Multi-Hit Models of Neurotoxicity: Implications for Understanding Disease and Improving Risk Assessment

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Designing Better Animal Models and Epidemiological Studies

• Current experimental models for neurotoxicology are generally of limited utility
  ▪ They don’t reflect current understanding of the complex basis of CNS disease and disorders or the complexity of human physiology
  ▪ They typically presume single mechanism of effect for an environmental chemical exposure
  ▪ They assume that an environmental exposure alone is sufficient to produce a phenotype; if it does, it will likely require high doses, leading to the comment that it is environmentally irrelevant
  ▪ For similar reasons, current models slow the process of therapeutic and neuroprotective discovery

• More complex models that incorporate multiple risk factors are critical to move forward our understanding of the contribution of environmental chemicals to CNS diseases and disorders
  ▪ They more accurately model characteristics of diseases and disorders and of the human environment
  ▪ They will therefore provide a truer assessment of the risks posed by environmental chemicals for neurodegenerative diseases and disorders and thus to public health protection
  ▪ They will provide a more realistic basis for therapeutic and neuroprotective strategies
Disease Complexity

• It is increasingly apparent that the most intractable diseases and disorders are complex and multifactorial in nature rather than the product of a single etiological factor.

• The importance of non-genetic contributions, including environmental risk factors in complex disease is underscored by estimates that single gene mutations account for less than 5% of incidence, at least in the case of cancers and cardiovascular-related diseases (Willet, Science, 2002; 296:695). Similar considerations apply to diseases of the nervous system.

• Human function reflects the integrated activity of multiple complex biological systems, generally not the actions of single molecules or pathways.
Interactions of Multiple Disease and Protective Factors Over Time Determine an Individual’s Health Status

Each Individual Has a Unique Set of Risk Factors
Behavioral function requires communications across multiple pathways and systems utilizing common neurotransmitters, increasing the matrix for damage, and producing an infrastructure that can amplify adverse effects. Since neurotransmitters are common to multiple regions, insults targeting neurotransmitter function can impact multiple regions and thereby influence a wider array of behavioral functions.
In Contrast, Most Neurotoxicology Models Study Toxicants as Risk Factors in Isolation

Study of one chemical in isolation in a healthy young organism, maybe examining e.g., gender
Not Just a Problem in Experimental Models

- Epidemiological studies generally focus on ‘main effects’ of an exposure
  - Often impose ‘statistical control’ for potential modifiers to minimize their influence on outcome
  - Often underpowered and thus can’t examine risk factor interactions
Potential Consequences of Multiple Risk Factors

- **Multiple Hits and Impairment of Homeostasis**
  Exposure to an individual chemical may be insufficient to induce overt effects, whereas multiple risks, by provoking changes concurrently at multiple different target sites of the dopamine system, would impair the operation of homeostatic mechanisms, leading to dopamine dysfunction and neuronal cell death.

- **Multiple Hits Converging on a Common Adverse Outcome**
  Single insults act by different mechanisms that converge upon a common outcome resulting in cumulative toxicity; e.g., impaired cognitive development
Models Based on Single Risk Factors

- Effectively examine the contribution of a chemical as a ‘sole etiology’ for an adverse outcome.
  - This assumes that the disease phenotype results from a single mechanism, which increasingly appears to be inconsistent with many neurological diseases and disorders.
  - This assumes that the impact of a chemical exposure is so severe that it alone produces the phenotype. Under such conditions, much higher exposure levels are likely to be required than would be the case when exposure occurs in conjunction with other known risk factors.

- Consequently:
  We’re picking the low-hanging fruit, i.e., the chemical contributions with the largest effects
Improving Experimental Models of CNS Diseases And Disorders

• Does studying chemical exposures in the context of other pertinent risk factors:
  – Provide more realistic models of the disease/disorder phenotype?
    • an outcome with implications for neuroprotective and therapeutic strategies
  – Demonstrate effects of chemical exposures at lower levels or in a synergistic or potentiated capacity?
    • outcomes with significant implications for risk assessment and public health protection
Two Examples Related to Brain DA Systems

• Pesticides and Parkinson’s Disease (PD)
  ▪ Humans are simultaneously exposed to multiple pesticides, along with other host and environmental insults that affect brain dopamine systems involved in PD

