Characterization of signaling pathways in *in vitro* developmental neurotoxicity battery

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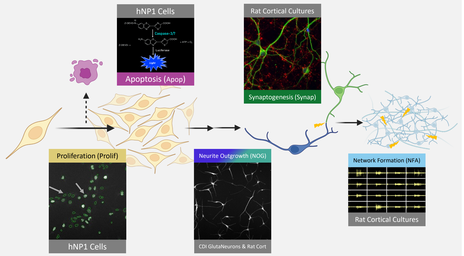
P331

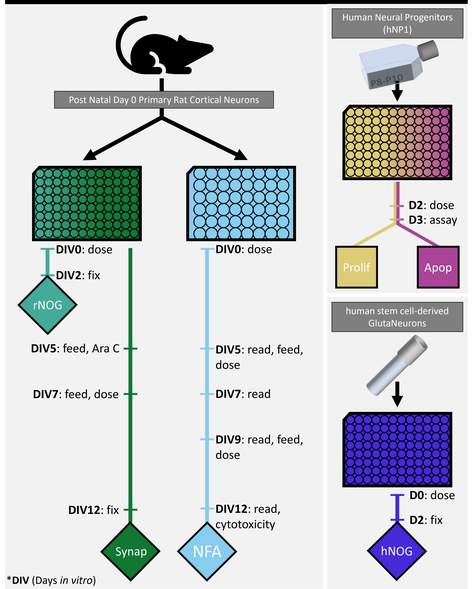
Background

- The EPA is moving towards cost effective and efficient methods for characterizing chemical hazard for risk assessment.
- Our lab uses a battery of cell-based assays to identify and characterize developmental neurotoxicity (DNT) hazard.
- These phenotypic assays evaluate chemical effects on key neurodevelopmental processes.



Objective:
Characterize the presence of important neurodevelopmental signaling pathways in our *in vitro* assay battery using specific chemical pathway modulators.

Methods



Pathway	Compound		Concentration
	Activator	Inhibitor	
BMPR	BMP2		2.5µg/mL
	BMP7		2.5µg/mL
Notch	DAPT		1nM - 30µM
EGFR	PD153035		0.3nM - 10µM
	AG1478		1nM - 30µM
Wnt	CHIR99021		0.8nM - 25µM
	IWP2		1nM - 30µM
PPAR-γ	Pioglitazone		1nM - 30µM
COX-2	Celecoxib		1nM - 30µM
mTORC	MHY1485		1nM - 30µM
	Rapamycin		1nM - 30µM
AKT	MK-2206		1nM - 30µM
	SC79		1nM - 30µM
PDGFR	CP-673451		0.3nM - 10µM
PKC	BIS-1/BIM-1		1nM - 30µM
RHO	Narciclasine		1nM - 30µM
EP1-4	PGE2		1nM - 30µM
CREB	KG-501		1nM - 30µM
ETC I	Rotenone		0.01nM - 1µM
NO-cGMP	ODQ		1nM - 30µM
ROCK	Y27632		1nM - 30µM
Protein Syn.	Cycloheximide		1nM - 30µM
HDAC	Vorinostat		1nM - 30µM
PARP1	Talazoparib		0.3nM - 10µM
ERK/MAPK	SCH772984		1nM - 30µM

