)	3	5	7	2 (1)	4 (1)	6	7	1	4
Clonic convulsions (irregular jerking, limbs)	2						1	1	1		7	7	3	2
Clonic convulsions (whole body)				2			1	3				3		1
Slight tremors	1 (1)	3 (1)	6 (1)	3 (1)	6 (1)	6	4	3	2 (1)	1 (1)	8	4	2	3
Moderately coarse tremors			3	3	3	1	1		2	5	1	4	1	
Markedly coarse tremors		1		1	1		1	3				2		
Extremely coarse tremors				2				1						
Low arousal level							1			2				
Stereotypic behavior (head flick)														

Quiz #2

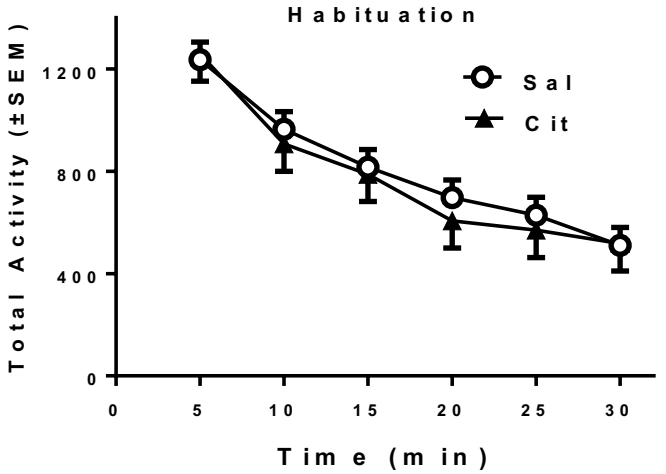
- What were the findings?

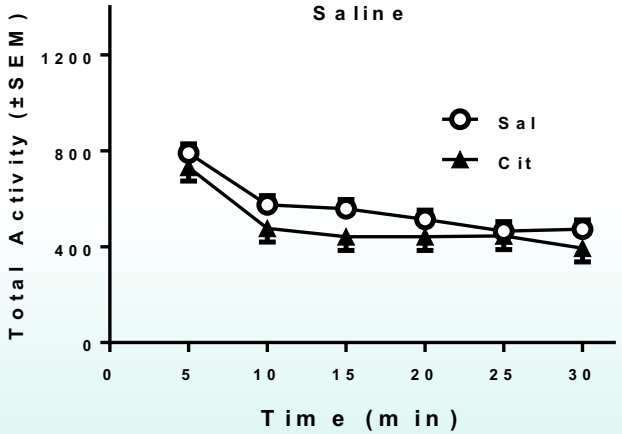
Hard to know

FOB problems:

1. Subjective; not quantitative
2. Data analysis: Usually none
3. Unrelated items; time-consuming
4. No underlying construct
5. Sensitivity (see below)
6. Reliability (?)
7. Validity (?)

Locomotor/Motor Activity, Open-Field





Sensitivity: high
Reliability: moderate
Validity: moderate

Type I errors: moderate
Apical (non-specific)

Locomotor Activity: Interlaboratory Reliability

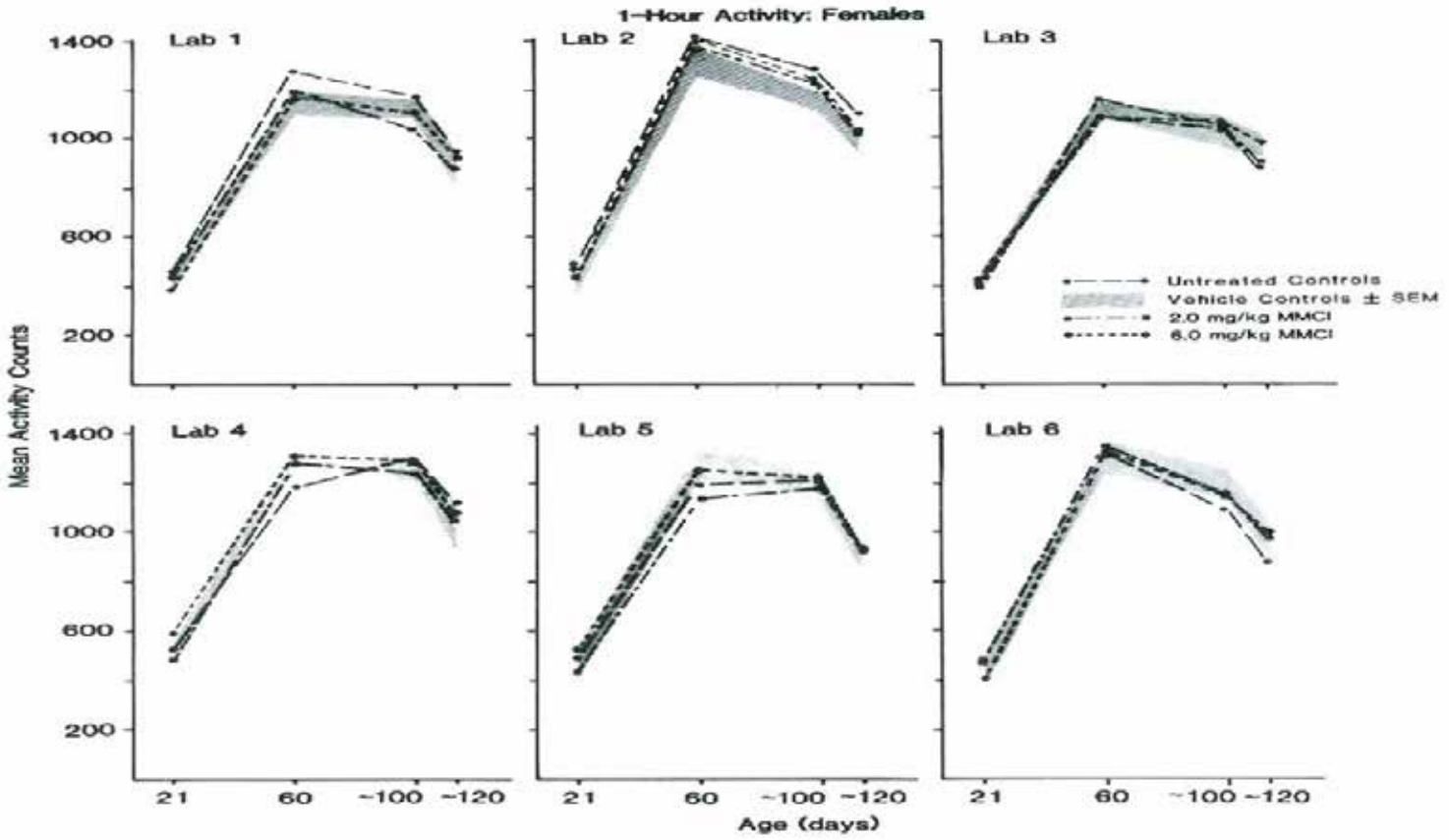


FIG. 22. Female 1-hr activity levels in the Figure-8 maze in the MMCl study at each laboratory.

