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Re-testing of false negative compounds in new approach methods (NAMs) for developmental neurotoxicity screening

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3828/P330

March 12th, 2024; 2:15 – 4:15 PM

Society of Toxicology Annual Meeting

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Introduction and Background

DNT NAMs have been developed and evaluated

- EPA developed an vitro battery of assays to evaluate chemicals for potential DNT
- The Organization of Economic Cooperation and Development has approved an initial guidance document on the use of data from in vitro DNT assays, including EPA's assays.
 - This will facilitate their incorporation into regulatory decisions.
- Carstens et al., 2022 evaluated of the sensitivity and specificity of EPA's assay battery.
 - Sensitivity = 74 % selective hits
 - 14/53 DNT Reference chemicals were not selectively active in DNT IVB assays.

GOAL: Re-screen false negative compounds in the EPA DNT assays to better understand why they may have tested negative.

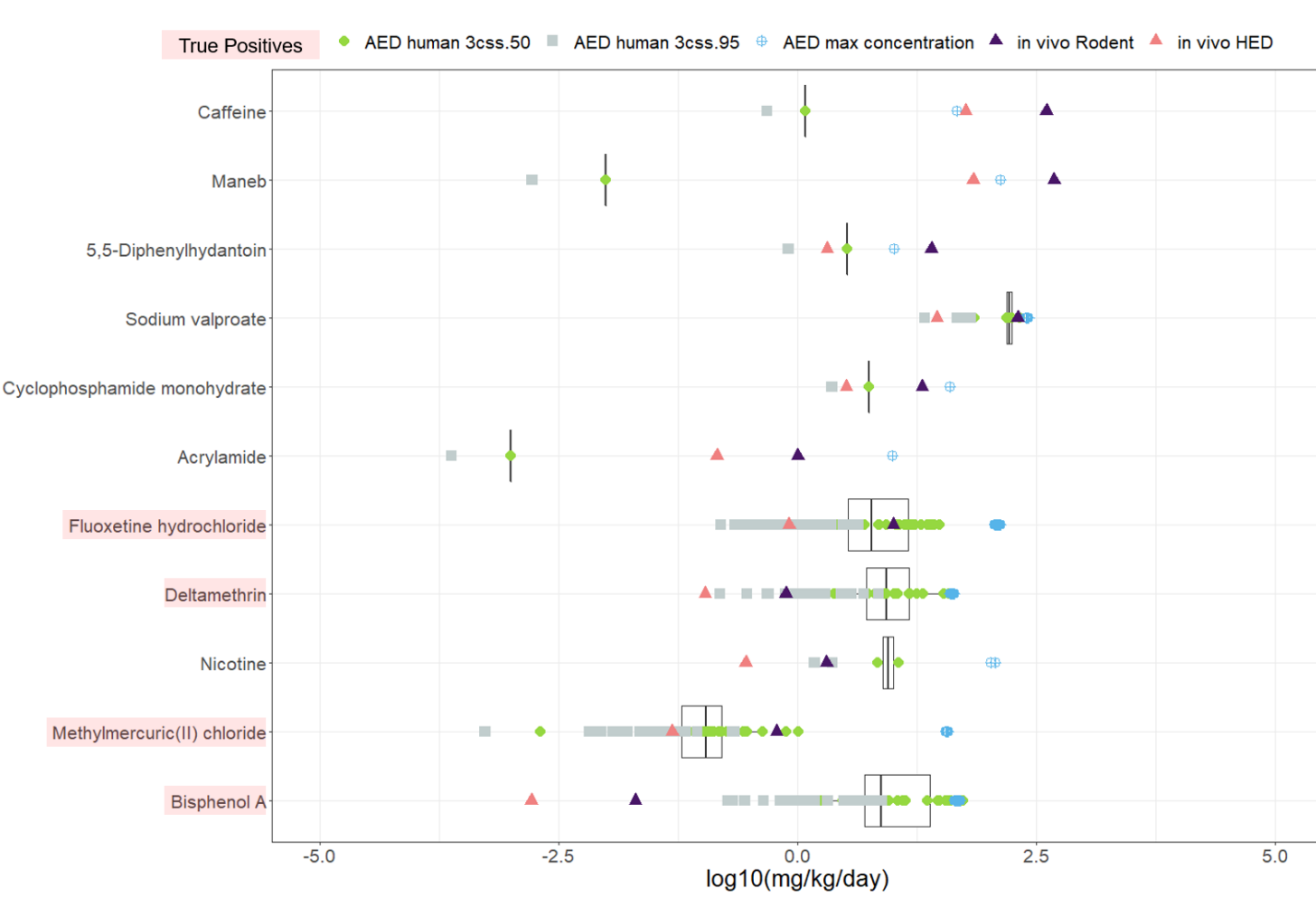
Carstens et al., Tox Sci, 187(1), 2022, 62–79

