NAFTA Developmental Neurotoxicity Guidance Document

Case Study – Chemical X

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EFSA DNT Lecture Series
April 2021

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Outline

- Lecture Series on NAFTA Guidance Continues
  - Past lectures
    - NAFTA Background and FOB & Clinical Observations
    - Motor Activity
    - Startle
    - Leaning and Memory
    - Neuropathology and Morphometrics
- Current Lecture – Case study for Chemical X
  - Study background
  - Individual methods
    - Review of methods and data
    - Study report vs our interpretations
  - Interpretation and recommendations for entire study

Disclaimer

In order to provide a more ‘real-life’ exercise methods and data have been selected from one or more studies for exemplary purposes.

No comparison to any actual chemical or chemical class should be made.

Case Study - Chemical X

- Chemical is a pesticide
- Mode of action – anti-fungal agent

- DNT study was conducted to address the need for a complete database for determination of whether there is an additional risk to children
- Dose selection was based on a preliminary DNT study (not used – doses where excessive body effects in dams and pup mortality)

DNT Guidelines

Testing Requirements for Motor Activity

	EPA 870.6300	OECD 426	OECD 443
Test species	Rat	Rat	Rat
Exposure	GD6 to weaning	GD6 to weaning	2 weeks pre-mating to weaning
Motor activity	Preweaning ontogeny and adult	Preweaning and adult	Preweaning and adult
Neuromotor ontogeny	None	Preweaning	None
Functional/Clinical observations	Throughout	Throughout	Adult
Auditory startle response	Weaning and adult	Weaning and adult	Weaning
Learning and memory	Weaning and adult	Weaning and adult	None
Neuropathology and morphology	Weaning and adult	Weaning and adult	Adult

Chemical X

Courtesy V. Moser

Chemical X
Method Summary

- Standard EPA 870.6300 (also meets OECD 426)
- Dietary exposure to the dams GD7-PND22, N=30/dose
- Litters standardized to 8 pups 4 male, 4 female on PND5
- N= 10 pups/sex/dose) *males and females were from different litters*
- Historical control data were submitted only for L&M and morphometrics
- No positive control data submitted

Body Weights

Body Weights

- Maternal
  - 4-5 % decrease in body wt gain – high dose only and only during lactation
- Offspring
  - Body weight gain was reduced in the mid and high dose groups in both males and females
  - Middle dose
    - Average 3-5 % lower than controls PND 12- PND50
    - Recovered at PND57
  - High Dose
    - Ranged from 3-14% with peak decrease PND21-29
    - Recovered by PND63

FOB

FOB – Methods

- Dams and pups tested on specific days
- "outside home cage"
  - But no details
- Observations listed, no specific protocol
  - "Assessment of signs of autonomic function…"
  - "Description, incidence, severity of convulsions, tremors, abnormal movements"
  - "Description, incidence of posture or gait abnormalities"
  - "Description, incidence of unusual or bizarre behaviors…"
- No statistical analyses
- No historical or positive control data submitted

FOB – Chemical X
Did Methods Present Necessary Information?

- List of signs to be observed
  - Very general, data tables include tests that were not mentioned in methods **X/✔**
- Defined scoring criteria or explicit descriptions of “normal” and “abnormal”
  - Not present **X**
- Observations made blind with respect to treatment
  - Specified for pups not dams **X/✔**
- Training and experience of observer
  - Not mentioned **X**
- Whether same animal is tested each time, especially pups
  - Not mentioned but data tables show this ✔
- Accounting for age of subject, e.g., underdeveloped motor and physical function in pup
  - Not mentioned **X**

Results

- Every animal had “X” for “no abnormalities observed”, followed by list of observations with “N” (normal)
- Hundreds and hundreds of pages of this
- Summary: “No treatment-related clinical observations”

Concerns with the Data

- No variability in any animals – all “normal”

- All data same regardless of pup age
  - PND5 data include “normal” pupil size and constriction – but PND5 rats’ eyes are not open!
  - PND5 data include “normal” gait and no ataxia – but PND5 pups can barely walk!
  - Suggests no actual thought going into evaluations

FOB Interpretations and Recommendations

- Request actual protocol to understand what was observed and how (but probably will not make a difference in outcome)
- Request positive control study to show they can actually pick up neurotoxicity

Conclusion

- Likely that severe toxicity of dams would have been detected
- But low confidence that meaningful, less than severe, changes in dams or pups, if present, would have actually been detected

Motor Activity

Motor Activity - Methods

- Multiple test days – PND 14, 18, 22, 60; same rats at all ages
- Very brief description of device
  - “automated recording device”
  - “small and large movements”
  - “separate room”
- Statistical analysis
  - ANOVA followed by Student’s t-test
- No historical or positive control data submitted with report

Motor Activity
Did Methods Present Necessary Information?

