Comparisons of network formation and acute *in vitro* assays for evaluating neurotoxicity

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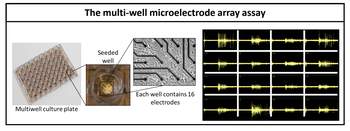
Introduction and Background

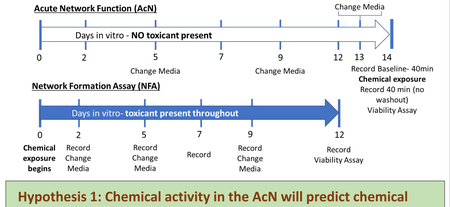
Need for new approach methodologies (NAMs):

- With > 30,000 chemicals in commerce, < 200 chemicals have been tested in EPA and/or OECD guideline DNT studies.
- Current guideline developmental neurotoxicity studies are costly, time-consuming, use large numbers of animals and are subject to methodological and scientific uncertainties.
- New approach methodologies (NAMs) have been developed to address the existing need for information on the potential neurotoxicity or developmental neurotoxicity hazard for thousands of chemicals in the environment.

The multi-well microelectrode array (MEA) screening approach measures neuronal electrical activity following chemical exposure.



- The acute network function (AcN) and network formation (NFA) MEA assays are both included in the ToxCast data resource and use P0 dissociated rat cortical cells.
- The AcN assay measures potential acute hazard in a model of a mature brain.
- Whereas the NFA measures potential developmental neurotoxicity hazard in a model of a developing network.



Hypothesis 1: Chemical activity in the AcN will predict chemical activity in the NFA.

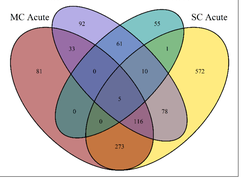
Hypothesis 2: Disruption of network formation occurs at lower concentrations than disruption of acute function.

Hypothesis 3: the NFA will have a greater number of cytotoxic minimum AC50 values

Data analysis approach:

- A total set of 1377 chemicals were tested in both MEA assays.
- A tiered screening strategy employed single concentration (SC) preliminary screening followed by multi-concentration (MC) response.
- 154 chemicals were screened in multi-concentration (MC) response in both the NFA and AcN.
- Computational methods were used to compare the chemical activity in the NFA and AcN for these 154 intersecting chemicals.

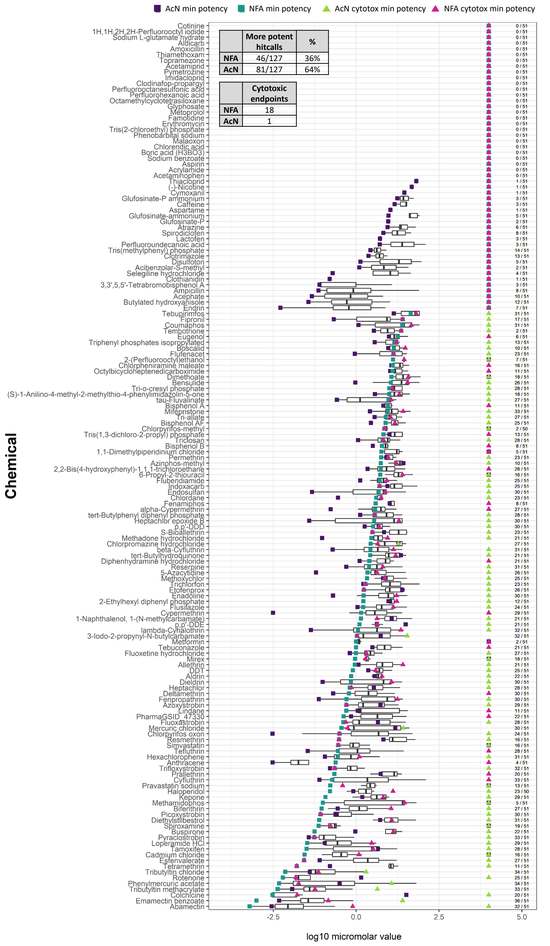
Overlap of MC and SC AcN and NFA chemicals tested



Main findings

In the multi-concentration screens, the AcN detects perturbations at lower concentrations for 64% of the tested substances