Identified “False Negative” Compounds and potential reasons for outcome

Chemical Name	CAS#	DSSTOX_ID	Concentration Tested				Selectivity
			min.uM.hci	min.uM.nfa	max.uM.hci	max.uM.nfa	
5,5-Diphenylhydantoin	57-41-0	DTXSID8020541	0.001	0.03	100	30	False Negative
Acrylamide	79-06-1	DTXSID5020027	0.001	0.03	100	30	False Negative
Caffeine	58-08-2	DTXSID0020232	0.001	0.03	100	30	False Negative
Carbamazepine	298-46-4	DTXSID4022731	0.001	0.03	100	30	False Negative
Cocaine	50-36-2	DTXSID2038443	0.001	0.03	100	30	False negative
Cyclophosphamide monohydrate	6055-19-2	DTXSID6024888	0.001	0.03	100	30	False Negative
Dexamethasone	50-02-2	DTXSID3020384	0.001	0.03	100	30	False Negative
Dextroamphetamine sulfate	51-63-8	DTXSID2057865	0.001	0.03	100	30	False Negative
Hydroxyurea	127-07-1	DTXSID6025438	0.001	0.03	100	30	False Negative
Esketamine	643-47-9	DTXSID601027734	0.001	0.03	100	30	False Negative
Maneb	12427-38-2	DTXSID9020794	0.001	0.01	10	10	False Negative
Manganese dichloride	7773-01-5	DTXSID9040681	0.001	0.03	100	30	False Negative
Naloxone hydrochloride dihydrate	51481-60-8	DTXSID90199452	0.001	0.03	100	30	False Negative
Nicotine	54-11-5	DTXSID1020930	0.001	0.03	100	30	False Negative
Phenobarbital sodium	57-30-7	DTXSID0021123	0.001	0.03	100	30	False Negative
Sodium fluoride	7681-49-4	DTXSID2020630	0.001	0.03	100	30	False Negative
Sodium valproate	1069-66-5	DTXSID5037072	0.03	0.03	3000	3000	False negative
Terbutaline hemisulfate	23031-32-5	DTXSID3045437	0.001	0.03	100	30	False Negative
Thalidomide	50-35-1	DTXSID9022524	0.001	0.03	100	30	False Negative

From: Carstens et al., Tox Sci, 187(1), 2022, 62–79

IVIVE estimation of in vivo doses indicates that five of the false negative compounds may have been tested at too low of a concentration in vitro