- Test device description – inadequate X
  - no information on shape, size, what type of detection system
  - no information how “small” and “large” movements were derived, which values (or sum?) were used in report
- Protocol description – inadequate X
  - no information whether test chambers isolated from each other, how long after placement did data collection begin
- Treatment groups counterbalanced across chambers ✔
- Statistical analysis – inadequate X
  - No repeated measures over test session (habituation)
  - Sex not included as a factor in analysis
- No historical or positive control data submitted with report X

Motor Activity Results
Control Data

- Activity be age-appropriate with expected ontogeny
  MAYBE
  - Activity peaks at PND22 (not PND18) in both sexes – does this agree with historical control?
- Variability decrease with age
  YES
- Variability not excessive YES

Control session counts
X±SD (CV)

Males

PND 14 167.0 ± 121.1 (72%)
PND 18 233.7 ± 153.5 (66%)
PND 22 370.7 ± 202.4 (54%)
PND 60 516.3 ± 131.0 (25%)

Females

PND 14 144.0 ± 149.9 (104%)
PND 18 265.4 ± 234.2 (88%)
PND 22 481.5 ± 163.5 (34%)
PND 60 597.2 ± 72.2 (12%)

Motor Activity Results Control Data (con't)

- Habituation evident on PND22 and 60 YES (males), NO (females)

PND60

Male Controls

Interval (min)	X±SD
1-5	65.6 ± 9.7
6-10	67.5 ± 8.5
11-15	59.3 ± 20.3
16-20	54.6 ± 23.0
21-25	60.2 ± 10.9
26-30	43.5 ± 29.9
31-35	44.3 ± 27.5
36-40	47.4 ± 31.3
41-45	37.2 ± 28.1
46-50	36.7 ± 29.5

Female Controls

Interval (min)	X±SD
1-5	63.4 ± 11.8
6-10	60.9 ± 13.6
11-15	60.6 ± 9.3
16-20	64.4 ± 8.3
21-25	63.3 ± 8.4
26-30	58.6 ± 12.3
31-35	57.1 ± 11.5
36-40	55.3 ± 17.9
41-45	54.5 ± 19.9
46-50	59.2 ± 21.4

Motor Activity Treatment Effects

- Total session counts
  - Decrease (~30%) in PND22 female counts at low and middle dose only (high dose, 11% decrease)
- Habituation
  - Some spurious blocks show significance (<4% of all blocks analyzed), no clear pattern
  - PND60 female high dose group showed no habituation, but neither did controls!

BUT

- Statistical analyses did not include sex or look at repeated measures

Motor Activity
Interpretations and Recommendations

- Require appropriate statistical analyses
  - No way to know whether there are sex or treatment differences
- Request more information on methods
- Request historical and positive control data
  - No decisions can be made due to lack of habituation in adult females, and unusual ontogeny of pups
- Check individual data for outliers that may skew group means

Conclusion

- At most, small changes maybe in females
  - But no statistical support
  - Not likely to be biologically significant, but…

Startle

Startle - Methods

- Testing on PND23 and 61
- One male and one female from each dose group from different litters

- Methods description - Only two sentences that state:
  - An automated recording apparatus use used.
  - Recorded variables: Mean response amplitude and time to max amplitude for 5 blocks of 10 trials

- No historical or positive control data included

Startle

Did Methods Present Necessary Information?

- Methods
  - Type of device used (and calibration) X
  - Treatment balanced across time of day and test boxes X
  - Good environmental control (e.g., animal handling, noise) X
  - Training and experience of technical staff X
  - Experimenter blind with respect to treatment X
- Control Data
  - Amplitudes should be age-appropriate ✔
  - Adult animals should be higher than weanling animals ✔
  - Habituation should be present to some extent at PND24? ✔
  - Variability not excessive, declines with age ✔
  - Historical and positive control data X

Startle - Results

- First look at controls – look for
  - Summary by and across blocks - NO
  - Smaller response in young vs old - YES
  - Higher amplitude in adult males compared to females - YES
  - Evidence of habituation - YES
  - Variability of controls - okay

		Males	Females
PND 23	1-10	379.6 ± 98.8	374.9 ± 121.3
	11-20	266.6 ± 64.6	332.9 ± 143.1
	21-30	233.3 ± 63.1	267.5 ± 67.0
	31-40	223.7 ± 50.9	234.7 ± 58.3
	41-50	208.0 ± 55.4	226.3 ± 44.1
PND 61	1-10	1351.5 ± 370.6	1064.0 ± 225.1
	11-20	898.6 ± 258.7	874.2 ± 170.2
	21-30	861.2 ± 346.1	772.0 ± 234.1
	31-40	769.7 ± 279.9	677.8 ± 270.4
	41-50	701.9 ± 307.9	652.6 ± 185.4

Startle Results

- Next – Look for treatment effects on amplitude
  - Report: Significance in high dose (3 out of 5 blocks) for males only and PND23 only
    No effect in adult males or female
  - But interaction of sex and treatment was not tested

		Control		Low		Medium		High		High Dose %
MALES	Trial Blocks	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Control
	1	380	99	439	149	373	99	333	122	88
	2	267	65	298	66	297	103	211	34	79
	3	243	63	261	53	266	91	177	48	73
	4	224	51	235	47	251	73	145	54	65
	5	220	55	221	46	222	63	128	51	58
FEMALES	1	375	121	320	177	353	108	385	63	103
	2	333	143	296	130	281	123	361	46	108
	3	268	67	294	175	253	165	323	55	121
	4	235	58	230	129	190	59	288	54	123
	5	226	44	249	140	199	73	290	56	128

Red text = Significant difference from control

Startle Results
A Closer Look

- **Lack of session averages**
  - Generate averages per session for males and females
  - Males increased 26 percent in high dose, females decreased 15%
- **But interaction of sex and treatment was not tested**
  - Generate session averages for males and females combined
  - Combined decrease was only 4.7%