• Environmental Lead (Pb) Exposure and Stress
  ▪ Highest levels of Pb exposure occur in low SES communities, the same populations already thought to have the highest levels of stress, making Pb and stress co-occurring risk factors. They also share biological substrates and produce common adverse outcomes. Both Pb and stress act on brain dopamine systems and the HPA axis, systems key to cognitive functions
Parkinson’s Disease as a Complex Disorder

• **Vulnerability Factors**
  - Age
  - Genetic background (e.g., α-synuclein, DJ1, parkin)
  - Boxing
  - Farming
  - Drinking Well Water
  - Pesticide Exposure
  - Gender
  - Diet

• **Protective Factors**
  - Gender
  - Smoking
  - Caffeine
  - Exercise
  - Diet
Parkinson’s Disease: A Disorder of the Nigrostriatal Dopamine System

• Loss of dopamine (TH+) cells in substantia nigra
• Loss of dopamine terminals and associated markers
• Reduction in levels of dopamine and metabolites and dopamine turnover
• Symptoms and signs occur with loss of approximately 80% of dopamine neurons
• Characteristic pathology: Lewy bodies, intracytoplasmic inclusions containing alpha-synuclein, ubiquitin and parkin proteins

Lewy bodies
Pesticide Exposure Link to PD

- Similarity of the herbicide paraquat to MPP+, the active metabolite of MPTP, a component of illegally manufactured heroin that produced severe progressive PD in young drug addicts (Barbeau, 1983).
- Subsequent associations of pesticide exposure with PD in numerous epidemiological studies and case reports.
Humans are Exposed to Multiple Pesticides that can Influence Dopamine Function
Dopaminergic Effects of the Ethylenebisdithiocarbamate Fungicide Maneb

- Decreases locomotor activity
- Potentiates MPTP effects on locomotor activity and catalepsy
- Enhances the uptake of MPTP into brain
- To date, two incidences of Parkinsonism in humans have been related to occupational manebo exposures
Risk Factor Interactions: Pesticides and Parkinson’s Disease

- Pesticide A
- Pesticides A+B
- Pesticides A+B+Genetic Background
- Pesticides A+B+Aging
- Pesticides A+B+Cumulative Toxicity
- Pesticides A+B+Development+Gender
Young Adult Model: Multiple Chemical Exposures that Affect DA Systems

Schedule of i.p. injections

Dose Groups:
- Saline
- 10 mg/kg paraquat
- 30 mg/kg maneb
- 10 mg/kg paraquat + 30 mg/kg maneb

Thiruchelvam et al., 2000
Combined PQ+MB Selectively Reduced Nigral Dopamine Neuron Immunoreactivity

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Thiruchelvam et al., 2000

Subsequently confirmed using unbiased stereology.
MB Increases PQ Accumulation and Delays its Clearance from Brain

What other food, chemical, drug etc might cause similar toxicokinetic interactions?

Barlow et al., 2003
Levels of lipid peroxidation in various brain regions in the young adult model with exposures to saline, PQ alone, MB alone or combined PQ+MB

Thiruchelvam et al., 2005
Gene-Environment Interactions

Normal (TG-)

+ human normal $\alpha$-synuclein (Hw5)

+ human double mutant $\alpha$-synuclein (HM$^2$)
Mutated Alpha-Synuclein Enhances Vulnerability to PQ+MB

Loss of TH+ Neurons Two Weeks Post-Treatment

* From corresponding saline; + from Hw5; # from TG-

Thiruchelvam et al., 2004
Aging and PQ+MB: Enhanced DA Cell Loss and Permanent and Progressive Effects

Measured Two Weeks and Three Months Post-Dosing

Thiruchelvam et al., 2003
Early Development and PD Risk?

The etiological risk factors for the disease may occur at a far different time than that at which the phenotype is expressed.

Cory-Slechta et al., 2005
Experimental Design: Postnatal Pesticide Exposure

- Can developmental insults lead to a PD phenotype?
- Can they alter vulnerability to risk factors later in life?

Thiruchelvam et al., 2002
Decreases in DA neurons following developmental exposures are significantly enhanced when followed by an adult exposure to pesticides as compared to adult only exposures. No changes in numbers of TH- neurons.

