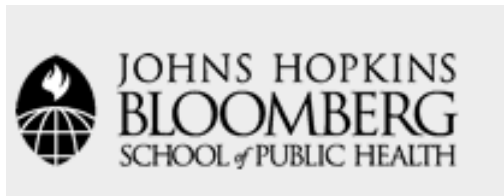
MOUSE EMBRYONIC STEM CELLS AND ENVIRONMENTAL HEALTH SCIENCES

Joseph Bressler



Why study environmental health sciences

- To identify environmental pollutants and occupational hazards that affect human health
- To uncover mechanisms underlying the toxicity of environmental pollutants
- To characterize biomarkers that will identify populations exposed to these pollutants and hazards
- To use the information from mechanistic studies to identify populations at risk. For example, genes (polymorphisms), gender, and development (children and seniors)

Identifying Toxicants: Technological Challenges

- New chemicals and drugs are introduced yearly that must be screened for toxicity.
- In Europe, screening must be conducted using in vitro methods
- In neurotoxicity, in vitro models must be developed that display the functions performed by neurons, astrocytes, oligodendrocytes, the blood brain, and choroid brain barriers. Also, in vitro models must be developed for identifying toxicants that are effective during development
- High input screening methods will also be developed for testing the high number of chemicals that need to be tested

Why Is Autism Increasing? Hg In Vaccines

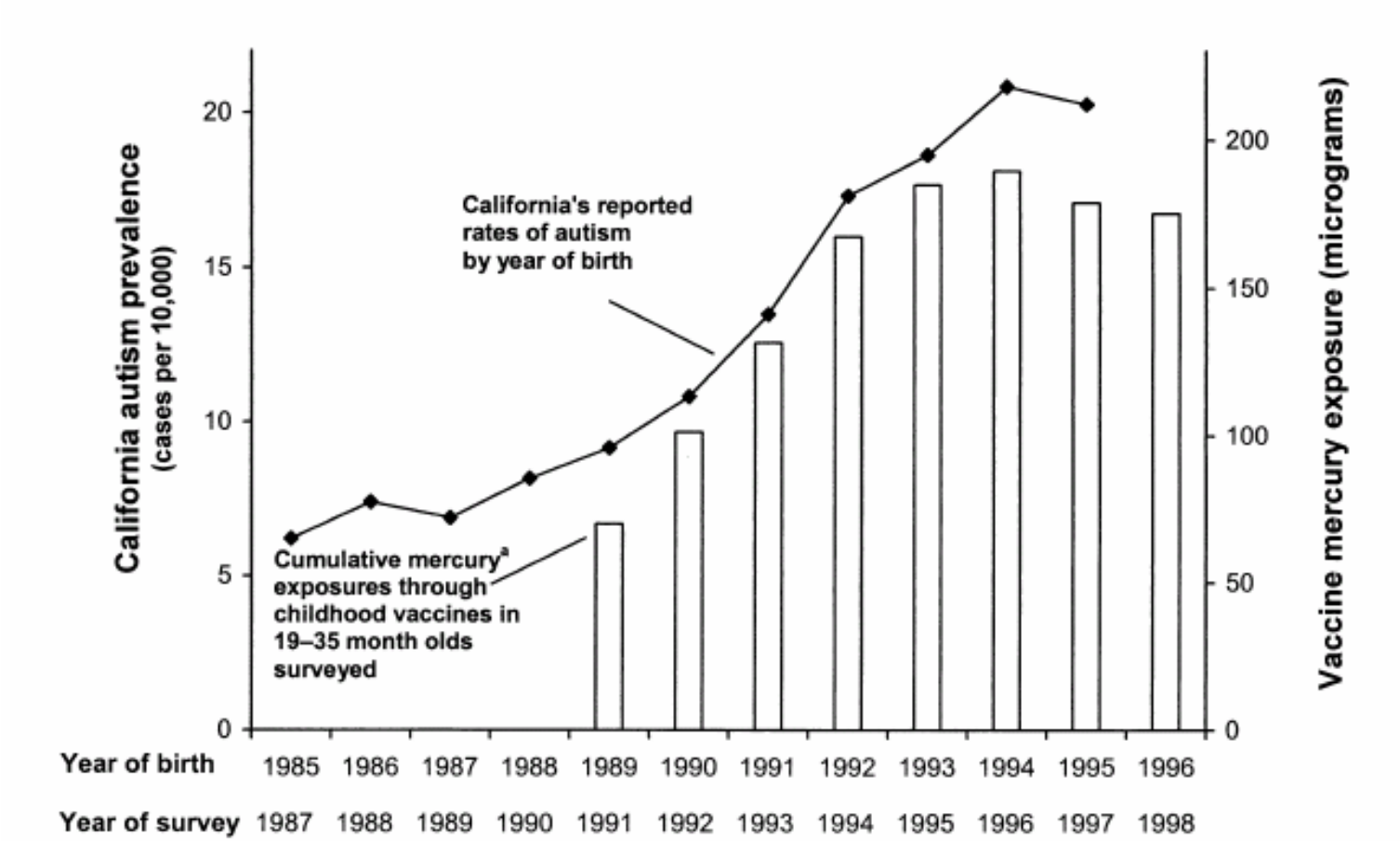
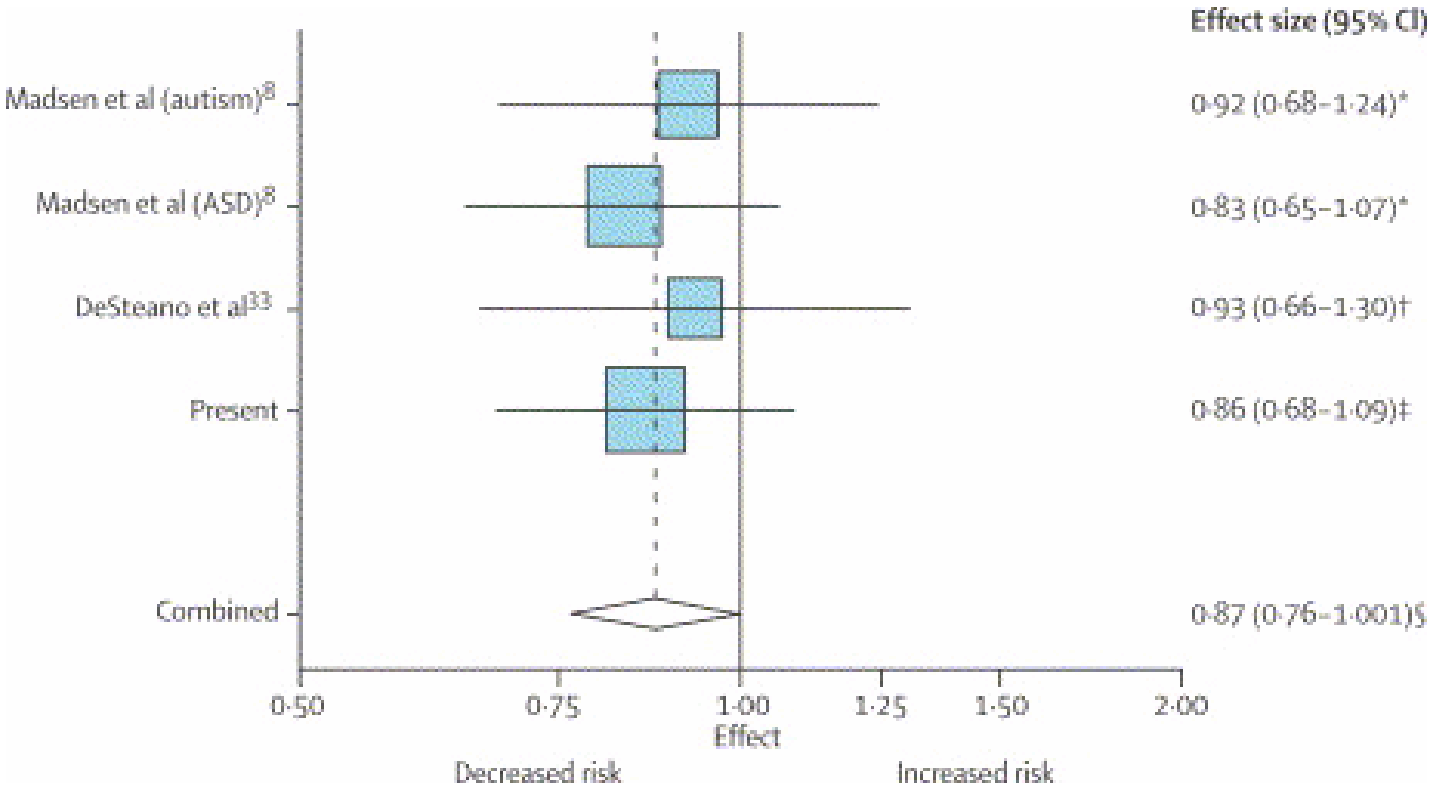