	Control		Low		Medium		High		High Dose % Control
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Males	266.6	66.6	290.9	72.3	281.8	85.8	198.6	61.8	74.5
Female	287.3	86.8	277.9	150.1	255.2	105.5	329.4	54.6	114.7
Combined	277.0	76.7	284.4	111.2	268.5	95.7	264.0	58.2	95.3

Data should be reanalyzed to determine it there was a real sex-specific effect

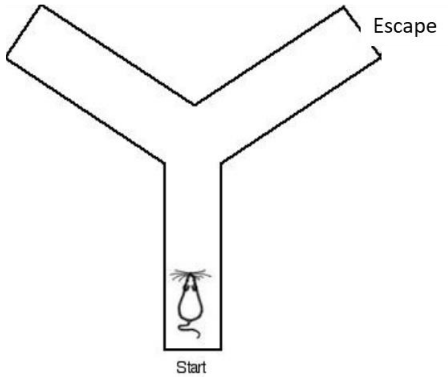
Startle Interpretations and Recommendations

1. Male only decrease may or may not be significant ….
2. Require adequate information on methods
3. Require submission of positive and historical controls
   - *Can they actually detect a change? Without it you cannot rule out a possible false-negative*
4. Require appropriate statistical analysis

Learning and Memory

Learning and Memory - Methods

- One male, one female from each litter tested on PN21, different pair at PN59
  - Implications for statistical analysis
- Y-shaped water maze
  - Dimensions? Water Temp?
  - Scaled to animal size?
- 6 trials/day
  - Inter-trial Interval? Inter-trial Housing?
  - Consistent time of day for testing?
  - Environmental conditions in test room?

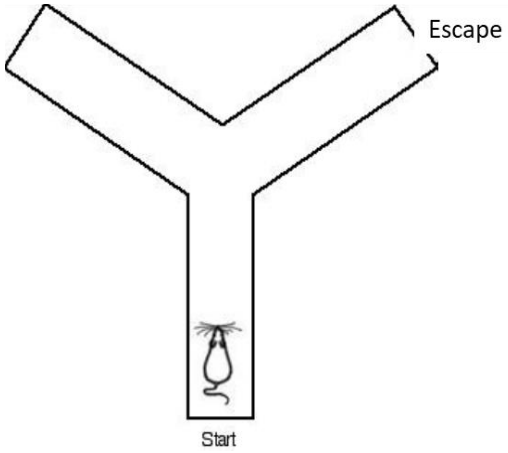


- Straight alley swim to evaluate motor competence was given after completion of 6 trials on each test day
  - Dimensions of alley relative to Y-Maze?
  - Why tested twice?
  - **After** learning test so not used to acclimate/reduce stress

- Historical Controls Provided for Trials 1 and 3
  - No Positive Controls provided – can this task detect anything?

Learning and Memory – Y-Maze
Did Methods Section Present Necessary Details?

Position
Discrimination
Y-Maze Water Maze



PROCEDURAL DETAILS THAT SHOULD BE INCLUDED

trials/day
acquisition days
Maximal Trial length
Interval between trials
Retention Interval i.e., Memory
retention trials - should be 1
Criterion performance defined
Error defined
Are Errors/Time Outs 'corrected'?
Maze Room Described -sensory cues- visual? olfactory?
Maze Size reported? Scaled to age?
Water Temperature
Control for side preference bias
Motoric competence assessed
Acclimation to stress before testing

Chemical 'X' Procedure Reporting Details - How Did They Do?

PROCEDURAL DETAILS FOR CHEMICAL 'X'

- # trials/day ✔
- # acquisition days ✔
- Maximal Trial length X
- Interval between trials X
- Retention Interval – Memory ✔
- # retention trials, but should be 1 ✔/ X
- Criterion performance defined X
- Error defined X
- Are Errors/Time Outs 'corrected'? X
- Maze Room Described -sensory cues- visual? olfactory?- X
- Maze Size reported? Scaled to age? X X
- Water Temperature- X
- Control for side preference bias X
- Motoric competence assessed ✔
- Acclimation to stress before testing- X

Bottom Line: Methodological details not adequate

Results - Weanlings

Male Controls 18 to 9 sec

Males-Weanling		Control	Low	Medium	High
Learning phase (PND 21)	Straight channel	4.49 ± 2.06	5.01 ± 2.70	4.47 ± 1.74	5.42 ± 2.54
	Trial 1	18.05 ± 7.97	15.65 ± 7.90	15.88 ± 8.31	21.14 ± 7.34
	Trial 2	10.29 ± 5.40	8.60 ± 4.78	12.64 ± 8.29	11.40 ± 5.27
	Trial 3	14.10 ± 6.49	9.92 ± 6.20* (↓30)	11.10 ± 6.97	12.43 ± 6.63
	Trial 4	10.35 ± 7.33	10.13 ± 5.51	9.99 ± 4.79	11.20 ± 7.33
	Trial 5	8.91 ± 4.01	6.56 ± 3.35	9.18 ± 4.52	9.50 ± 5.32
	Trial 6	9.22 ± 7.42	6.63 ± 3.85	9.39 ± 5.97	10.48 ± 6.29
Memory phase (PND 24)	Straight channel	3.46 ± 1.06	3.81 ± 2.51	3.41 ± 1.50	4.44 ± 2.20
	Trial 1	8.46 ± 3.46	9.16 ± 5.83	8.72 ± 5.63	7.40 ± 4.96
	Trial 2	5.33 ± 2.09	6.27 ± 4.48	5.30 ± 2.67	5.69 ± 2.61
	Trial 3	6.41 ± 4.13	4.44 ± 2.63* (↓31)	4.40 ± 3.07* (↓31)	5.37 ± 3.18
	Trial 4	5.55 ± 4.67	4.62 ± 2.51	5.52 ± 3.10	5.17 ± 2.55
	Trial 5	4.42 ± 2.07	4.81 ± 2.55	4.65 ± 2.15	3.89 ± 1.01
	Trial 6	5.33 ± 4.04	4.41 ± 1.63	5.08 ± 3.65	5.58 ± 2.88