Startle Reflex

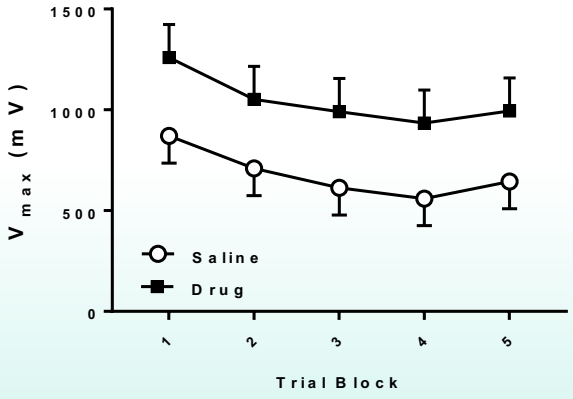
Startle apparatus

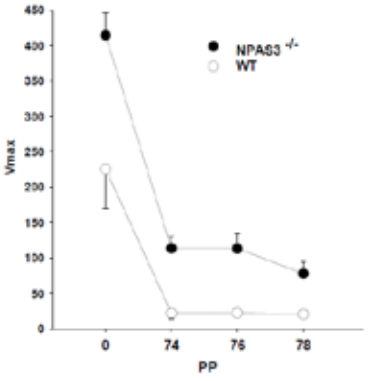
- Key words
  - PPI
  - acoustic startle response (ASR)
  - tactile startle response (TSR)

Acoustic Startle (ASR)

Validity
Construct : Yes, neural pathway known
Predictive: Human to rodent homologous response
Content/face: Yes, and quantitative
Empirical: EPA point of departure in 19% of OPPS OP studies