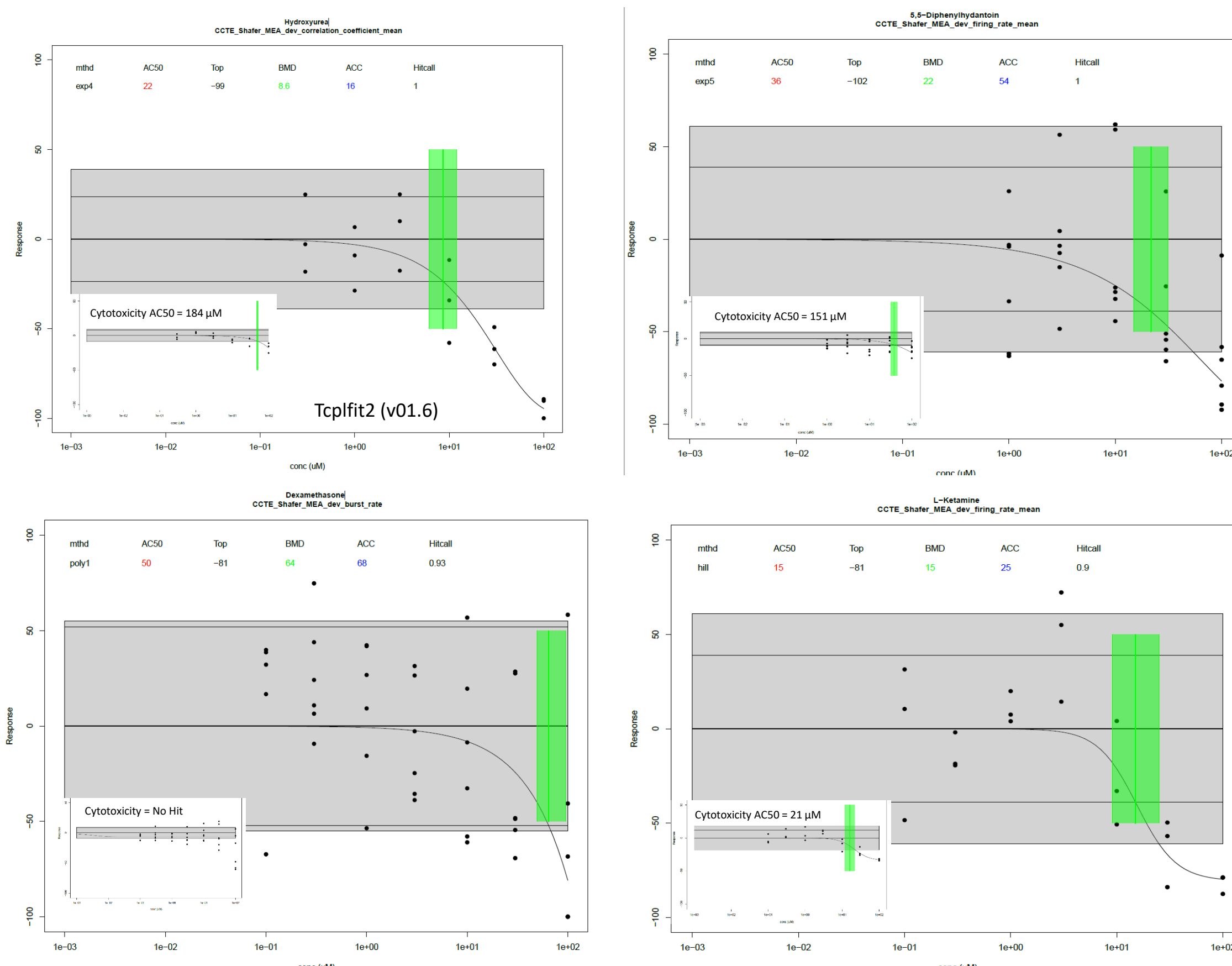
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Review of Laboratory Notebooks and the Literature indicated several additional potential issues, including:

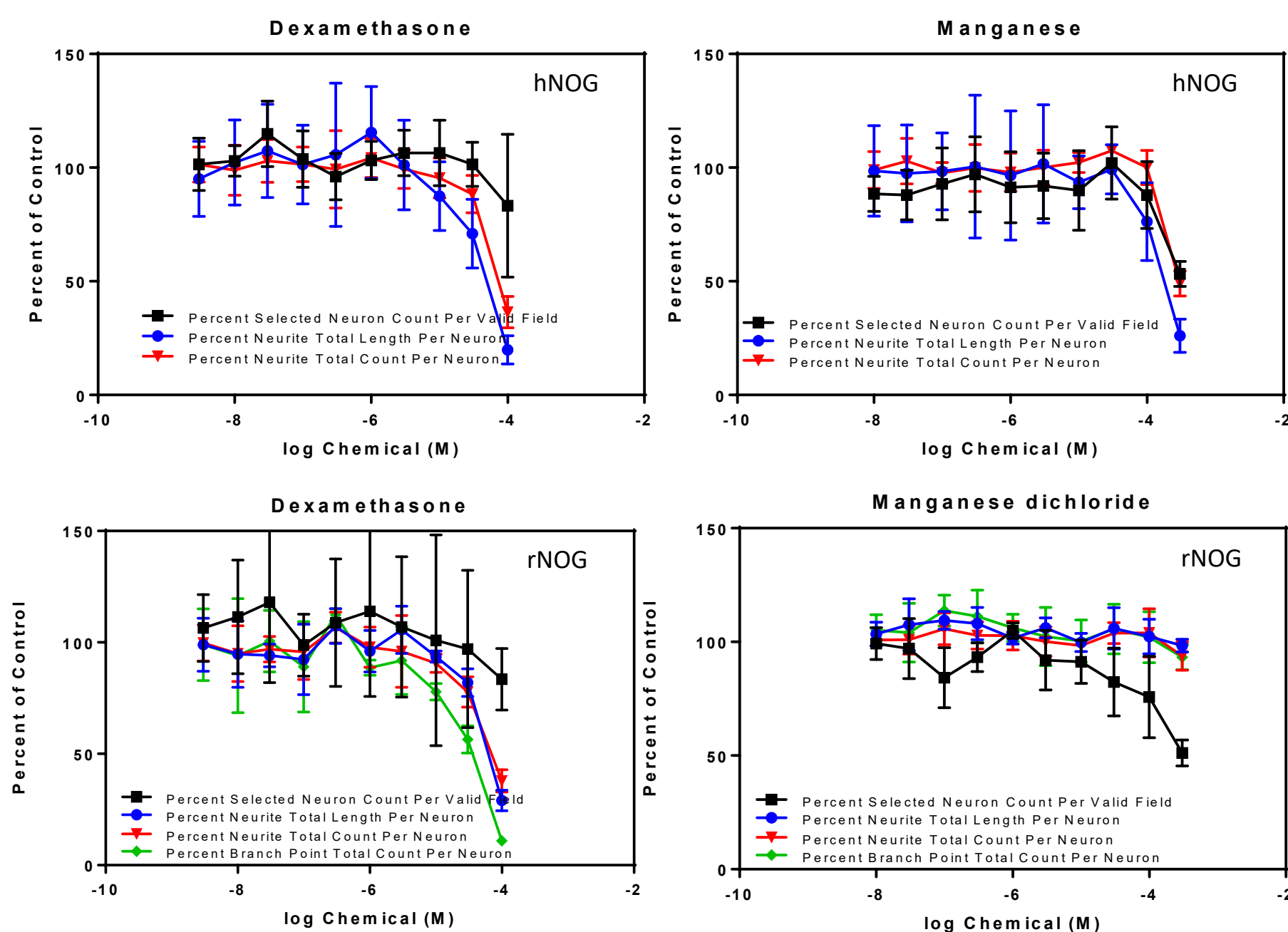
- Chemical instability/insolubility/wrong solvent
- Need for metabolism
- Testing of less active or inactive enantiomers
- Lack of necessary biology (e.g. critical receptor not expressed in the assay)

Chemical Name	CAS#	dsstox_substance_id	Possible Issues Identified
5,5-Diphenylhydantoin	57-41-0	DTXSID8020541	Tested concentration too low
Acrylamide	79-06-1	DTXSID5020027	
Caffeine	58-08-2	DTXSID0020232	Tested concentration too low; More soluble in H2O
Carbamazepine	298-46-4	DTXSID4022731	
Cyclophosphamide monohydrate	6055-19-2	DTXSID6024888	Tested concentration too low; Potential Stability Issues
Cocaine	50-36-2	DTXSID2038443	Tested free base; retest hydrochloride (CAS# 53-21-4; DTXSID2048903)
Dexamethasone	50-02-2	DTXSID3020384	Old Chemical stock; Obtain new chemical
Dextroamphetamine sulfate	51-63-8	DTXSID2057865	
Hydroxyurea	127-07-1	DTXSID6025438	Improper storage
Esketamine	643-47-9	DTXSID601027734	Test another enantiomer; L-ketamine (CAS#33795-24-3; DTXSID90187455)
Maneb	12427-38-2	DTXSID9020794	Tested concentration too low; insoluble higher than 10 mM (10 uM final)
Manganese dichloride	7773-01-5	DTXSID9040681	Old chemical, hygroscopic
Naloxone hydrochloride dihydrate	51481-60-8	DTXSID90199452	Biology? (agonist loperamide active in many assays)
Nicotine	54-11-5	DTXSID1020930	Potential Stability Issues; Old Chemical stock; Wrong Solvent?
Phenobarbital sodium	57-30-7	DTXSID0021123	
Sodium fluoride	7681-49-4	DTXSID2020630	Old chemical, hydroscopic?
Sodium valproate	1069-66-5	DTXSID5037072	Tested concentration too low
Terbutaline hemisulfate	23031-32-5	DTXSID3045437	
Thalidomide	50-35-1	DTXSID9022524	