No motor issues 4-5 sec

Female Controls 22 to 7 sec

Females Weanling		Control	Low	Medium	High
Learning phase (PND 21)	Straight channel	4.05 ± 1.37	4.03 ± 1.50	5.17 ± 3.59	4.19 ± 1.70
	Trial 1	22.37 ± 6.76	14.27 ± 6.83** (↓36)	15.80 ± 6.39** (↓29)	17.48 ± 6.94* (↓22)
	Trial 2	10.57 ± 7.23	10.41 ± 7.23	12.42 ± 7.79	12.90 ± 6.90
	Trial 3	7.32 ± 4.36	8.91 ± 5.96	11.35 ± 7.23* (↑55)	11.17 ± 7.49* (↑53)
	Trial 4	6.80 ± 3.74	10.50 ± 5.93* (↑54)	8.83 ± 5.93	7.50 ± 5.69
	Trial 5	7.85 ± 4.99	8.12 ± 7.29	9.10 ± 6.11	7.00 ± 5.90
	Trial 6	10.04 ± 7.57	5.61 ± 3.15** (↓44)	7.92 ± 4.97	7.12 ± 5.96
Memory phase (PND 24)	Straight channel	4.08 ± 1.77	3.38 ± 1.12	3.74 ± 1.58	3.63 ± 1.24
	Trial 1	7.83 ± 3.67	8.18 ± 5.85	8.63 ± 4.61	7.08 ± 3.29
	Trial 2	5.26 ± 3.49	3.87 ± 1.91	5.00 ± 2.67	4.94 ± 2.29
	Trial 3	4.01 ± 1.61	5.21 ± 3.23	5.36 ± 2.57	4.62 ± 3.45
	Trial 4	4.73 ± 2.43	5.37 ± 5.04	5.00 ± 3.71	3.76 ± 0.90
	Trial 5	5.49 ± 3.93	5.92 ± 4.66	5.36 ± 3.50	4.45 ± 2.48
	Trial 6	4.88 ± 2.92	7.08 ± 6.38	5.70 ± 2.96	5.09 ± 2.91

No motor issues 4-5 sec

Results- Adults

Male Controls 13 to 4 sec

Males-Adult		Control	Low	Medium	High
Learning phase (PND 59)	Straight channel	4.05 ± 1.40	4.11 ± 1.50	5.04 ± 3.82	3.82 ± 0.88
	Trial 1	13.74 ± 5.69	12.96 ± 3.71	12.31 ± 4.63	11.96 ± 3.89
	Trial 2	6.26 ± 4.72	6.67 ± 4.04	6.84 ± 4.08	5.28 ± 1.96
	Trial 3	4.71 ± 2.34	4.84 ± 2.26	5.14 ± 3.08	4.97 ± 2.04
	Trial 4	4.91 ± 2.35	4.88 ± 2.09	4.79 ± 2.70	4.88 ± 2.00
	Trial 5	4.19 ± 2.19	5.79 ± 3.44* (↑38)	4.43 ± 1.45	5.44 ± 3.05
	Trial 6	4.63 ± 2.35	6.15 ± 4.00	4.81 ± 2.16	5.63 ± 3.65
Memory phase (PND 62)	Straight channel	3.14 ± 1.24	3.15 ± 1.16	3.02 ± 0.99	3.48 ± 1.17
	Trial 1	4.95 ± 2.26	5.41 ± 2.58	6.12 ± 3.44	5.08 ± 2.24
	Trial 2	4.12 ± 2.33	5.66 ± 5.98	3.76 ± 1.36	5.81 ± 5.18
	Trial 3	5.14 ± 3.43	6.81 ± 4.66	6.91 ± 4.02	6.42 ± 7.22
	Trial 4	5.52 ± 3.66	7.11 ± 5.63	6.67 ± 3.39	7.48 ± 5.41
	Trial 5	5.04 ± 2.32	7.60 ± 6.08	6.17 ± 4.01	7.47 ± 5.39
	Trial 6	6.11 ± 3.06	6.85 ± 5.42	6.33 ± 5.90	6.72 ± 4.54