Tests of reflex modification
Acoustic startle
Tactile startle
Prepulse inhibition
Startle facilitation





1. Trials: 50 to 100
2. Habituation: within sessions: yes
   between sessions: minor
3. Retesting: Yes
4. Is trial-1 different? Yes
5. V_{max}, first V_{max}, V_{avg}, T_{max}

ASR Interlaboratory Reliability

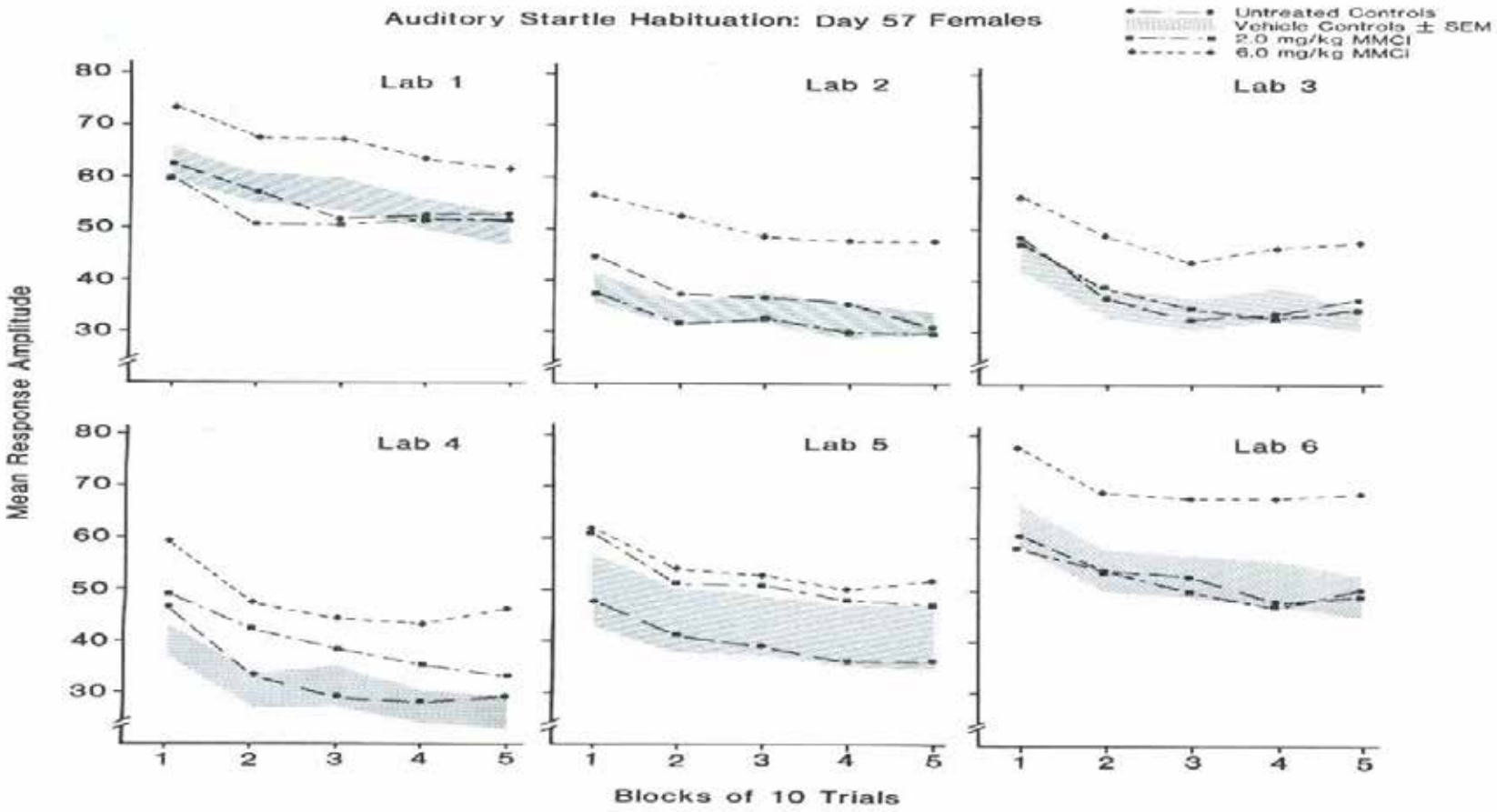
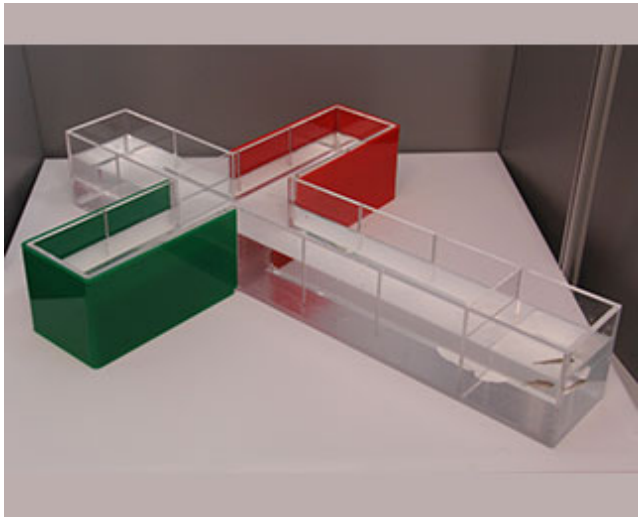
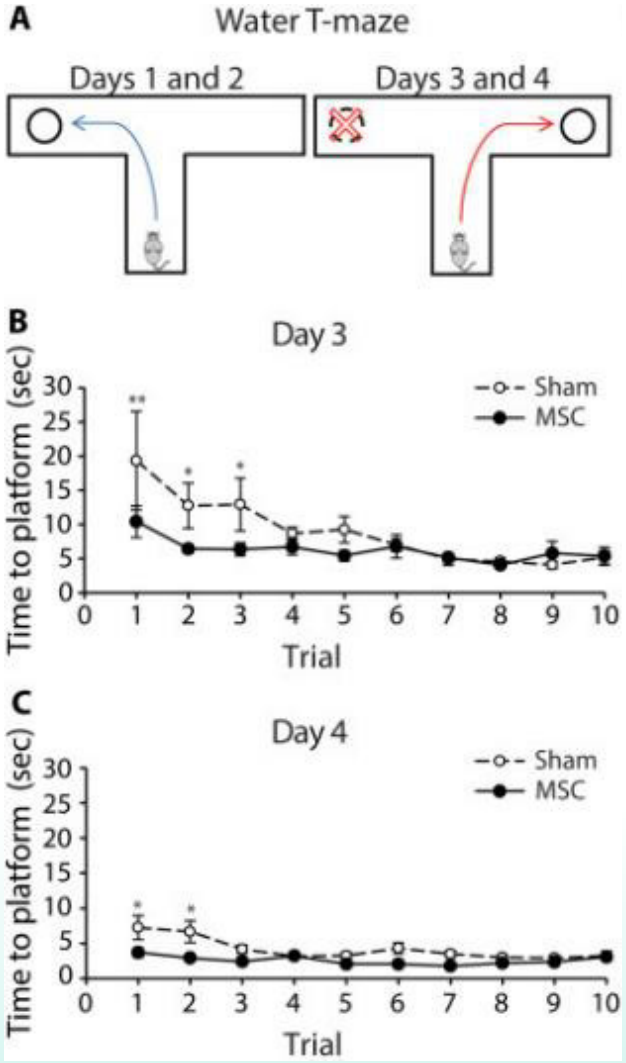