Thiruchelvam et al., 2002
Progressive Decline in DA Cell Loss With Age in Males Following Postnatal Exposure

Stereological counts of substantia nigral DA neurons across the lifetime following PND 5-19 exposure to saline, PQ alone, MB alone or combined PQ+MB

Thiruchelvam et al., ongoing
Progressive Decline with Age in Striatal Dopamine in Males Following Postnatal Exposure

Striatal dopamine levels across the lifetime following PND5-19 exposure to saline, PQ alone, MB alone or combined PQ+MB

Thiruchelvam et al., ongoing
Parkinson’s Disease and Residential Exposure to Maneb and Paraquat from Agricultural Applications in the Central Valley of California

- PD risk increased for DAT A clade diplotype OR=1.66) and for 3’VNTR (OR=1.8).

- High exposure to PQ + MB increased PD risk 3 fold in carriers of one susceptibility allele (OR=2.99)

- High exposure to PQ+MB increased PD risk >4 fold in carriers of 2+ alleles (OR=4.53).

- Same effects were found for occupational pesticide exposures

Ritz et al., 2009. Am. J. Epidemiology, vol. 169:919-926
Improving Experimental Models of CNS Diseases And Disorders

• Does studying chemical exposures in the context of other pertinent risk factors:
  – Provide more realistic models of the disease/disorder phenotype?
    • YES
  – Demonstrate effects of chemical exposures at lower levels or in a synergistic or potentiated capacity?
    • YES
Elevated Pb exposure in the US is now demographically circumscribed. Highest blood Pbbs occur in:

- Low socioeconomic status (SES)
- Minority children
- Living in old housing
- Medically-underserved
- Living in poverty
- In urban environments
Defined PbB of Concern for U.S. Children

Based on reported deficits in cognitive/neurological function in studies with children
Pb is absorbed from lung and GI tract and enters bloodstream.

- Plasma delivers Pb to soft tissue
- Soft tissue Pb half-life are approximately 30-40 days
- Most Pb is found in bone (95%) where it has a half life of decades but maintains a dynamic equilibrium with blood
- Pb passes unimpeded to fetus via placental transfer
Pb and Stress Are Co-Occurring Risk Factors

i.e., blood Pb levels are elevated in the same populations thought to experience the highest levels of stress.
Low Socioeconomic Status as a Risk Factor

• Low SES is a known risk factor for increased incidence of multiple diseases and disorders, even after controlling for access to medical care

• It has been hypothesized that this is due to higher levels of stress associated with such environments which results in chronic elevation of glucocorticoids

• Consistent with reports of increases in cortisol levels in low SES groups

• Both stress per se and chronic elevation of glucocorticoids are associated with increases in multiple diseases and disorders
Pb and Stress Share Biological Substrates and Produce Common Adverse Effects

- Both Pb and stress act on brain mesocorticolimbic dopamine and hippocampal systems
- Both Pb and stress alter the function of the HPA axis, the body’s major physiological response system for stress
  - HPA axis and mesocorticolimbic systems have extensive interactions
- Pb and stress are associated with increased incidence of similar diseases and dysfunctions (e.g., diabetes, obesity, hypertension, cardiovascular disease, schizophrenia)
- Pb and stress also evoke similar behavioral dysfunctions
  - Cognitive dysfunction, attention deficit

Rossi-George et al., 2008
Can Pb and Stress Act Synergistically?

**HPA Axis**
- Hippocampus/Amygdala
- Hypothalamus (PVN) → CRH, AVPP
- Anterior Pituitary → ACTH
- Adrenal Cortex

**Glucocorticoids/Catecholamines**
- CV Disease
- Hypertension
- Metabolic Disease
- Obesity
- Diabetes
- Metabolic Syndrome
- Inflammatory Diseases
- Arthritis
- Cognitive Decline
- Psychiatric Disorders
- Depression
- Anxiety