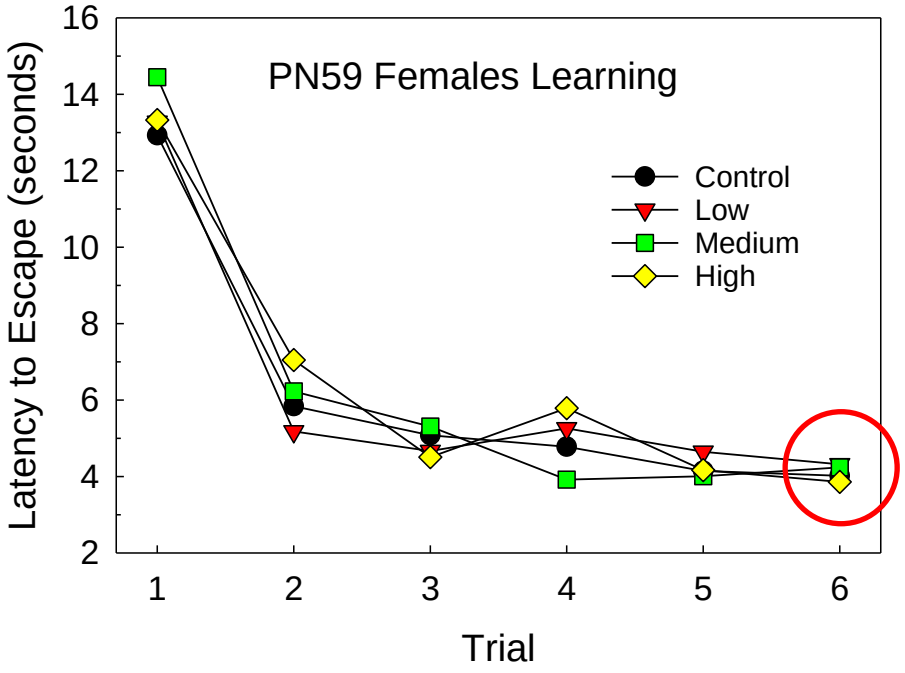
No motor issues ~4-5 sec

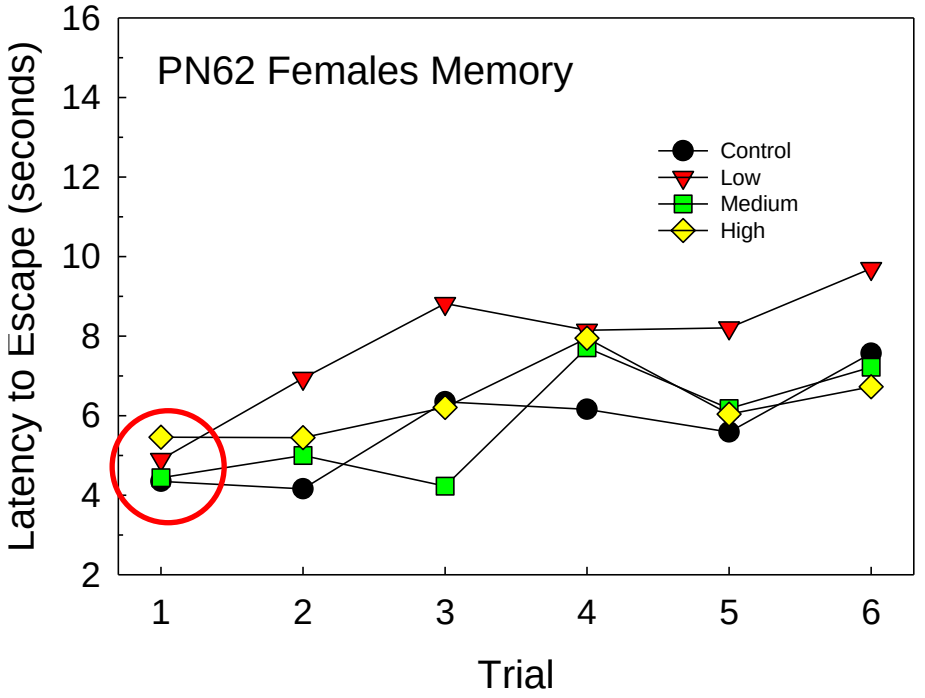
Female Controls 13 to 4 sec

Females-Adult		Control	Low	Medium	High
Learning phase (PND 59)	Straight channel	4.33 ± 2.42	4.30 ± 2.33	4.34 ± 1.79	4.34 ± 2.75
	Trial 1	12.93 ± 5.07	13.28 ± 5.00	14.45 ± 5.14	13.33 ± 5.58
	Trial 2	5.84 ± 3.27	5.18 ± 2.24	6.23 ± 4.15	7.05 ± 3.31
	Trial 3	5.08 ± 3.60	4.67 ± 1.76	5.31 ± 2.60	4.51 ± 2.05
	Trial 4	4.78 ± 2.69	5.26 ± 3.67	3.92 ± 1.61	5.79 ± 4.30
	Trial 5	4.15 ± 1.49	4.65 ± 2.71	4.01 ± 1.55	4.17 ± 1.26
	Trial 6	4.02 ± 2.26	4.32 ± 2.36	4.24 ± 2.25	3.86 ± 1.52
Memory phase (PND 62)	Straight channel	2.88 ± 0.73	3.14 ± 1.02	2.87 ± 0.69	3.55 ± 1.33* (↑23)
	Trial 1	4.35 ± 1.82	4.90 ± 2.29	4.44 ± 1.60	5.46 ± 2.80
	Trial 2	4.16 ± 2.73	6.94 ± 6.05* (↑67)	5.00 ± 3.52	5.45 ± 3.91
	Trial 3	6.35 ± 4.29	8.82 ± 7.35	4.23 ± 1.79	6.21 ± 6.36
	Trial 4	6.16 ± 4.91	8.15 ± 7.15	7.71 ± 4.93	7.95 ± 6.85
	Trial 5	5.59 ± 3.58	8.21 ± 5.49* (↑47)	6.18 ± 3.40	6.04 ± 3.74
	Trial 6	7.57 ± 7.07	9.70 ± 5.29	7.22 ± 5.82	6.73 ± 4.79