FIG. 13. PND 57 female auditory startle response amplitude at each laboratory in the MMCI study.

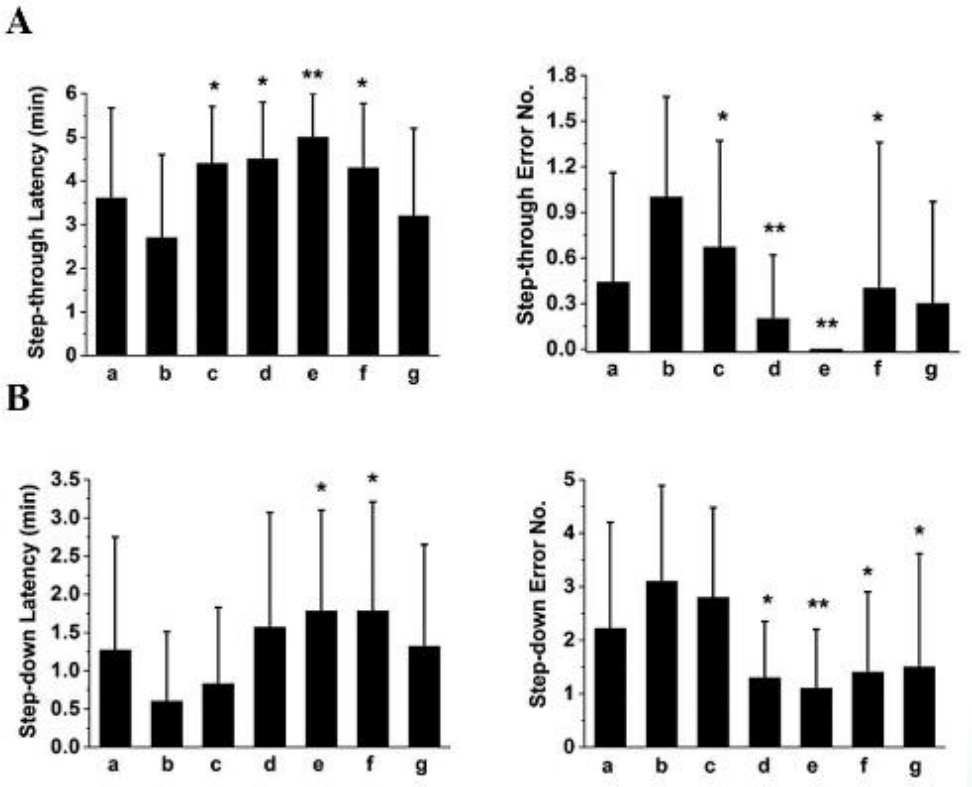
Water T-Maze



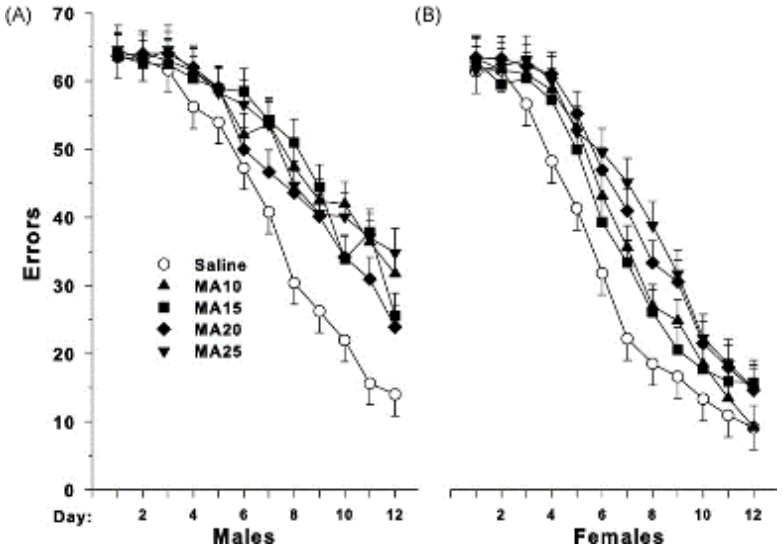


Segal-Gavish et al. (2015). Autism Res., 9(1)

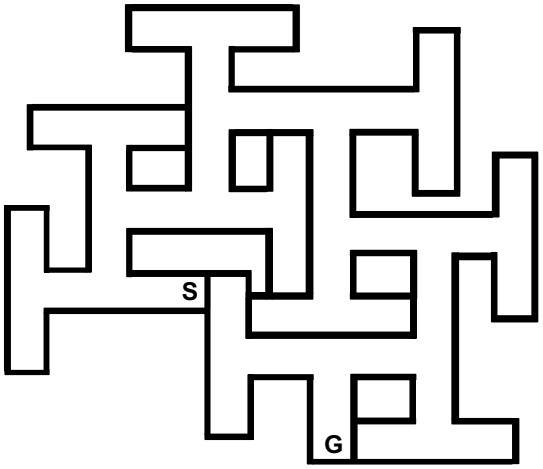
Passive Avoidance

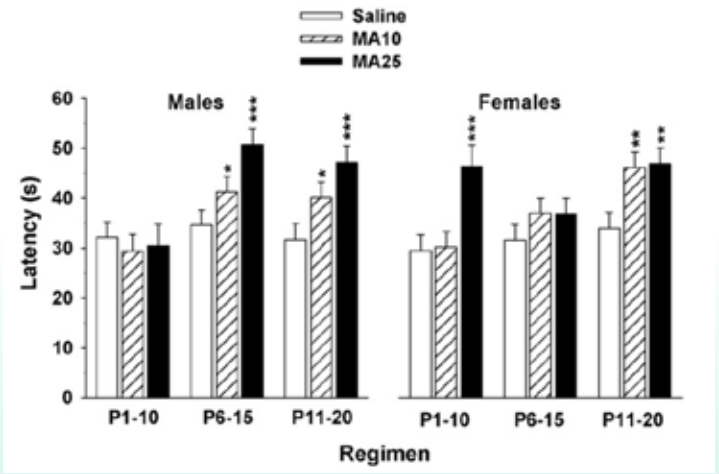


Developmental Effects of Methamphetamine

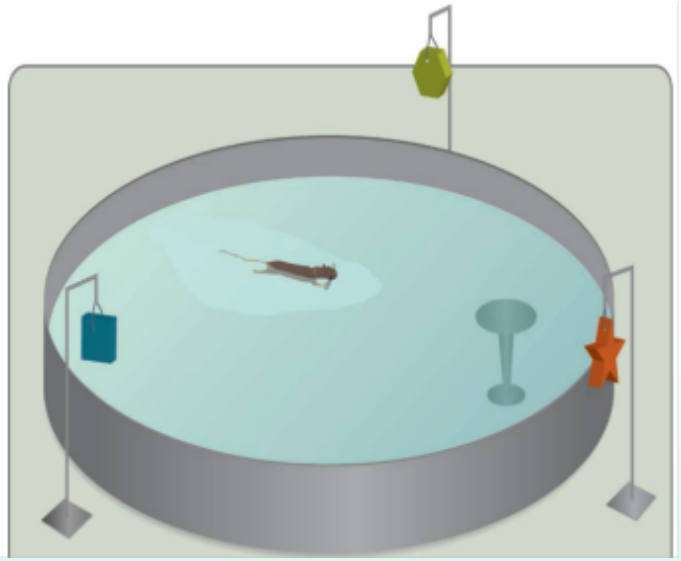


CWM





MWM

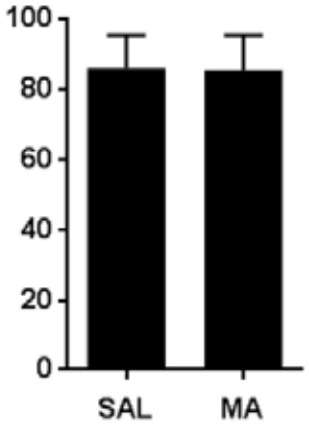


Developmental Meth and PA

Evidence that PA is insensitive

developmental methamphetamine

- ✓ Effects:
  - ✓ CWM
  - ✓ MWM
  - ✓ RWM
  - x PA No



How do these Tests Perform in EPA DNT Guideline Studies on 69 Neurotoxins?

Here's the EPA's own evaluation of DNT tests:

From: Raffaele et al. (2010). The use of developmental neurotoxicity data in pesticide assessments. *Neurotoxicol. Teratol.*, 32, 503-572.

Table 2

Data set of guideline[a] DNT studies on pesticide active ingredients

Data category	Number of studies
Submitted to OPP[b]	78
Final reviews available	72
Included in analysis	69
Regulatory impact as of 12/2008[c]	15
Potential regulatory impact[d]	13

[a]OPPTS 870.6300

[b]As of December 2008

[c]The DNT hazard value was selected as the point of departure for at least one exposure scenario in the final risk assessment.