Figure 1. Graphical ecologic analysis presented by Blaxill[3] to the Institute of Medicine on July 16, 2001, comparing the estimated average cumulative dose of mercury exposure in the United States from vaccines, and the estimated prevalence (per 10,000 population) of children diagnosed with autism-like disorders seeking special education services for autism in California from 1987 to 1998, by birth-year cohort.

[a]Includes DPT, *Haemophilus influenza* B, and hepatitis B exposures weighted by survey year compliance.

No association between vaccines



Smeeth et al., 2004 Lance

Measles-mumps vaccine (thimersol) not associated with autism

Environmental Involvement In Autism

- • Siblings 3 to 8% chance, whereas in the general population it is 0.16%-Genetic
- Penetrance 30-75%-Genetic maternal rubella infection (Chess, 1971);
- • ethanol (Nanson, 1992);
- • thalidomide (Strömland et al., 1994);
- • valproic acid (Moore et al., 2000);
- • misoprostol (Bandim et al., 2003).

Int. J. Devl Neuroscience 23 (2005) 189–199

Problems in identifying neurodevelopmental toxicants

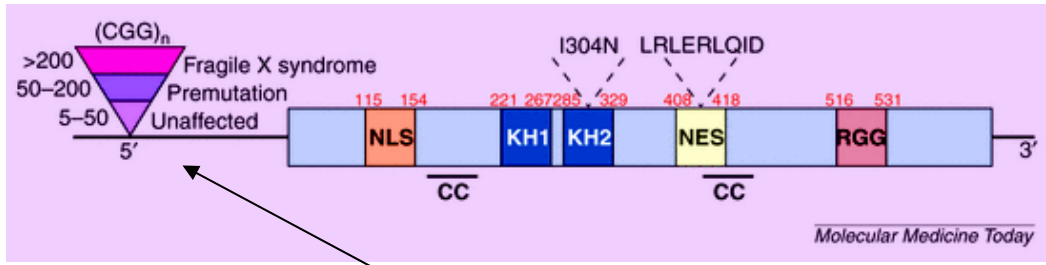
- Autism likely has a complex etiology involving interplay between genetics and exposures
- The behavioral outcomes of autism are first observed at 2 yrs of age but the biochemical and morphological mechanism affects took place in utero

Learn From What is Known
Fragile X
2 Developmental Stages Affected

Table 1 MAJOR EVENTS IN CORTICAL HISTOGENESIS AND TIMETABLE IN HUMANS

Event	Timing
Prosencephalic differentiation	1–3 prental months
Neuronal proliferation	2–5 prental months
Neuronal migration	3–5 prental months
Organization and neurite development	Third prental month–early childhood
Regressive events	? Early childhood–adolescence
Cortical glial development	2–5 prenatal months, fifth prenatal month–early childhood