No motor issues ~4.5 Sec

Problem with Y-Maze Dataset

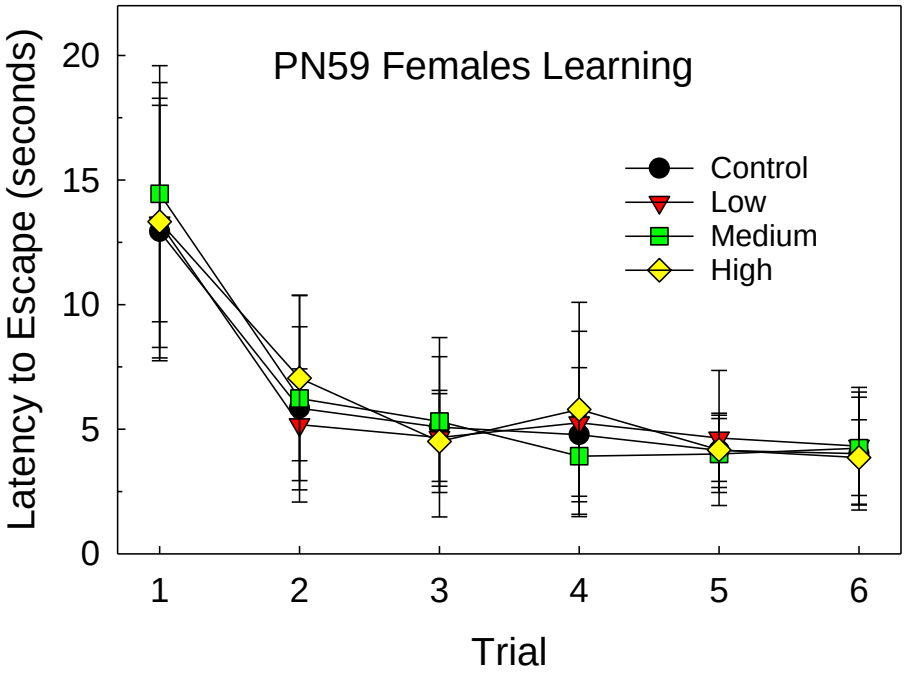


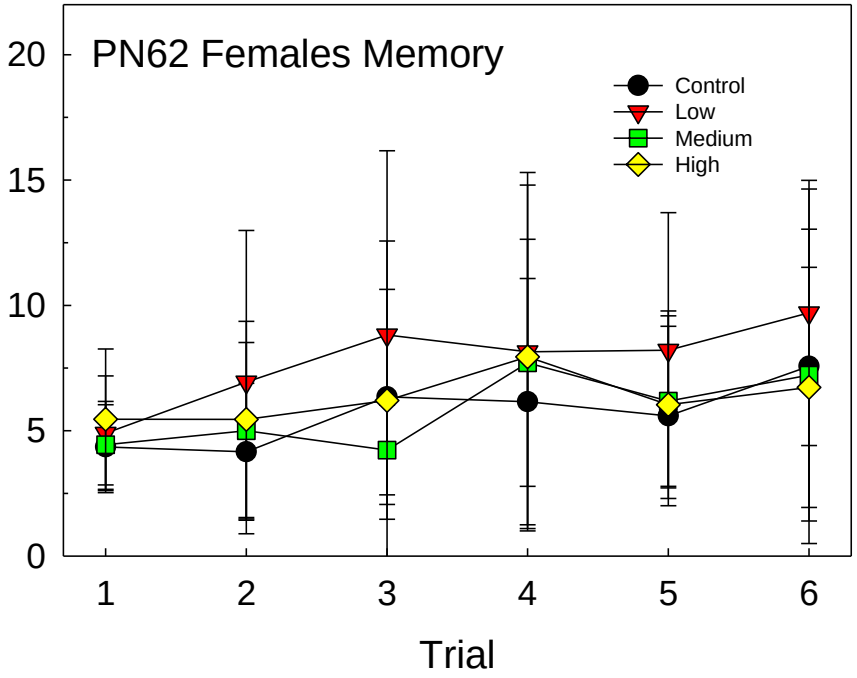


Learning: Learning curve BUT all learning happening in 1st trial - Limited dynamic range

Memory: Good retention at Trial 1 BUT what happened in the 5 trials? Relearning? Boredom? Enjoying spa day?

Problem with Y-Maze Dataset





Learning shows very shallow curve when variability included
Report claims - no treatment-related differences observed – true
Is there evidence of learning in controls?

'Memory' data are not interpretable beyond Trial 1

High variability – especially in 'memory phase' - animals not that motivated?