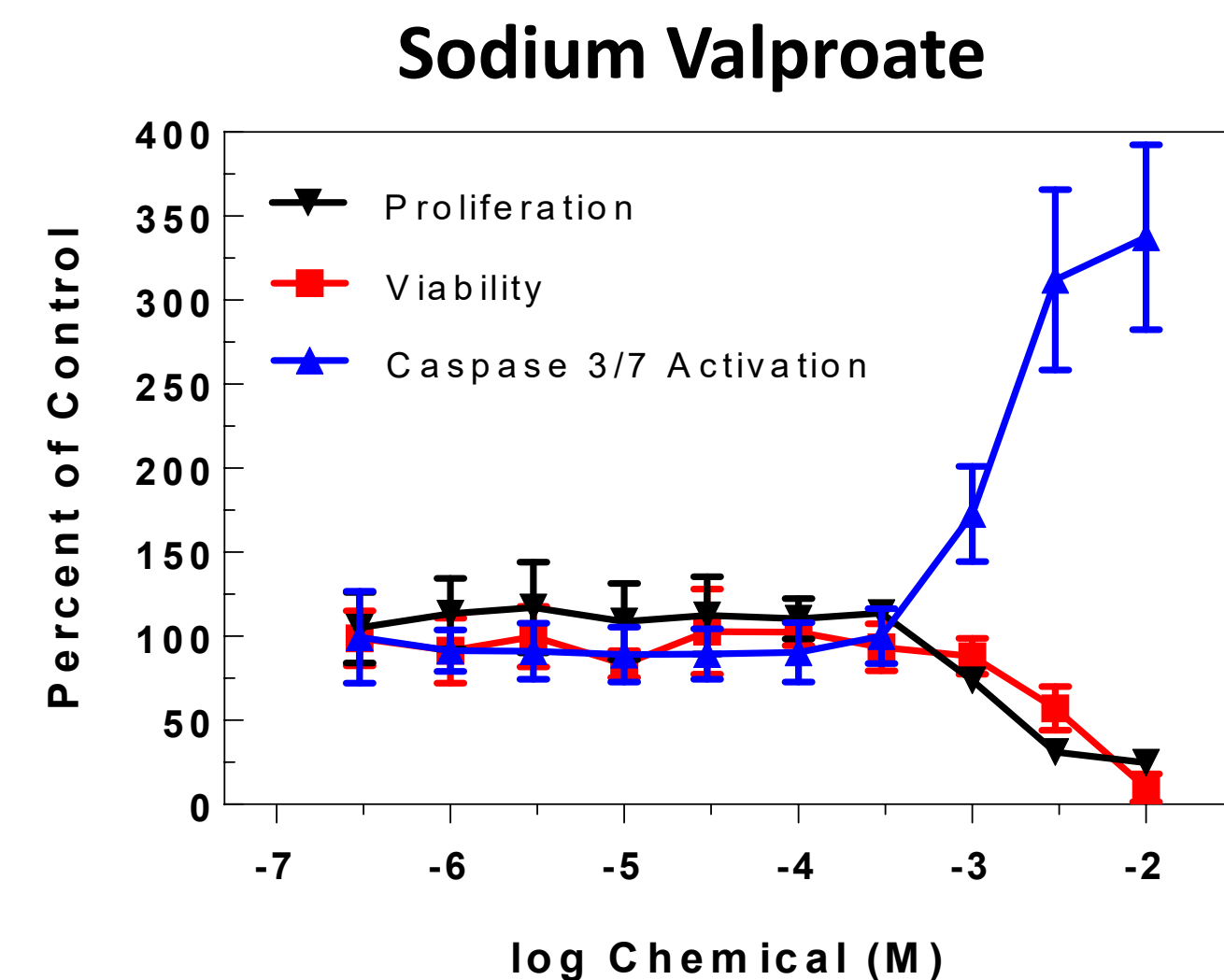
Hydroxyurea, 5,5'-Diphenylhydantoin and Dexamethasone appear to be selective in the NFA. L-Ketamine is active but not selective