***FMR1* gene/FMRP**



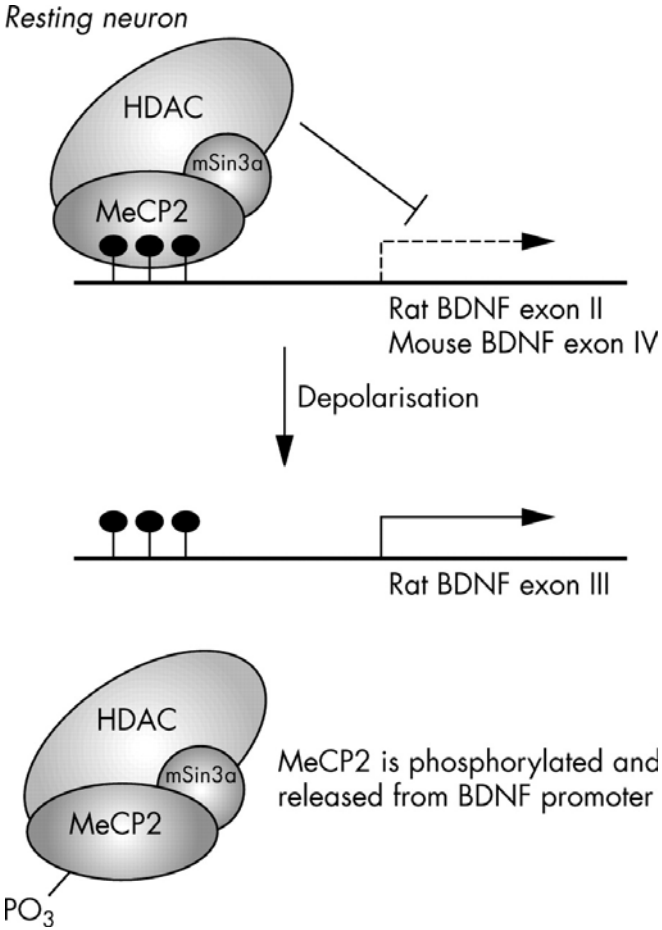
Expansion of CpG islands

Some physical features are disturbed
Variable mental retardation
Autistic like features
Seizures
Hyperactivity
DECLINE IN IQ

Hinton et al. Am J Med Genet 41:289-294, 1991

Rett Syndrome Also Involves DNA Methylation

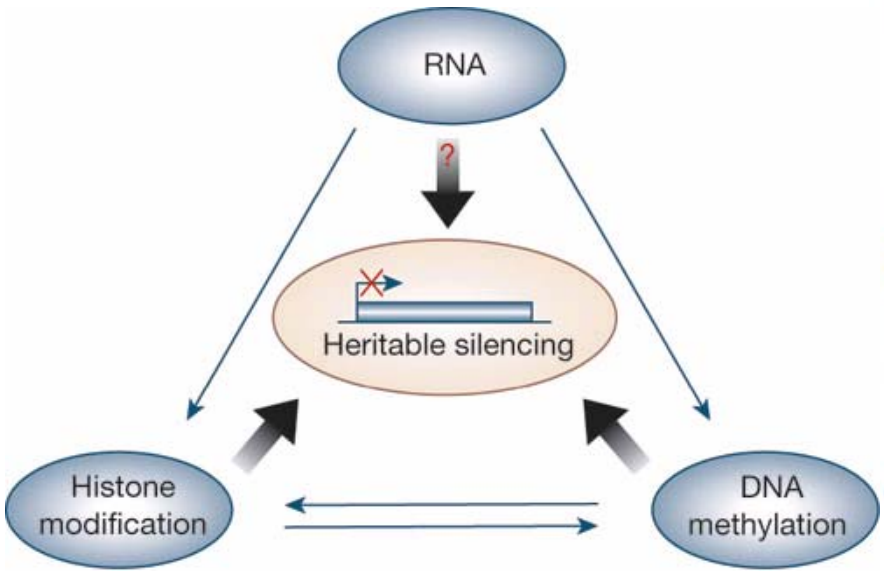
(Missense) Mutations in MeCP2, which binds to methylated DNA