Looking at Data in Different Way

Females P59: Number of Successful Trials*

*Percent of trials completed by define cut-off time across the 6-trial learning phase

Females-Adult		Control	Low	Medium	High
Learning phase (PND 59)	≤ 10 seconds	82.5 ± 13.4	81.7 ± 13.8	83.3 ± 11.5	83.3 ± 10.1
	≤ 2 x straight channel time	74.6 ± 18.7	76.2 ± 13.5	75.0 ± 19.0	73.2 ± 21.2
Memory phase (PND 62)	≤ 10 seconds	85.7 ± 18.5	75.4 ± 22.7	88.5 ± 13.1	87.0 ± 22.4
	≤ 2 x straight channel time	73.8 ± 22.7	50.8 ± 28.6** (↓31)	62.2 ± 22.9	67.4 ± 22.2

Learning defined as "*improved performance over time*"

- Collapsing learning single number – what does that mean?
- Six trials for 'memory/retention' ?

Possible Additional Measures - Set a 'Criterion':

- Trials to Criterion for Each Animal
- # of Animals Reaching that Criterion at each Trial
- Still single numbers for learning but incorporate a temporal component

To Evaluate L&M Data You're Looking For

No motor impairment
Decreased escape latency over trials
Decreased and errors over trials
Increased # animals attaining 'criterion' performance
Memory - Maintained reductions in latency and errors from end of learning phase
Data in line with historical controls

Data Reported for Chemical 'X'

✔/X No difference in straight alley latencies – no motor impairment

✔/X Mean latency at each of 6 trials in 'Learning Phase'

But all the learning happens in between Trial 1 and Trial 2!

✔/X Mean latency at each of 6 trials in 'Memory Phase'

Can look at Trial 1 – but why run 6? After Trial 1 this is 'relearning'

X Number of animals reaching a nominal 'cut-off' latency

X Errors not reported – likely because there were very few!

X Criterion performance not reported – likely because everyone got there in 2 trials!

- Some/all (?) of this information *could* possibly be extracted from the individual animal data for each trial are provided

Learning and Memory
Interpretations and Recommendations

Possible Conclusions and Course of Action:

1) No Learning Impairment Evident

2) Unable to make definitive decision
   - Dynamic range too small to effectively probe for changes in cognitive function

3) Request more information on methods
   - Method description scarce but not likely to improve ability to make a decision

4) Require submission of positive control data
   - Task is too easy
   - *Can this lab actually detect a change in learning with this method under any circumstances?*

Morphometrics

Morphometrics - Methods

- PND12
  - 10 males and 10 females from different litters
  - Immersion fixed
  - Brain weight 24 hours after fixation

- PND63
  - 10 males and 10 females from different litters
  - PND63 10 males and 10 females from different litters
  - Perfusion fixation with formalin
  - Brain weights
  - Histological Processing information
    - All brains embedded in paraffin
    - Brains from only the control and high dose were sectioned and stained with hematoxylin

Morphometrics (con't)

- Morphometrics
  - A short appendix (7 pages) provides details
  - 8 sections taken – diagrams provided for each section
  - *"where there was a degree of obliqueness, only the side exhibiting the features required for that level were measured"*
- Statistics for Brain weights and morphometric data
  - Males and females analyzed separately
  - *Used ANOVA, and ANCOVA on body weight*
- Historical control data provided, but no positive control data

Best Practices

- Two ages – PND11 (or 21) and 60-70 ✔
- N=10/sex/dose ✔
- Brain weights ✔
- Fixative
  - Early age - immersion ✔
  - Adult age – perfusion ✔
- Immediate embedding for all groups ✔
- Counterbalancing during processing ??
- Paraffin recommended ✔
- Slice thickness 4-5um ??

Best Practices (con't)

- Measurements – neocortex, hippocampus, cerebellum, striatum, cerebral cortex
- Blinding recommended X
- Proof of sensitivity via concurrent and historical positive control data X
- Need highly homologous sections ??
- Statistics "The choice of statistical analysis is properly left to the discretion of the laboratory conducting the DNT" ✔

Brain Weights

- Statistically significant in high dose females and middle dose males – adults only
- All high dose brain weights are lower
- PND63 averaged across sex shows dose response

		Control	Low	Middle	High	High Dose % Control
PND21	Male	1.13	1.15	1.14	1.10	97.35
	Female	1.10	1.10	1.10	1.08	98.18
	Aveage	1.12	1.13	1.12	1.09	97.76
PND63	Male	2.08	2.03	2.02	2.03	97.60
	Female	1.91	1.94	1.91	1.86	97.38
	Average	2.00	1.99	1.97	1.95	97.49

Morphometric Data

- Report provides summary and raw data tables.

Example Table PND63

F1 GENERATION - DAY 63

MALES	Dietary Concentration of 0(Control)	High dose
Level 5 - Dorsal Cortex - Thickness (5AB)		
MEAN	1.38	1.32*
S.D.	0.05	0.07
N	10	10
Level 5 - Piriform Cortex - Thickness (5BB)		
MEAN	1.22	1.11**
S.D.	0.06	0.08
N	10	10
Level 5 - Thalamus Width (5C)		
MEAN	7.75	7.71
S.D.	0.28	0.35
N	10	10
Level 5 - Hippocampus - Width Dentate Gyrus (5DB)		
MEAN	0.76	0.77
S.D.	0.06	0.05
N	10	10
Level 5 - Hippocampus - Width Overall (5EB)		
MEAN	1.54	1.57
S.D.	0.08	0.07
N	10	10

Unclear whether asterisk is for ANONA or ANCOVA

Group means and % control (n=10/sex/dose)