[d]The DNT study may have a future regulatory impact, but the current regulatory decisions were finalized prior to the receipt of the DNT study for these pesticides (see text).

i.e., known neurotoxins

Number of studies where DNT provided Point of Departure for risk assessment:

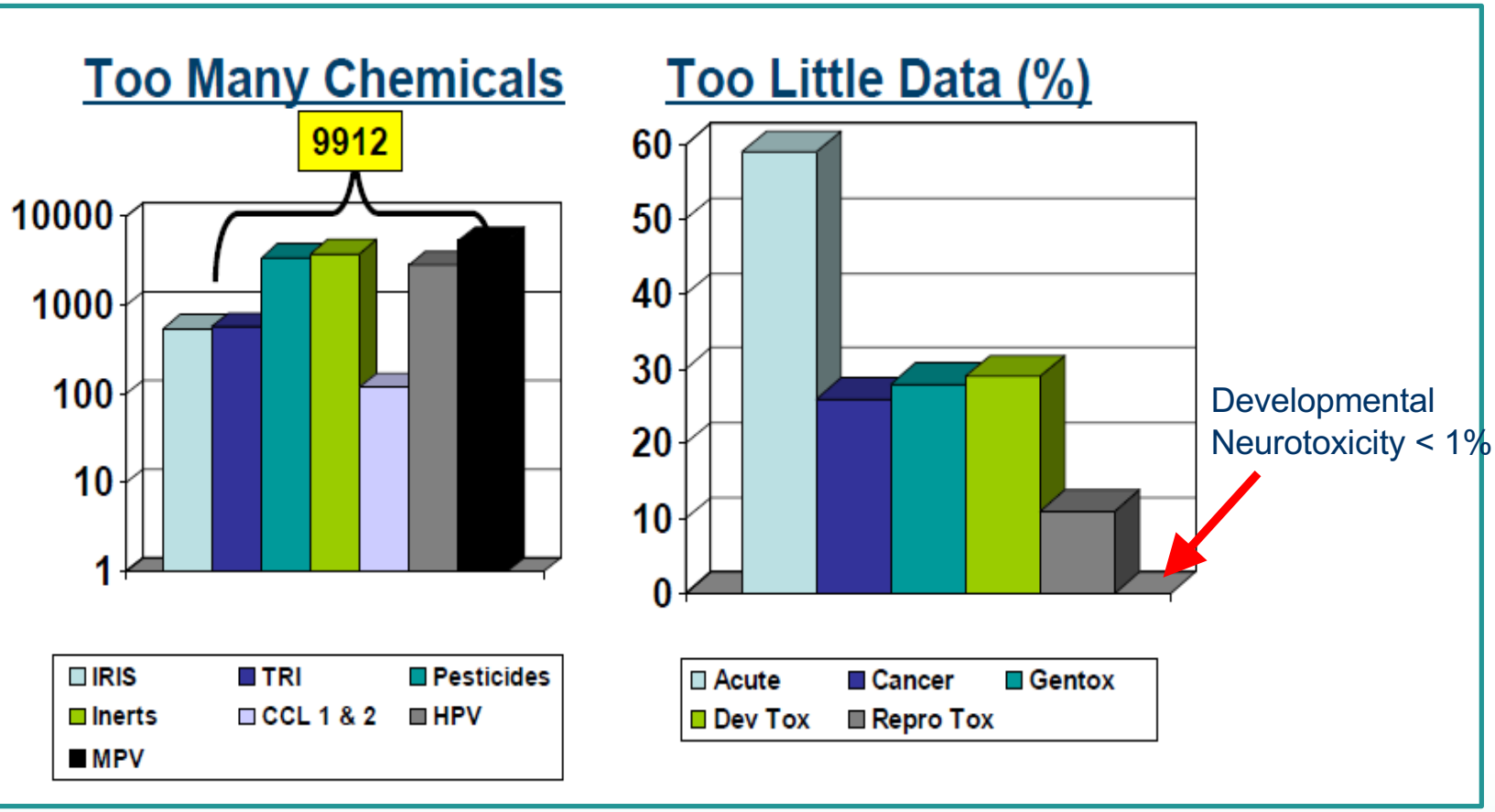
20/69 (29%) motor activity
13/69 (19%) ASR
3/69 (4%) FOB

If FOB and L&M Tests were Insensitive should They be Dropped?

- Not necessarily
- The problems are different
- FOB: Not one test; differs by lab. Needs revision
- L&M: problem is the tests; choose better tests

Why does the DNT evoke concern?

Scale of the Problem for Chemicals in Commerce



Scale of problem for pesticides: ~100

Slide courtesy of Tim Shafer, Ph.D., EPA

Scale of the Problem for FDA:

Food additives: ~2000 direct, "secondary" direct, color additives, GRAS and prior-sanctioned substances: manageable.

Drugs: ~1500; ~30/yr; quite manageable for DNT and necessary.