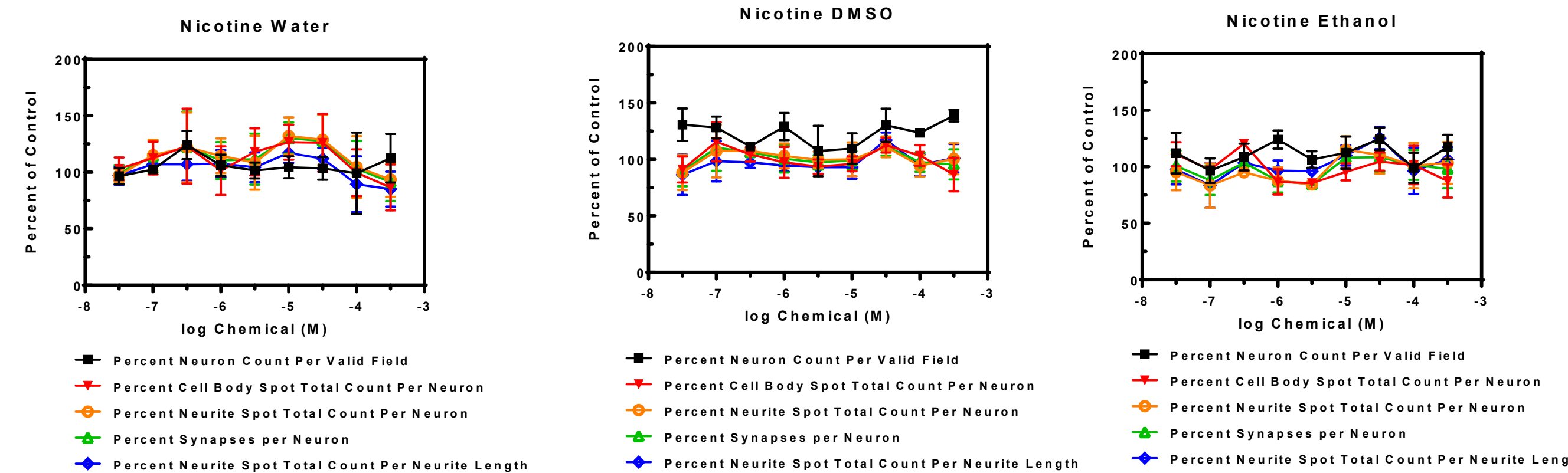
Dexamethasone, but not Manganese, is selective in the neurite outgrowth assay



Sodium valproate at higher concentrations causes apoptosis and decreases proliferation in hNP1 cells



Nicotine at higher concentrations and in different solvents is still inactive



Summary and Conclusions

Chemical Name	Highest Concentration (uM)	NFA	Cortical	CDI	hNOG	hNP1	hNP1	Overall Hit
5,5-Diphenylhydantoin	1000	Selective	Active	Active	N	N	N	Selective
Acrylamide	300	N	N	N	N	N	N	Active
Caffeine	300	N	N	N	N	N	N	N
Carbamazepine	100	N	N	N	N	N	N	N
Cocaine Hydrochloride	100	N	Active	N	N	N	N	Active
Cyclophosphamide monohydrate	300	N	Active	N	N	N	N	Active
Dexamethasone	100	Selective	Selective	N	Selective	N	N	Selective
Dextroamphetamine sulfate	100	Selective	N	N	N	N	N	Selective
Hydroxyurea	300	Selective	Active	Active	N	Selective	Active	Selective
L-Ketamine	100	Active	N	N	N	N	N	Active
Maneb	10	Active	Active	Active	N	N	N	Active
Manganese dichloride	300	Active	Active	Active	N	N	N	Active
Naloxone hydrochloride	300	N	N	N	N	N	N	Active
Nicotine	300	N	N	N	N	N	N	N
Phenobarbital sodium	100	N	N	N	N	N	N	N
Sodium fluoride	100	N	Active	Active	N	N	N	Active
Sodium valproate	10000	Active	Active	Active	Selective	Active	N	Selective
Terbutaline hemisulfate	100	N	N	N	N	N	N	N
Thalidomide	100	N	N	N	N	N	N	N

Green text = Different salt or isomer tested vs previous evaluations

- Previous evaluation of EPA DNA NAMs sensitivity were likely influenced by chemical stability, concentration ranges and chemical salts/isomers tested.
 - Consider expanding concentration range for unknown testing to maximum solubility in the absence of toxicokinetic data
- These data improve the selective sensitivity of the EPA DNT NAMs from 74% to 83%.