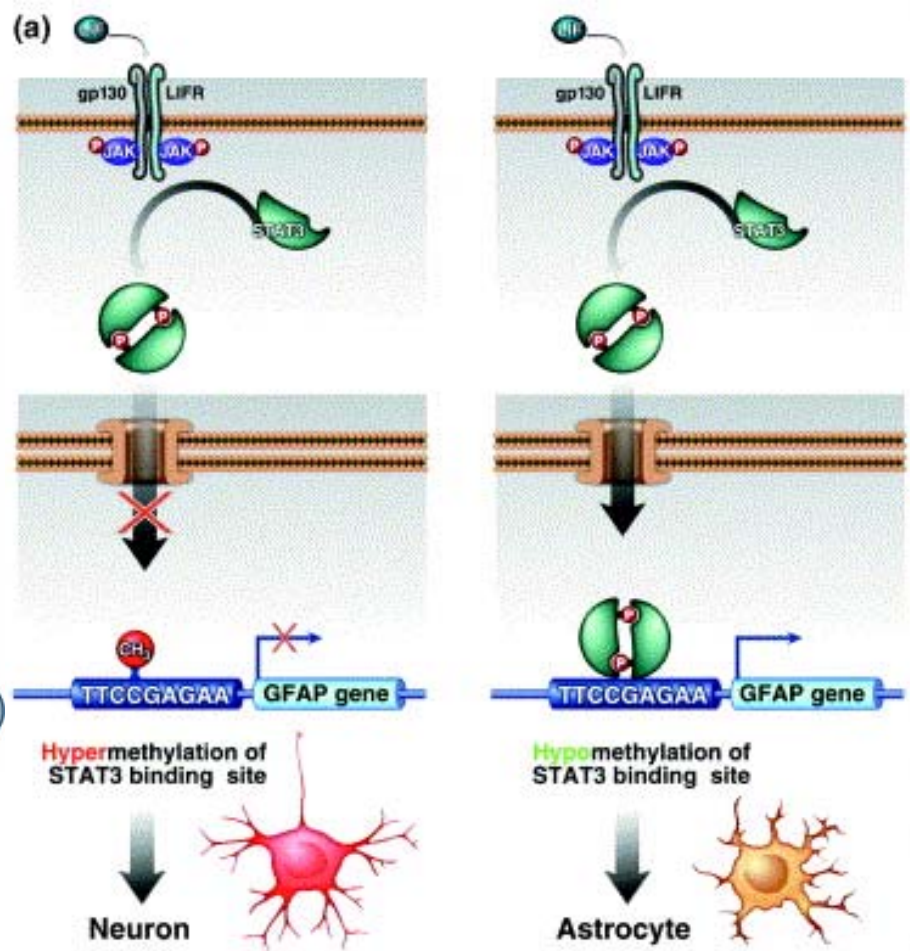
Two isoforms:
MeCP2B>>MeCP2A
In Brain

Weaving, L S et al. J Med Genet 2005;42:1-7

Astrocyte: Glial Fibrillary Acidic Protein (GFAP) Is Regulated By DNA Methylation



deacetylase



In Vivo Models For Studying Developmental Neurotoxicants

- Zebrafish- genetics, in vivo localization
- Xenopus- knock ins, large oocytes for injections
- Mice- genetics, conventional

Regulating Use Of Experimental Animals

- Animal Welfare Act-U.S.D.A.-focuses on refinement not replacement or reduction and does not cover rodents or non mammals
- Public Health Service-through the Office of Laboratory of Animal Welfare-adopts the policies of the act but includes rodents etc. It is a policy of the PHS not a law. PHS funds NIH! PHS requires animal use committee.
- Toxicant Control Act-EPA follows guidelines similar to the AWS. Again, no stipulation about alternatives

Animal Use Committee Application

- Description of procedure
- Explain number of animals planned to be used
- Category of pain (will the animal experience pain, will you alleviate pain)
- Repeated procedures on the same animal
- All drugs and surgeries need to be recorded.
- Not duplicating other studies
- Are there alternatives?

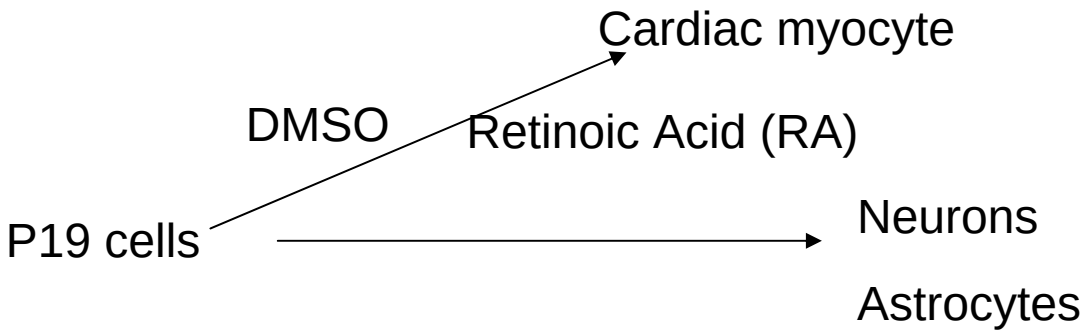
Research In Alternatives In US

- Interagency Coordination Committee Validation Alternative Methods
- does not generate own data
- does not fund
- located within NIEHS/EPA
- CAAT (maximum US $20,000)
- Proctor Gamble (maximum grant is $50,000)
- Alternatives In Research Development Program (maximum US $40,000)

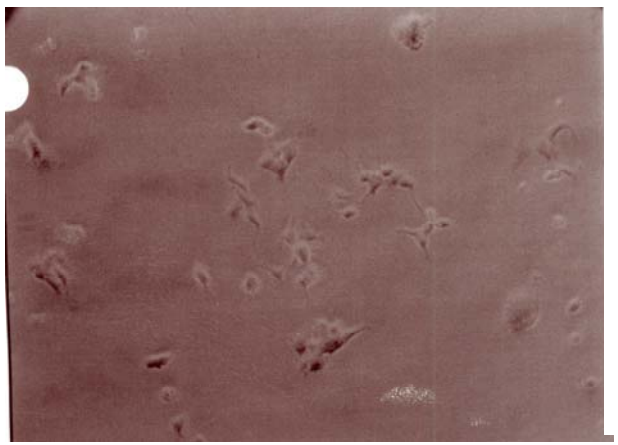
In Vitro Model: To identify neurodevelopmental toxicants

- Must represent stem cells before commitment to neural lineage
- Must be sensitive to chemicals that alter DNA methylation and/or histone modifications