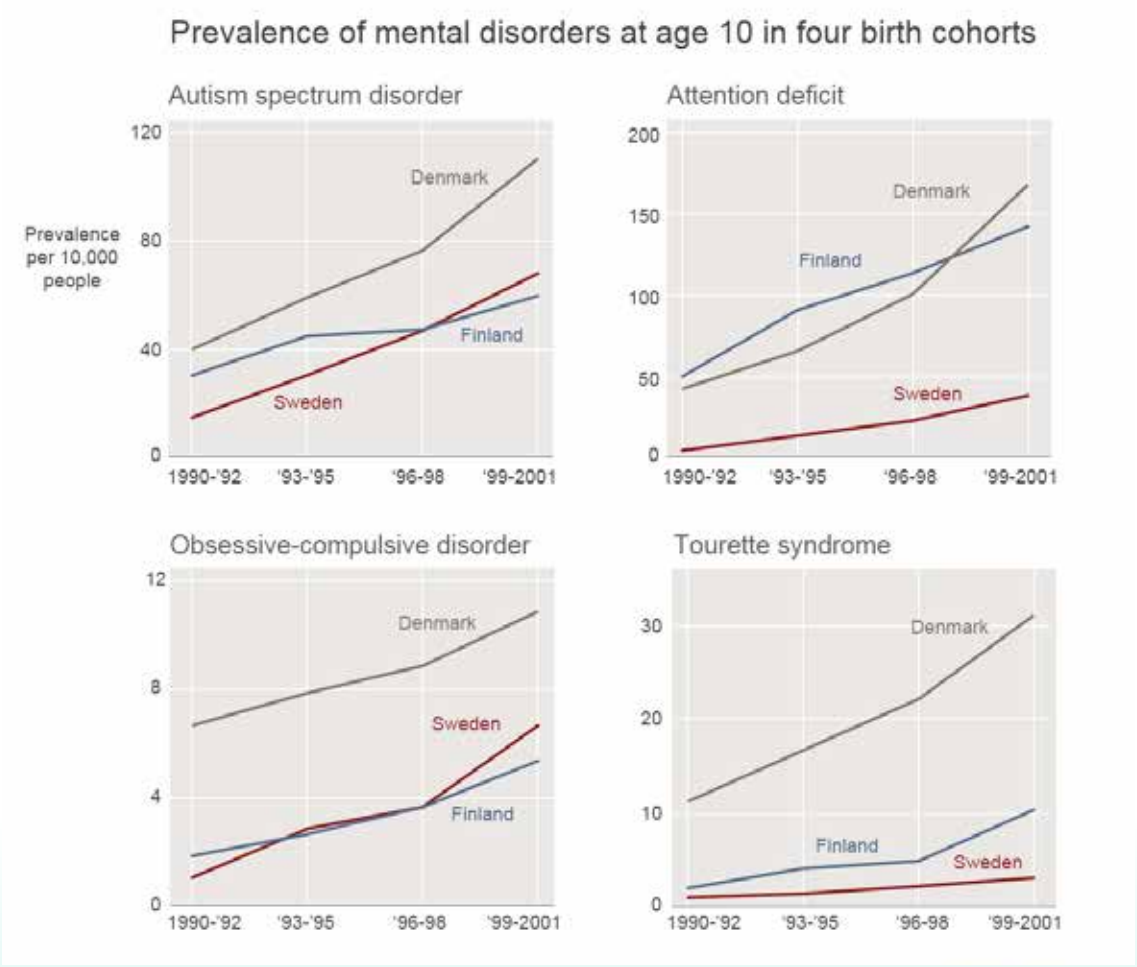
Residues: 1000's; problematic

Hence, the problem of assessing DNT across agencies and programs varies widely

Where does DNT Fit?

- FDA
  - All drugs: yes
  - Food additives: yes if prioritized
  - Residues in food: Selectively only
- EPA
  - All pesticides: yes
  - Chemicals in commerce: no
    - Need high-through-put screens, AOPs; then high priority subset for DNT confirmation

Need for DNT Studies



ASD = 1/68 (CDC); having risen steadily over 20 years

Atladottir et al., Eur Child Adolesc Psychiatry. 2015 Feb;24(2):173-83; slide courtesy of Tim Shafer, Ph.D., EPA

Is Current DNT the Best We Can Do? No

- FOB: Inconsistent content, subjective, insensitive
- Activity: pretty good
- ASR habituation: pretty good; could improved by adding PPI (would add 20 min to protocol).
- L&M: Needs major improvement; discontinue T-mazes and PA; switch to better tests (see below).

Learning: What Makes a Good Test?

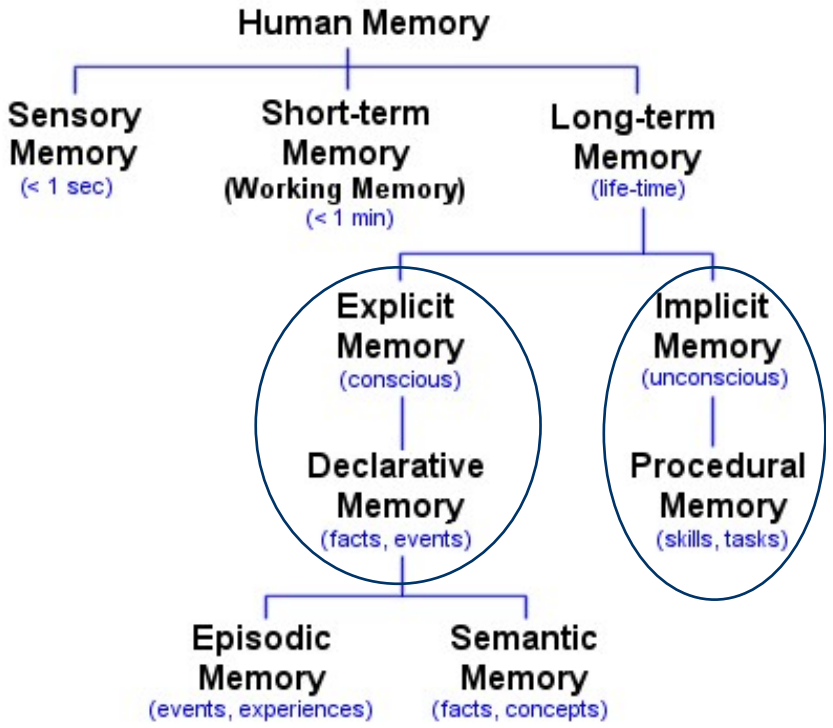
- Not too easy (steep learning curve)
- Not too hard (flat learning curve)
- Not too aversive
- Easy to motivate
- Able to equalize motivation
- Control for performance factors
- Relevant types of L&M

What Kind of L&M should be Assessed? What's Important for People?

Learning

Classical Conditioning (Pavlov)	Operant (Instrumental) Conditioning (Skinner)
Unconditioned Stimulus (food) —> Unconditioned Response (salivation)	Response (press lever) —> Stimulus (reward) (food)
Unconditioned Stimulus (food) + Conditioned Stimulus (bell) —> Unconditioned Response (salivation)	TIME
Conditioned Stimulus (bell) —> Conditioned Response (salivation)	Conditioned Response (press lever) —> ConditionedStimulus (Reward)(food)
In classical conditioning, a neutral stimulus becomes associated with a reflex. The bell, a neutral stimulus, becomes associated with the reflex of salivation.	In operant conditioning, the learner "operates" on the environment and receives a reward for certain behavior (operations). Eventually the bond between the operation (pressing the lever) and the reward stimulus (food) is established.

Memory



What are the Equivalent Forms of Memory in Rodents?

- Explicit/Declarative/Allocentric memory (people, places, things, and events) = orientation to surroundings: distal cues. Brain region = hippocampus; entorhinal cortex

- Implicit/Egocentric memory: (skills, paths, event order) = self-orientation: proximal and internal cues Brain region: striatum; presubiculum; other

How are These Assessed in Rodents?