Values are sorted (from top to bottom) less potent to more potent by the minimum AC50 values for the NFA. Boxplots represent the range of potency values for both assays. The minimum AC50 values for non-active chemicals were set to a value of 4; 27 chemicals were not active in either assay. Of the 127 active chemicals, 36% of the chemicals were more potent in the NFA, whereas 64% of the chemicals were more potent in the AcN assay. 18 chemicals were cytotoxic in the NFA, and 1 chemical was cytotoxic in the AcN (with no overlap). **These analyses reveal that of the 127 active chemicals, that the AcN has a greater percentage of more potent chemicals.**

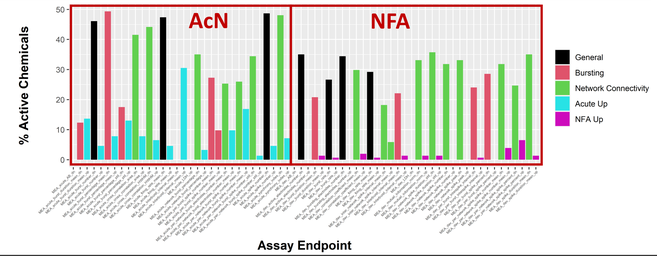


More potent hitcalls	%
NFA	46/127	36%
AcN	81/127	64%

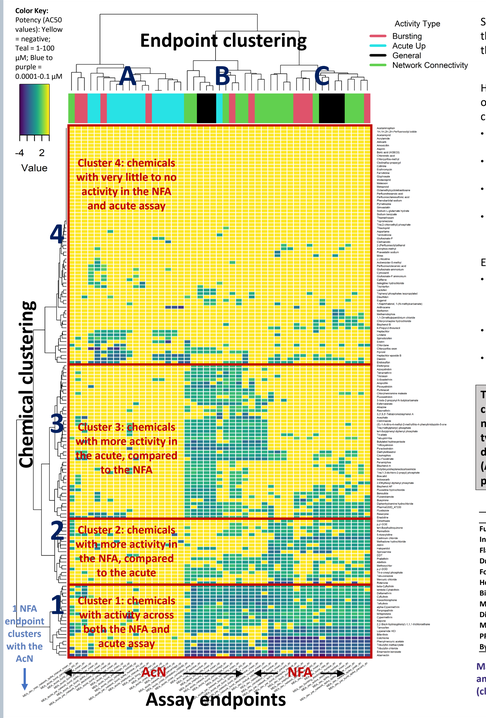
Cytotoxic endpoints	
NFA	18
AcN	1

The percent of selectively active chemicals was reduced in "Acute Up" and "NFA Up" endpoints for both the AcN and NFA.

The percent of selectively active chemicals for each assay endpoint was determined for the NFA and AcN. There were very few NFA "up" endpoints and, since these endpoints need further assay validation, they were removed from our analyses.



Selective potency (AC50 values) informs differential bioactivity patterns of acute (AcN) vs. developmental (NFA) exposure in the multi-concentration screens.



Selective potency was defined as those AC50 values that were lower than the minimum cytotoxicity.

Hierarchical clustering for the 154 overlapping chemicals reveals that chemicals cluster in 4 groups (y-axis):

- Cluster 1.) Chemicals with activity across both the NFA and AcN assay.
- Cluster 2.) Chemicals with activity in the NFA.
- Cluster 3.) Chemicals with activity in the AcN.
- Cluster 4.) Chemicals with very little to no activity in the NFA and AcN assay.

Endpoint clustering (x-axis) reveals:

- Cluster A.) AcN up endpoints cluster separately from the AcN down and NFA endpoints with one exception (an NFA endpoint).
- Cluster B.) AcN down endpoints cluster together.
- Cluster C.) NFA endpoints cluster together.

These analyses indicate that specific chemical clusters uniquely inform neurotoxicity hazard based on the type of exposure (acute or developmental). Selective potency (AC50 values) informs potential patterns of activity.

Cluster	1	2	3	4	Total
Fungicide	1	2	10	2	15
Insecticide	13	10	12	25	60
Flame retardant	0	1	4	3	8
Drug	4	4	10	16	34
Food	0	1	1	5	7
Herbicide	0	0	4	9	13
Bisphenol	0	0	2	1	3
Manufacturing	0	1	1	2	4
Disinfectant	1	0	0	0	1
Molluscicides	2	0	0	0	2
PFAS	0	0	1	5	6
Byproduct	0	0	0	1	1

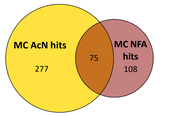
Many of the fungicides, flame retardants, and drugs were detected in the AcN (clusters 1 and 3).