Mouse Embryonic Carcinoma Cell Line P19 cells

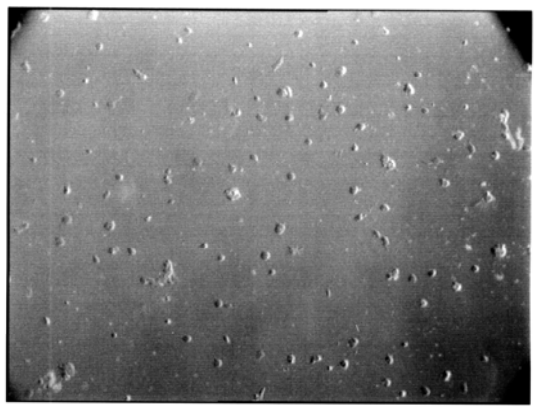


Before RA



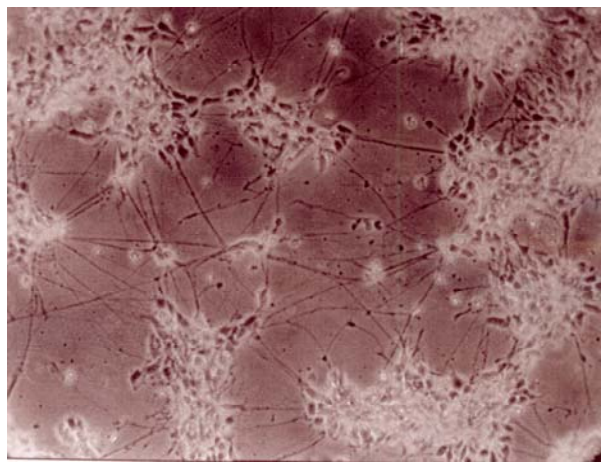
pre-neuralation

1 day after RA



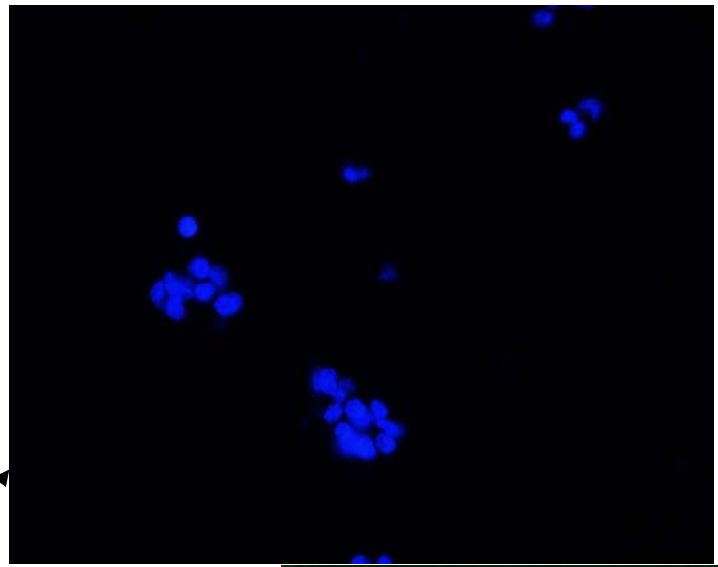
neural stem cell

4 d after RA

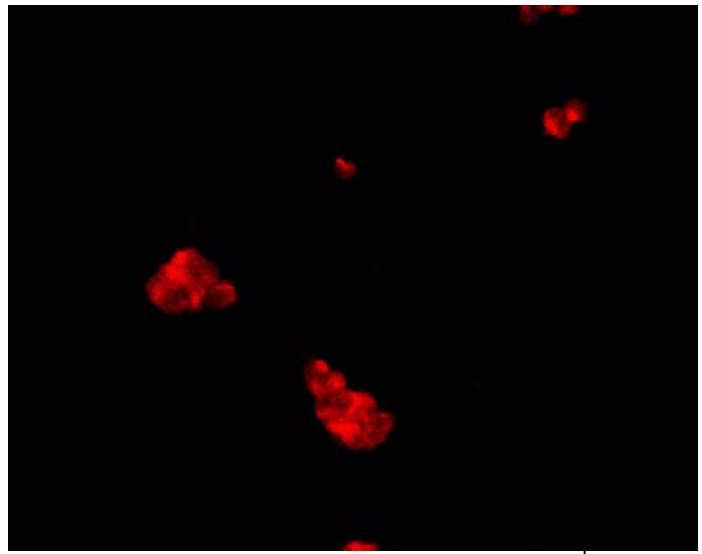


differentiating neurons

Differentiation to Neurons and Astrocytes

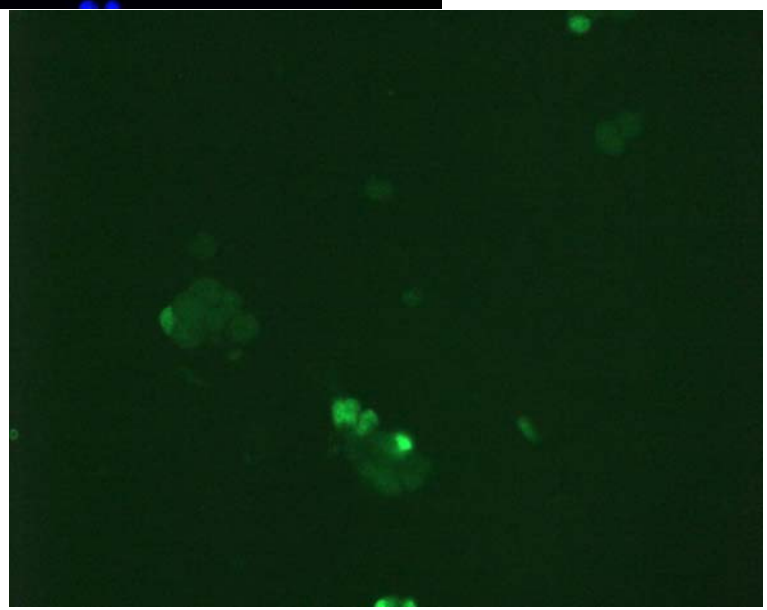


DAPI



GFAP

NueN



P19 Cells Expresses Zinc Transporter 4 Only Expressed Early Embryogenesis

(RA) **Before** **After**

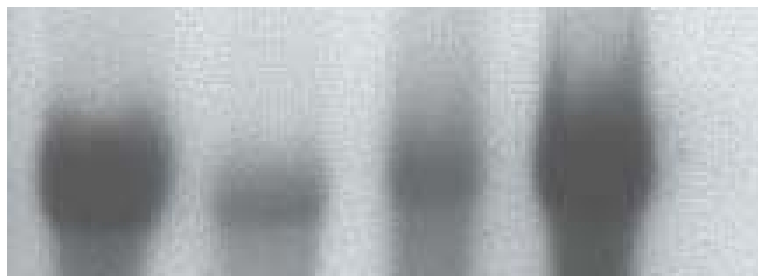