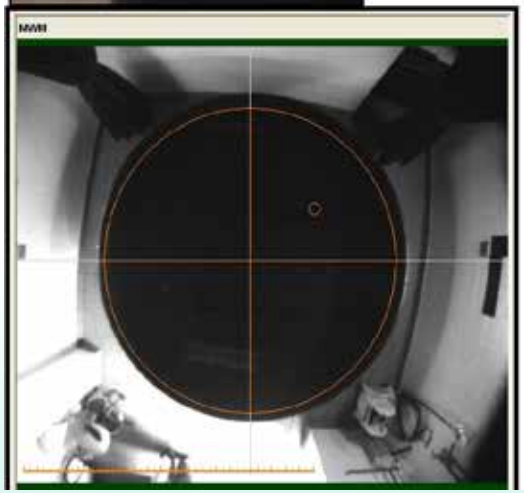
- Allocentric: Morris water maze (MWM); radial-ram maze (RAM); radial water maze (RWM); Barnes maze.
- Egocentric: Cincinnati water maze (CWM); Cued MWM, foraging maze, Wishaw method

Why Water Mazes?

Advantages of water mazes

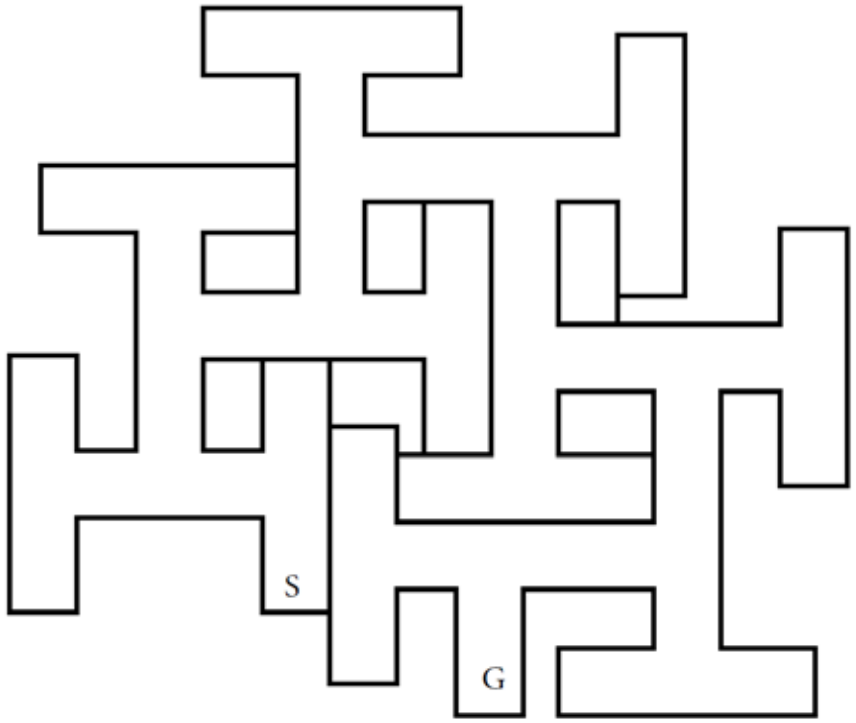
- No training (for MWM)
- Minimal training (labyrinthine mazes)
- Immune to appetite differences
- Immune to body weight differences
- Intrinsic motivation
- 100% of animals finish
- Nearly 100% master these tasks

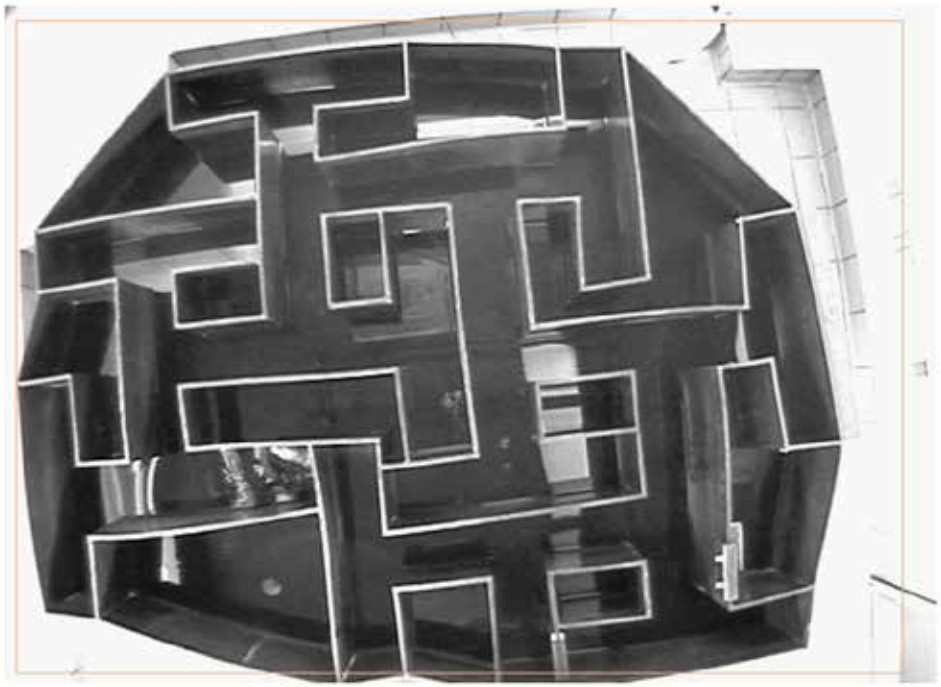
MWM



Morris water maze (MWM)

CWM





Vorhees & Williams (2016). ***Neurotoxicol. Teratol***., ePub head of print.

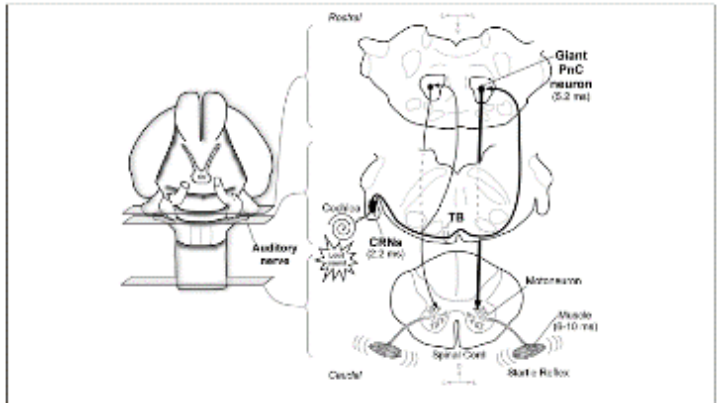
Perspective

- **DNT:**
- Needs 2 types of improvement
  - Revised tests
  - Revised ages
- Fit to agency needs
- Not for mechanisms
- Only for outcomes