Additional Results

There were 75 overlapping selective potency hitcalls in the MC NFA and AcN assays.

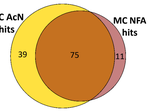
For all tested chemicals, the percentage of selective activity in the AcN and NFA are similar.

Assay	# selective chemicals	%
AcN	300/508	59%
NFA	215/395	54%



For just the 154 intersecting chemicals, the AcN has a higher percentage of selective activity.

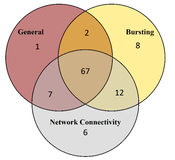
Assay	# selective chemicals	%
AcN	114/154	74%
NFA	86/154	56%



Overlap among the three broad categories of activity in the NFA and AcN were roughly similar.

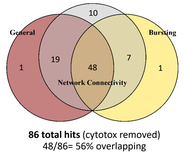
Assay Endpoint Categorizations
General
Mean firing rate
Burst number
Spike #
Active electrodes #
Burst rate
Bursting electrodes #
Bursting
Burst duration, mean
Burst %, mean
Burst % std
Per burst inter spike interval
Per burst spike # mean
Per burst spike %
Inter burst interval mean
Network Connectivity
Cross correlation area
Cross correlation HWHM
Network burst %
Per network burst, # electrodes mean
Per network burst, spike # mean
Per network burst, spike # std
Synchrony index
Spike duration mean
Per network spike, spike %
Per network spike, spike # mean
Correlation coefficient mean
Inter network spike interval mean
Mutual information norm
Network spike duration , std
Network spike #
Network spike peak

67 of the 103 selective chemicals in the AcN were active in all 3 broad categories



103 total hits (cytotox removed)
67/103= 65% overlapping

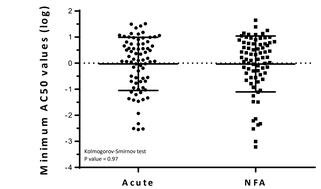
48 of the 86 selective chemicals in the NFA were active in all 3 broad categories



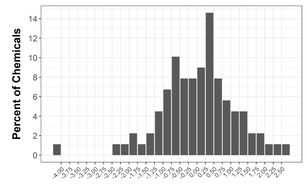
86 total hits (cytotox removed)
48/86= 56% overlapping

The distribution of the selective potency values and functional use categories in the NFA and AcN were similar.

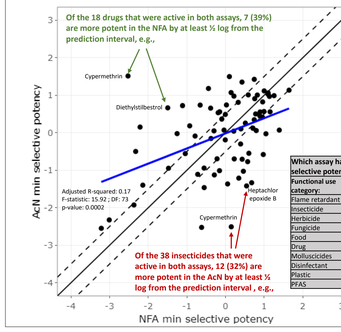
Selective potency (AC50) values for the intersecting chemicals from the NFA and AcN are similarly distributed.



Most chemicals tested in both the NFA and AcN are within a 1-log difference from each other.



Linear regression analysis indicates that the NFA and AcN are significantly correlated but not equivalent.



Which assay has a lower selective potency value?		
Functional use category:	AcN	NFA
Flame retardant	1	7
Insecticide	18	17
Herbicide	2	0
Fungicide	0	2
Food	0	1
Drug	9	8
Molluscicides	0	2
Disinfectant	1	0
Plastic	1	0
PFAS	3	0

Conclusions

- These data support our hypothesis that there were a greater number of chemicals with cytotoxic minimum AC50 values in the NFA compared to the acute (AcN). Overall, the AcN has a greater selective activity.
- Chemical activity in the AcN assay did not always predict activity in the NFA. The NFA detected 11 chemicals that the AcN did not detect whereas the AcN detected 39 chemicals that the NFA did not detect. In part, this analysis indicates the AcN and NFA may compliment one another to inform chemical neurotoxicity hazard as well as likely probe distinct neurobiological substrates and specific functional processes.
- The minimum potency (AC50) values were evenly distributed. Therefore, the disruption of network formation in the NFA did not occur at lower concentrations than disruption of network function in the AcN.