zip4

zip1

zip4

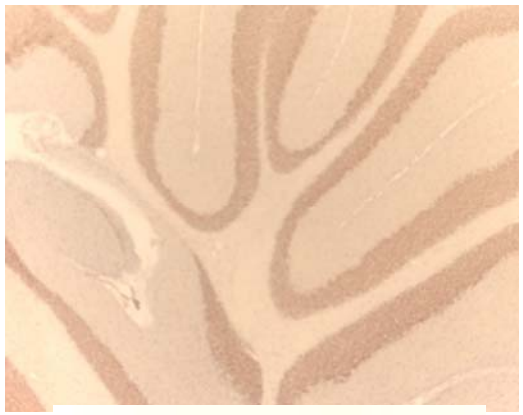
actin

cer hip embryo intes



Zip1 Not Zip4 Is Expressed In Mature Cerebellar Granule Cells

zip1

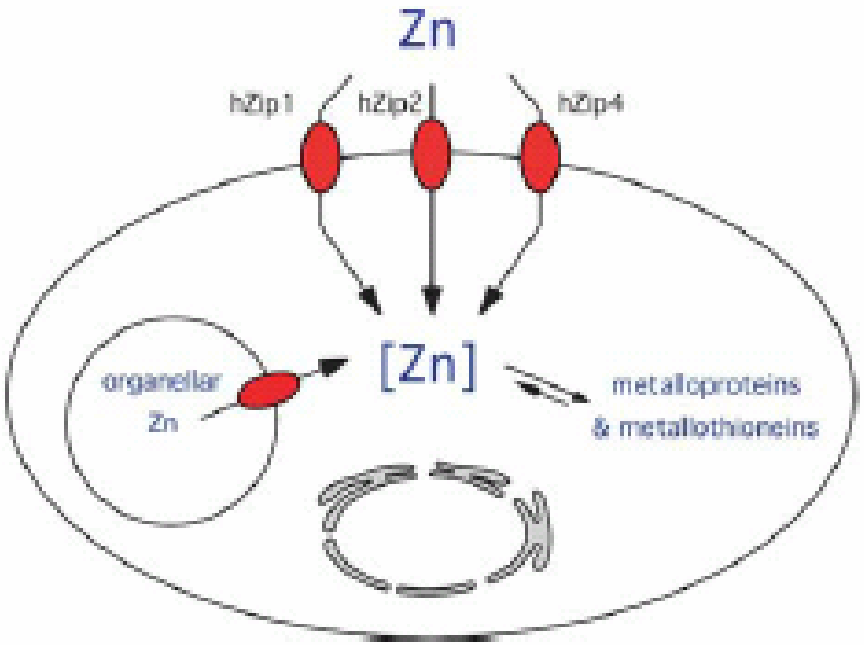


zip4

antisense

sense

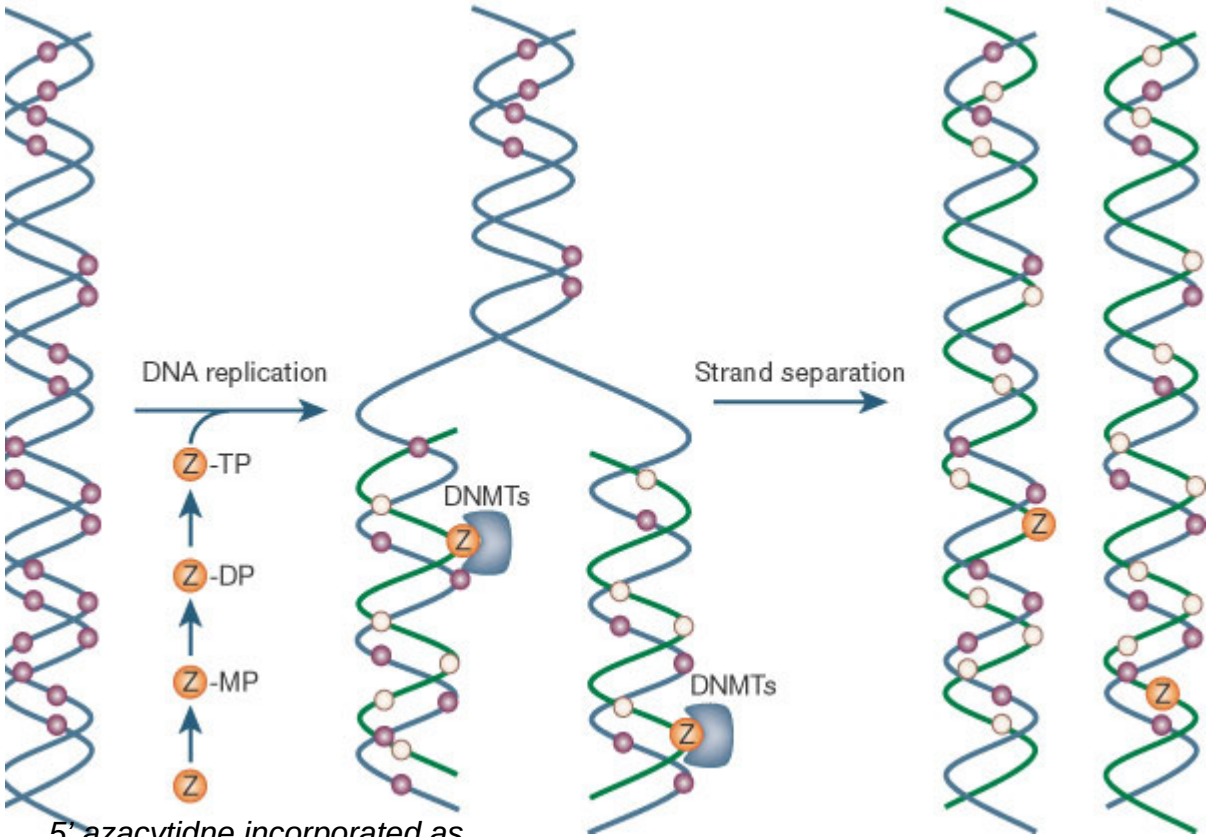
Zip4 Response Quickly To Changes In Zn States



Zip 1 not responsive to zinc status; ubiquitous expression

Zip 4 responsive to zinc status; found in intestine, pancreas, embryo

Can Chemicals That Interfere With DNA Methylation Affect Differentiation In P19 Cells?