**Factors**
- Pb
- Low SES Communities
- Stress

**Pathways**
- Glucocorticoids
- Catecholamines
Experimental Design: Pb and Stress

**Pb Exposure**
- Initiated 2 mos prior to dam breeding

**Maternal Pb**
- Ended at pup weaning

**Continuous Pb**
- Exposure continued in pup postweaning

**Prenatal Stress**
- 45 min immobilization 3x/day on GD16-17

**Maternal Pb Exposed Groups**
- 0 ppm
  - No Stress (NS)
  - Prenatal Stress (PS)
  - Prenatal + Offspring Stress (OS)
- 50 ppm
  - No Stress (NS)
  - Prenatal Stress (PS)
  - Prenatal + Offspring Stress (OS)
- 150 ppm
  - No Stress (NS)
  - Prenatal Stress (PS)
  - Prenatal + Offspring Stress (OS)
Maternal Pb Alone Permanently Alters Corticosterone, the Body’s Primary Stress Hormone

Increases in basal levels were seen in both genders, and were of comparable magnitude in response to Pb alone, and Pb+stress

Cory-Slechta et al. 2004
Corticosterone in females is increased by Pb at 2 mos of age (basal), but decreased at 10 mos of age (final), with further suppression produced by maternal stress, and even further by offspring stress.

Cory-Slechta et al., 2010
Fixed Interval Schedule of Reinforcement

The first response occurring after a fixed interval of time has elapsed (60 sec in this example) results in reward delivery and the initiation of the next fixed interval of time. The schedule produces a pattern of responding that is very low early in the interval but increases as the time to reward approaches. Human example: studying for an exam.
Measures of FI Performance

Fixed Interval Performance

Overall Rate:  Total number of responses by total session time

Postreinforcement Pause (PRP):  Time to the first response in an interval

Run Rate:  Rate of responding with PRP time subtracted out
• Pb routinely impacts FI performance
  ▪ Occurs across species and across developmental periods of exposure
  ▪ Inverse U-shaped dose-effect curve

Cory-Slechta, 1995
FI Response Rates Predict Impulsivity (Self Control) in Children

**Impulsive choice:**
Short delay followed by small reward

**Self-control choice:**
Long delay followed by large reward
Fl Response Rates Predict Impulsivity in Children

Darcheville et al., 1992
Maternal Pb + Stress: ‘Exposure and Stress Condition’-Related Potentiated Effects in Females

Females exhibit ‘Pb-concentration and stress-condition’-related increases in FI response rates: potentiated effects of 50 ppm and maternal and offspring stress

Virgolini et al., 2008
Potentiated Effects of Pb and Stress Condition on Brain Neurotransmitters: Frontal Cortex

Marked neurochemical changes occur in female offspring, and, importantly, in some cases changes reflect combined Pb concentration-stress condition effects.

Virgolini et al., 2008
Potentiated Effects of Pb and Stress Condition on Brain Neurotransmitters: Frontal Cortex

NS=No stress; MS=maternal stress; MS+OS= maternal and offspring stress

Virgolini et al., 2008
Multiple Schedule of Repeated Learning & Performance

Accuracy Component: Requires completion of a new 3 member response sequence that changes with each daily session

Performance Component: Requires completion of a 3 member response sequence that remains constant across the experiment
Combined Lifetime Pb & Stress Effects on Learning

Preferential Impairments in Females: Lower accuracy and delayed improvement over time, requiring more responses to achieve a completed sequence, an effect that cannot be attributed to lowered response rates.

Cory-Slechta et al., 2010
Repeated Learning: Lifetime Pb + Stress and Sequence-Specific Learning Deficits in Females

For sequence LRC, a synergistic reduction in accuracy was evident in training, with selective reductions in the 50-PS group, and not with 50-NS or 0-PS. No corresponding changes in response rates were found.

Cory-Slechta et al., 2010
But Other Sequences Reveal Individual Subject Vulnerability

Thus, of the total subjects, 1 of 8 (13%) 0-PS subjects, 5 of 11 (45%; chi square=17.6, p<0.0001) 50-NS subjects, and 6 of 9 (67%; chi square=28.13, p<0.0001) 50-PS subjects exhibited behavioral deficits when considered across sequences.

Cory-Slechta et al., 2010
Importance of Corticosterone and Mesocortical Dopamine to Learning Deficits in Females

As yet unclear is whether corticosterone/HPA changes induce neurochemical modifications that underlie behavior or vice versa

Cory-Slechta et al., 2010
Hippocampal NGF Was Reduced by Continuous Pb and PS in Females but Not Males

NGF is a molecule with clear significance for learning processes given its critical role in synaptic plasticity and the stabilization of synaptic structures, especially of cholinergic neurons.