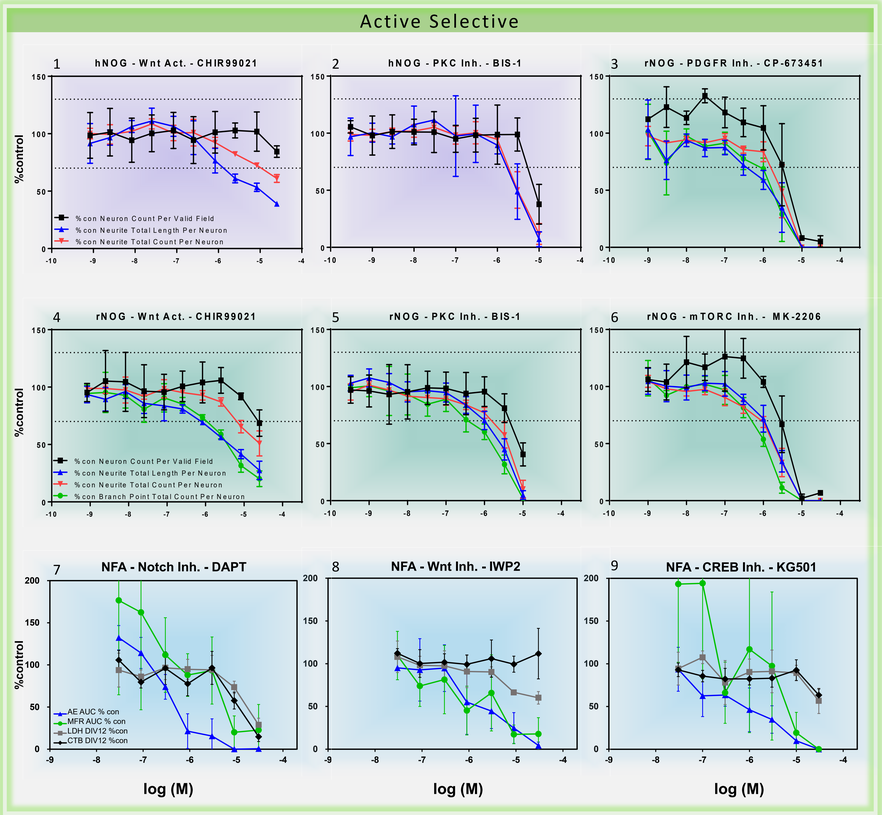
Work in progress
Evaluated qualitatively while awaiting quantitative analysis in ToxCast.

	BMPR	Notch	EGFR	Wnt	PPAR-γ	COX-2	mTORC	PDGFR	PKC	RHO	EP1-4	CREB	AKT	ETC I	EGFR	NO-cGMP	ROCK	Protein	HDAC	PARP1	ERK/MAPK	p38/MAPK
hNP1 Apoptosis		10						11														
hNP1 Proliferation																						
h. Neurite Outgrowth			1					2														
r. Neurite Outgrowth			4			6	3	5														
Cortical Synap.																						
Network Formation		7		8							9											

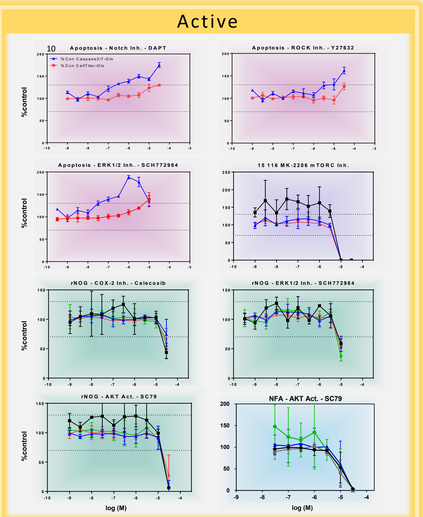
Active Selective
Active/Cytotoxic
Inactive
Awaiting Data
To be tested

*Numbers correspond to figures below

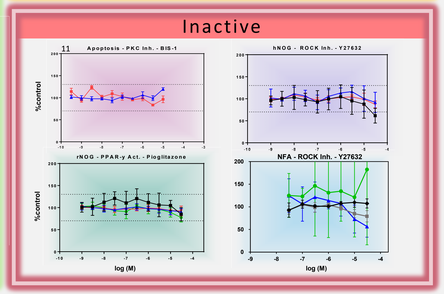
Active Selective



Active



Inactive



Preliminary ToxCast Analysis

- The current NOG data (15 chemicals) has been analyzed using ToxCast R package tcplfit2 (v0.1.6). The computed AC_{50} for each chemical and endpoint are given in the figure below.
- The color scale indicates selectivity, which is determined as the ratio between the cytotoxic AC_{50} and the AC_{50} for the endpoint of interest.

$$\text{Selectivity Ratio} = \frac{AC_{50}Cyto}{AC_{50}Endpoint} \geq 3, \text{ Selective Effect}$$

Computed AC_{50}

AC_{50}: Concentration (µM) at which 50% maximum activity is observed.