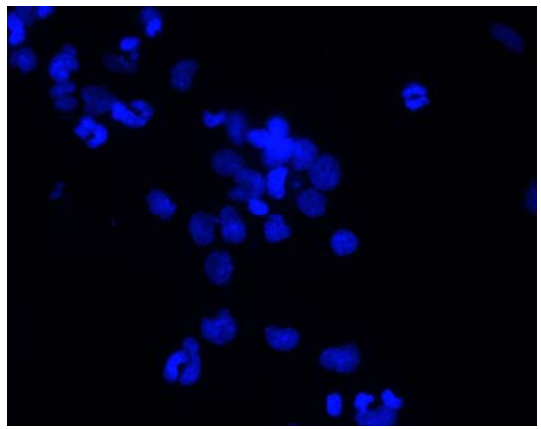
5' azacytidne incorporated as cytosine resulting in hypomethylated DNA

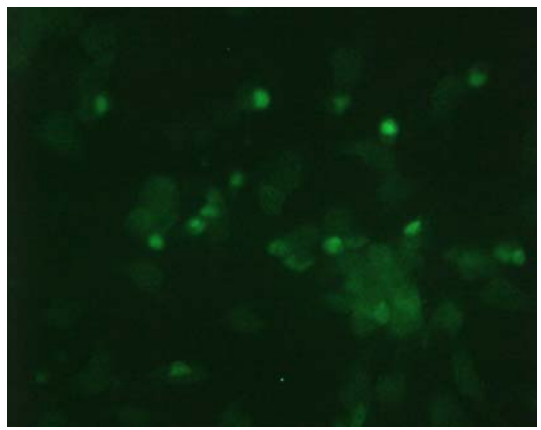
Nature **429**, 457-463 (27 May 2004)

5’ AZACYTIDINE INCREASES NUMBERS OF NEURONS

Control

Methytrans ferase inhibitor





NeuN

5 %

11%

Nuclei Stain

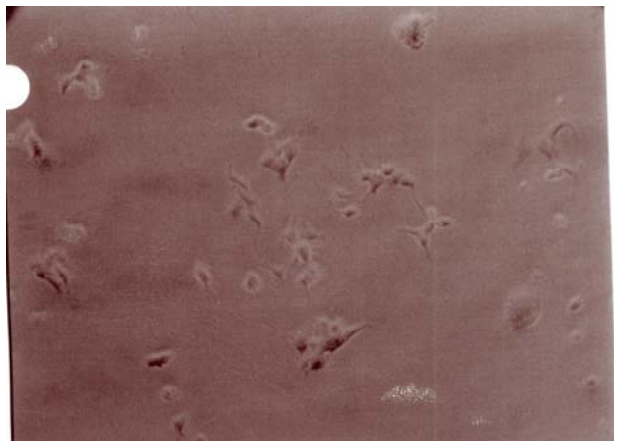
Neuronal stain

Environmental Chemicals Affecting DNA Methylation/Histone Modification Interfere With Differentiation?

- Nickel, occupational carcinogen, (mM) increases H3 methylation and decreases H4 acetylation (hypoxia…)
- Cadmium, carcinogen, kidney and bone toxicant, decreases DNA methylation (oxidative stress…)
- Arsenite, human, carcinogen decreases DNA methylation found in ground water (also increases oxidative stress)
- Trichloroacetic acid (TCA) and dichloroacetic acid, rodent carcinogen, decreases DNA methylation are by-products of water chlorination (also peroxisome proliferator)

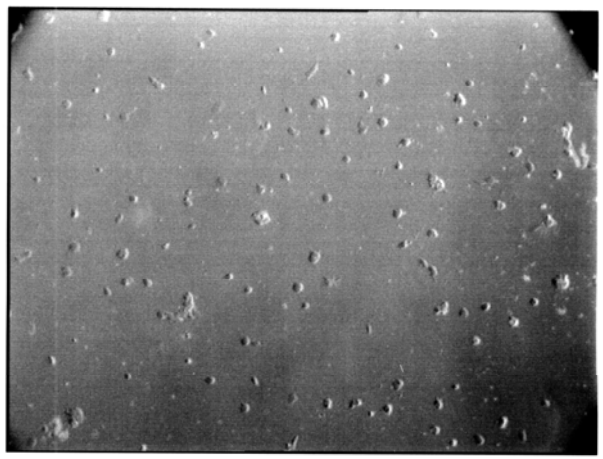
What is the effect of nickel, cadmium, arsenite and TCA on neural differentiation?