But can provide hints of mechanisms if the right tests are chosen

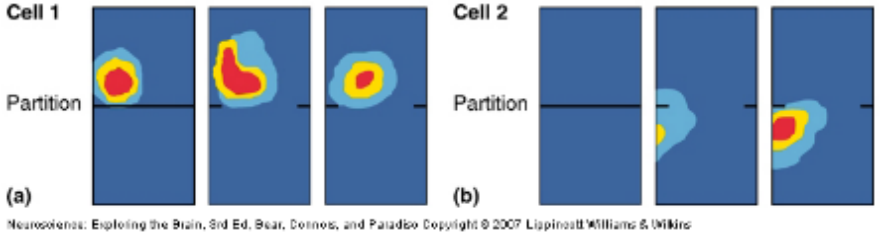
Top Down Mechanistic Inference

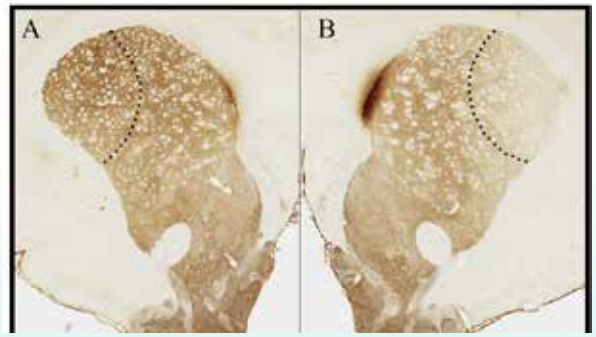
- ASR
- MWM
- CWM



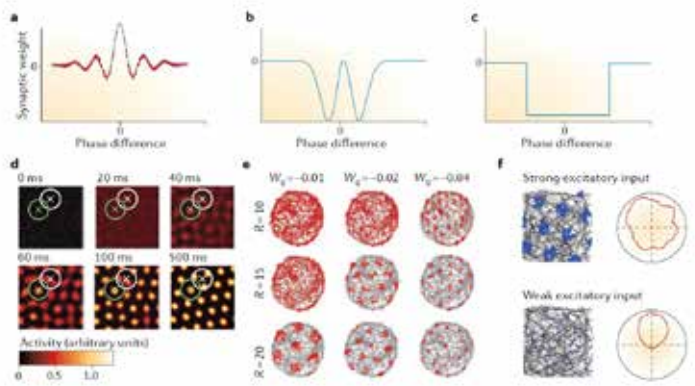
From Gomez-Nieto et al. (2014). ***Front. Neurosci.,*** **8**: 216. Illustration of ASR trisynaptic pathway.

CRN = cochlear root neuros; PnC = giant neurons of the caudal pontine reticular nucleus (also called the n. reticularis pontis caudalis)





Braun et al. (2015). ***Neurobiol. Learn. Mem.,*** **118**, 55-63.



Grid cells and cortical representation
Edvard I. Moser, Yasser Roudi, Menno P. Witter, Clifford Kentros, Tobias Bonhoeffer and May-Britt Moser1. Nature Reviews Neuroscience, 2014, 15, 466–481.

Future Directions for DNTs

- Not appropriate in all contexts
- Often necessary; rarely sufficient
- Non-mechanistic but often the only option
- NTP: new DNT (includes PPI, MWM, etc.)
- Should be in other study designs too: Extended one-generation reproductive (EOGRT) guideline (not cut L&M out).

Keep Goal in Sight

- Brain development is complex; many things can go wrong
- Effects in humans are often about higher functions: forgetting, poor recall, impaired learning, impaired problem-solving, attention problems: all cognitive; but the DNT is weak on cognitive assessment.
- FOB does not address these
- PPI is one aspect of attention
- MWM/CWM assess learning and 2 forms of memory
- 69 DNT studies of neurotoxins tell us something important. Are we listening? Or are we going to do 69 more the same way before we take action?

I hope not!

References

- Atladottir HO, Gyllenberg D, Langridge A, Sandin S, Hansen SN, Leonard H, Gissler M, Reichenberg A, Schendel DE, Bourke J, Hultman CM, Grice DE, Buxbaum JD, Parner ET (2015) The increasing prevalence of reported diagnoses of childhood psychiatric disorders: a descriptive multinational comparison. Eur Child Adolesc Psychiatry 24:173-183.
- Buelke-Sam J, Kimmel CA, Adams J, Nelson CJ, Vorhees CV, Wright DC, St.Omer V, Korol BA, Butcher RE, Geyer MA, Holson JF, Kutscher CL, Wayner MJ (1985) Collaborative behavioral teratology study: Results. Neurobehav Toxicol Teratol 7:591-624.
- Clancy B, Kersh B, Hyde J, Darlington RB, Anand KJ, Finlay BL (2007) Web-based method for translating neurodevelopment from laboratory species to humans. Neuroinformatics 5:79-94.
- Herlenius E, Lagercrantz H (2004) Development of neurotransmitter systems during critical periods. Exp Neurol 190:S8-S21.
- Maurissen JP, Hoberman AM, Garman RH, Hanley TR, Jr. (2000) Lack of selective developmental neurotoxicity in rat pups from dams treated by gavage with chlorpyrifos. Toxicol Sci 57:250-263.
- Raffaele KC, Rowland J, May B, Makris SL, Schumacher K, Scarano LJ (2010) The use of developmental neurotoxicity data in pesticide risk assessments. Neurotoxicol Teratol 32:563-572.
- Rodier PM (1980) Chronology of neuron development: Animal studies and their clinical implications. Dev Med Child Neurol 22:525-545.
- Tsuji R, Crofton KM (2012) Developmental neurotoxicity guideline study: issues with methodology, evaluation and regulation. Congenit Anom (Kyoto) 52:122-128.
- Vorhees CV (1986) Principles of behavioral teratology. In: Handbook of Behavioral Teratology (Riley EP, Vorhees CV, eds), pp 23-48. New York: Plenum Press.
- Vorhees CV (1994) Behavioral and functional ontogeny: Biomarkers of neurotoxicity. In: Principles of Neurotoxicology (Chang LW, ed), pp 733-763. New York: Marcel Dekker, Inc.
- Weiner ML, Nemec M, Sheets L, Sargent D, Breckenridge C (2009) Comparative functional observational battery study of twelve commercial pyrethroid insecticides in male rats following acute oral exposure. Neurotoxicology 30 Suppl 1:S1-16.

Acknowledgements

- I wish to thank Dr. Michael Williams for 20 years of collaboration and stimulating scientific dialogue.
- NIH for >30 years of grant support.

Thank You