	Endpoint	Chemical: DAPT-Notch Inh.	ODQ-NO-cGMP Inh.	Pioglitazone-HCl PPAR-γ	IWP2-Wnt Inh.	Y-27632-ROCK	KG-501-CREB Inh.	AG1478-EGFR Inh.	Celecoxib-COX-2 Inh.	SCH772984-ERK1/2 Inh.	SC79-AKT Act.	MK-2206-mTORC Inh.	CP-673451-PDGFR Inh.	MHY1485-mTORC Act.	BIS-1/BIM-1-PKC Inh.	CHIR99021-Wnt Act.
rNOG	Neuron count	Not Active							15.00	5.00	30.00	3.23	4.31	15.00	10.00	25.00
	Neurite count per neuron								9.27	22.67	1.68	2.22		3.36	5.00	
	Neurite length per neuron								5.00	28.09	1.42	1.24	4.64	3.88	2.15	
	Branch points per neuron							5.25	8.62		0.80	1.22	2.98	2.44	3.66	
hNOG	Neuron count					2.97	1.50	2.97	9.30	20.79	4.88	4.50	30.00	10.00	25.00	
	Neurite count per neuron									11.65	4.61	3.41	15.00	2.58	2.76	
	Neurite length per neuron									9.74	6.29	3.11	15.00	2.62	1.30	

Selectivity Ratio: Selective (≥3), Non-selective (1)

* indicates no hit for cytotoxic endpoint, thus highest tested concentration was used to determine selectivity.

- Eventually, the AC_{50} for each chemical and endpoint in the DNT battery will be computed, showing which pathways are best covered by the DNT battery and where improvements are needed.

Conclusions

- The DNT battery captures some important and highly conserved signaling pathways associated with various processes involved in neurodevelopment.
- Specifically, we demonstrated that:
  - **Wnt**, **mTORC**, **PKC**, and **PDGFR** pathway modulators have selective effects on neurite outgrowth.
  - ERK1/2, AKT, and COX-2 pathway modulators demonstrated non-selective effects on neurite outgrowth.
- To date, ROCK, NO-cGMP, and COX-2 do not seem to play a significant role in our assays.

Future Directions:

- Ten chemicals remain to be tested in the battery.
- After all chemicals have been tested, a concentration-response analysis will be performed using the ToxCast pipeline.
- If we find that developmentally relevant biology is not being adequately recapitulated in the battery, we may explore incorporating other assays that include these pathways.

Discussion

- These results work in conjunction with the full OECD DNT battery developed in collaboration with global partners.
- Our work expands upon previous *in vitro* DNT pathway characterizations especially when it comes to neurite outgrowth[1], demonstrating that our battery is sensitive to **Wnt**, **PKC**, **PDGFR**, and **mTORC** disruption.
- A more robust characterization of the biology captured by our assays will aid in the interpretation of results gathered using the DNT battery.

References

1. Masjosthusmann, Stefan, Jonathan Blum, Kristina Bartmann, Xenia Dolde, Anna-Katharina Holzer, Lynn-Christin Stürzl, Eike Hagen Keßel, et al. "Establishment of an a Priori Protocol for the Implementation and Interpretation of an In-vitro Testing Battery for the Assessment of Developmental Neurotoxicity." *EFSA Supporting Publications* 17, no. 10 (October 2020). https://doi.org/10.2903/sp.efsa.2020.EN-1938.
2. Harrill, Joshua A., Theresa Freudenrich, Kathleen Wallace, Kenneth Ball, Timothy J. Shafer, and William R. Mundy. "Testing for Developmental Neurotoxicity Using a Battery of in Vitro Assays for Key Cellular Events in Neurodevelopment." *Toxicology and Applied Pharmacology* 354 (September 2018): 24–39. https://doi.org/10.1016/j.taap.2018.04.001.
3. Johnstone, Andrew F.M., Guenter W. Gross, Dieter G. Weiss, Olaf H.-U. Schroeder, Alexandra Gramowski, and Timothy J. Shafer. "Microelectrode Arrays: A Physiologically Based Neurotoxicity Testing Platform for the 21st Century." *NeuroToxicology* 31, no. 4 (August 2010): 331–50. https://doi.org/10.1016/j.neuro.2010.04.001.
4. Some images made with the help of BioRender.com assets

* The views expressed in this poster are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.