		PND 12			PND63		
	Sex	**Control**	**High**	**% Control**	**Control**	**High**	**% Control**
Hippocampus Length	M	2.79	2.67	95.7	2.62	2.69	102.7
	F	2.83	2.61	92.2	2.62	2.58	98.5
Corpus Callosum	M	0.58	0.56	96.6	0.38	0.37	97.4
	F	0.64	0.59	92.2	0.34	0.42	123.5
Hippocampus Width	M	0.48	0.46	95.8	0.63	0.62	98.4
	F	0.49	0.46	93.9	0.62	0.63	101.6
Cortex Thickness 1	M	1.06	1.08	101.9	1.38	1.32	95.7
	F	1.12	1.1	98.2	1.3	1.34	103.1
Cortex Thickness 2	M	1.08	1.11	102.8	1.22	1.11	91.0
	F	1.1	1.09	99.1	1.21	1.22	100.8
Cerebellum Height	M	3.53	3.7	104.8	5.74	5.75	100.2
	F	3.66	3.74	102.2	5.43	5.6	103.1

Group means and % control (n=10/sex/dose)

		PND 12			PND63		
	Sex	**Control**	**High**	**% Control**	**Control**	**High**	**% Control**
Hippocampus Length	M	2.79	2.67	95.7	2.62	2.69	102.7
	F	2.83	2.61	92.2	2.62	2.58	98.5
Co Cal	M	0.58	0.56	96.6	0.38	0.37	97.4
							123.5
Hipp us Width							98.4
	F	0.49	0.46	93.9	0.62	0.63	101.6
Cortex Thickness 1	M	1.06	1.08	101.9	1.38	1.31	94.9
	F	1.12	1.1	98.2	1.3	1.34	103.1
Cortex Thickness 2	M	1.08	1.11	102.8	1.22	1.11	91.0
	F	1.1	1.09	99.1	1.21	1.22	100.8
Cerebellum Height	M	3.53	3.72	105.4	5.74	5.75	100.2
	F	3.66	3.74	102.2	5.43	5.6	103.1

FLAG - seven measurements changed by more than 5%

Morphometrics Interpretation and Recommendations

1. Without new data both brain weight and morphometrics should be considered positive
2. Re-analyze the brain weight data with sex in model
3. Ask for morphometrics on both low and middle dose
4. Recommendation – do not use body weight as co-variate for brain weight or morphometric measurements*
   - There is no clear correlation between mild to moderate decreases in body weight and brain weight (ie., brain sparing**)
   - Food restriction DNT guideline study: 10-15% decrease in body weight gain = no effects on brain weight, behavior or morphometrics
   - NAFTA (2016) "…effects on brain weight cannot be dismissed even in the presence of body weight differences, and should be considered treatment-related and adverse;

* NAFTA (2016)

** Peeling and Smart, Metab Brain Dis. 9:33-42, 1994.
Sellers et al., Tox Pathol. 35:751–755, 2007

Summary

Chemical X - Results Summary

Generation	Endpoint	Treatment Effect?	NOEL	LOEL	Notes
Maternal	Body Weight	Yes	Middle	High	4-6% decreases
	Food Consumption	Yes	Middle	High	5-6% PND8-23 only
Offspring	Body weight	Yes	Low	Middle	4-15% recovery starting at PND30
	FOB	No	-	-	All animals were normal' is not adequate
	Motor Activity	Yes	-	Low	Likely not significant after reanalysis for sex by treatment interactions
	Startle Response	Yes	Middle	High	Likely not significant after reanalysis for sex by treatment interactions
	Learning & Memory	No	High	-	Not deemed to be sensitive method
	Brain weight	Yes	Middle	High	Cannot dismiss due to changes in body weight
	Neuropathology	No	-	-	No effects
	Morphometrics	Yes	-	High	*Lack of low and middle doses and effects at high dose preclude any estimation of NOEL*

Red text = flag alerts on methods and/or results problems

How to judge the whole report

- Did the study follow guideline requirements? ✔/X
  - Most requirement met, but not all. Not apparent that all methods were valid. Lack of positive controls etc.

- Study conduct adequately reported?
  - This includes methods descriptions, statistics, results tables, QA, analytical data on the chemical, food analyses etc. ✔/X

- Confidence in data
  - Appropriate statistical analyses X
  - Adequate data reporting X
  - Historical Controls ✔/X
  - Lack of Positive Controls X
    - possible false negatives
    - Inability to judge dynamic ranges of methods

- Due to study deficiencies no definitive conclusions can be made

Overall Study Conclusions

- What to do with submitted study
  - Reject study due to severe problems
    - e.g., missing data, inappropriate methods
  - Accept study and report conclusions
  - Accept study and review/change interpretations
  - Postpone decision until additional information is received ✔


THANK
YOU
GRACIAS
BOLZÏN
MERCI
BÏYAN
ARIGATO
SHUKURIA
JUSPAXAR
GRAZIE
MEHRBANI
SUKSAMA
TASHAKKUR ATU
EKHMET
YAQHANYELAY
DANKSCHEEN
SHUKRIA
TINGKI
GOZAIMASHITA
EFCHARISTO
KOMAPSUMNIDA
MAAKE
PALDIES
SUKSAMA
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DENKAUJA
NENACHALHIYA
SPASSIBO
SNACHALHUYA
NUHUN
CHALTU
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UNALCHÉESH
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HATUR
EKOJU
SIKOMO
MAKETAI
MINMONCHAR
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