Cory-Slechta et al., 2010
Higher Pb Enhances the Cortisol Response to Acute Stress in Children

Figure 1. Children’s unadjusted initial salivary cortisol levels (log-ng/dL) and after an acute stress task as a function of quartiles of prenatal (A) and postnatal (B) Pb exposure.

Gump et al., 2007
Interactions of Stress, Lead Burden and Age on Cognition in Older Men: the VA Normative Aging Study

Figure 2a. Relationship between age and predicted Mini-Mental State Examination (MMSE) by combined high perceived stress (PSS) and high blood lead categories representing high on one or both.

Weisskopf et al., 2010
Improving Experimental Models of CNS Diseases And Disorders

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  – Provide more realistic models of the disease/disorder phenotype?
    • YES
  – Demonstrate effects of chemical exposures at lower levels or in a synergistic or potentiated capacity?
    • YES
Significance of Complex Models

- Multifactorial animal models are likely to more precisely reflect human nervous system disease and dysfunction, thereby facilitating:
  - the evaluation of mechanism(s) of effect and vulnerability
  - the understanding of different and common pathophysiological processes across neurological diseases and disorders
  - the discovery of therapeutic and neuroprotective strategies

- Refined animal models permit design of more focused epidemiological/cohort studies: forward translation
  - Information from more focused cohort studies can also ‘back translate’ to further refine experimental animal models

- More realistic models and epi studies increase precision of risk assessment estimates by reducing uncertainties
  - Therefore they increase prevention and enhance public health protection
Model Complexity Can Improve Understanding of Mechanisms

- Gender
- Ubiquitin
- Copper
- Dieldrin?
- Rotenone
- Paraquat
- Nurr1?
- Maneb
- Copper + Lead
- Parkin
- α-synuclein
- Aging
- Iron + Lead
- Manganese
- Prenatal LPS

Parkinson’s Disease Phenotype
Ongoing Insults Oxidative Stress Source

**Risk 1**
- Complex 1 Inhibition
- Increased Iron
- Reduced GSH
- DA Oxidation

**Risk 2**
- Complex 1 Inhibition
- Increased Iron
- Reduced GSH
- DA Oxidation

**Risk 3**
- Complex 1 Inhibition
- Increased Iron
- Reduced GSH
- DA Oxidation

**Scheme 1**
- Oxidative Stress
- DA Neuron Cell Death
- PD Phenotype

**Scheme 2**
- Oxidative Stress
- DA Neuron Cell Death
- PD Phenotype

PD phenotype results from a cumulative or net increase in oxidative stress arising from multiple sources.

**Scheme 3**
- Oxidative Stress
- DA Neuron Cell Death
- PD Phenotype

Activation of preferentially vulnerable pathway(s) leads to oxidative stress (heavy arrow) and its disruption is sufficient to initiate a process of neurodegeneration.

A set of primary oxidative stress pathways (solid weighted arrows) must be concurrently activated to tip the balance of oxidative and anti-oxidative events to increases rather than effective management.

Model Complexity Can Improve Understanding of Mechanisms
Implications for Risk Assessment Related to Environmental Chemicals that Influence Brain

• Potentiated effects (no effect of treatment A or B alone, but significant effects when A and B are combined) will never be captured by current risk assessment methodology
  ▪ Do 10-fold safety factors adequately encompass the risks?
• Cumulative/silent neurotoxicity are not captured in current neurotoxicology risk assessment
• How do we define which interactions to assess?
Improving Models of Toxicity: Where Do We Begin?

• Inclusion of:
  ▪ Co-occurring risk factors (chemical/non-chemical)
  ▪ That share biological substrates or targets
  ▪ That produce common adverse outcomes
  ▪ And/or have potential for toxicokinetic interactions

  – *Combining chemicals by their common adverse effects actually circumscribes and defines the a set of mixtures to study*

  – *Requires moving forward with cumulative risk paradigms*
• Mona J. Thiruchelvam
• Eric K. Richfield
• Miriam Virgolini
• Veronica Rodriguez
• Brian Barlow
• Alba Rossi-George

• Becky Goodman
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