Before RA



pre-neuralation

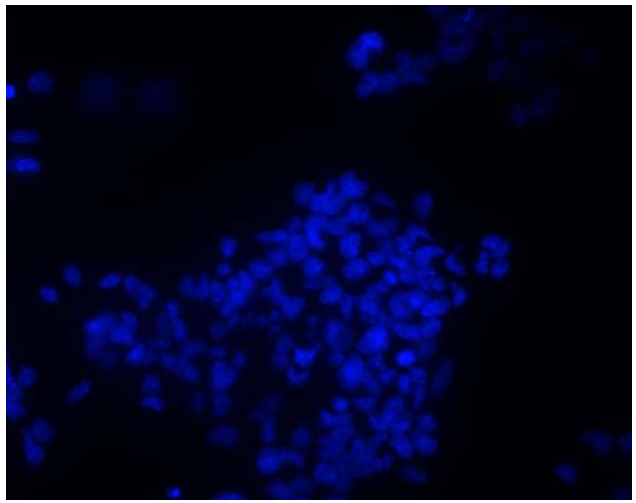
24 hr after RA

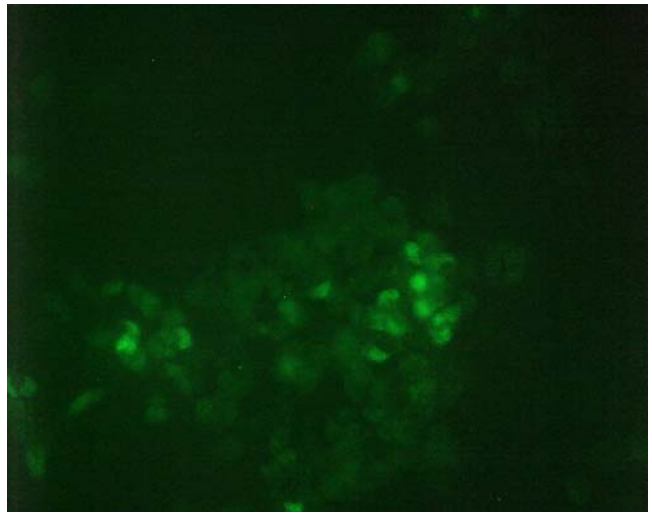


neural stem cell

Arsenite Treatment After RA

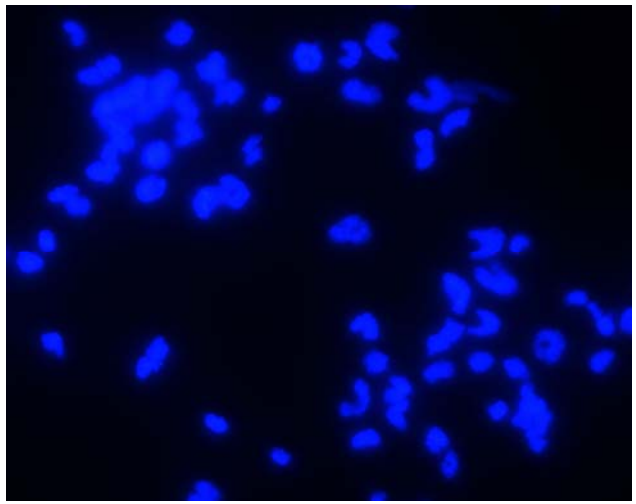
control

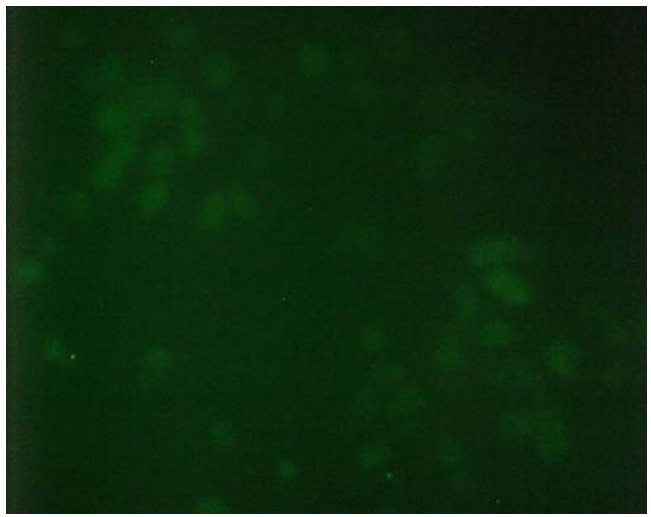




6.1%

0.2 uM arsenite

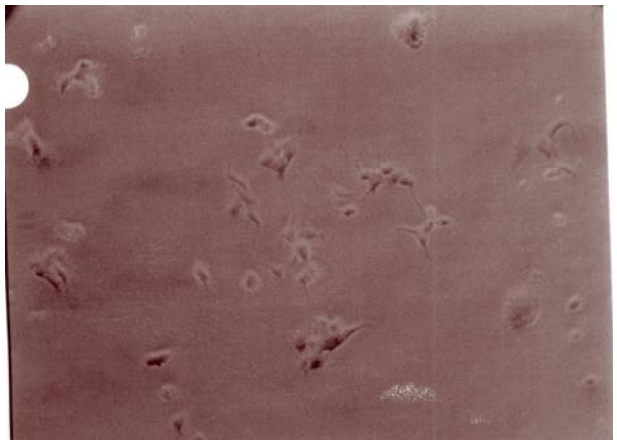




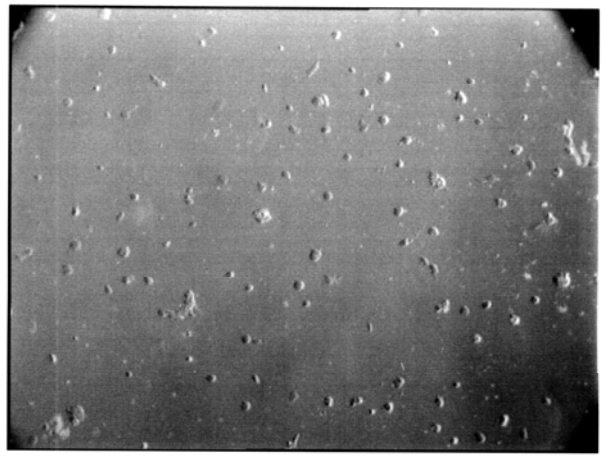
<1%

Arsenite Is Effective Only During Neuronal Differentiation (not DNA methylation)

Before RA



24 hr after RA



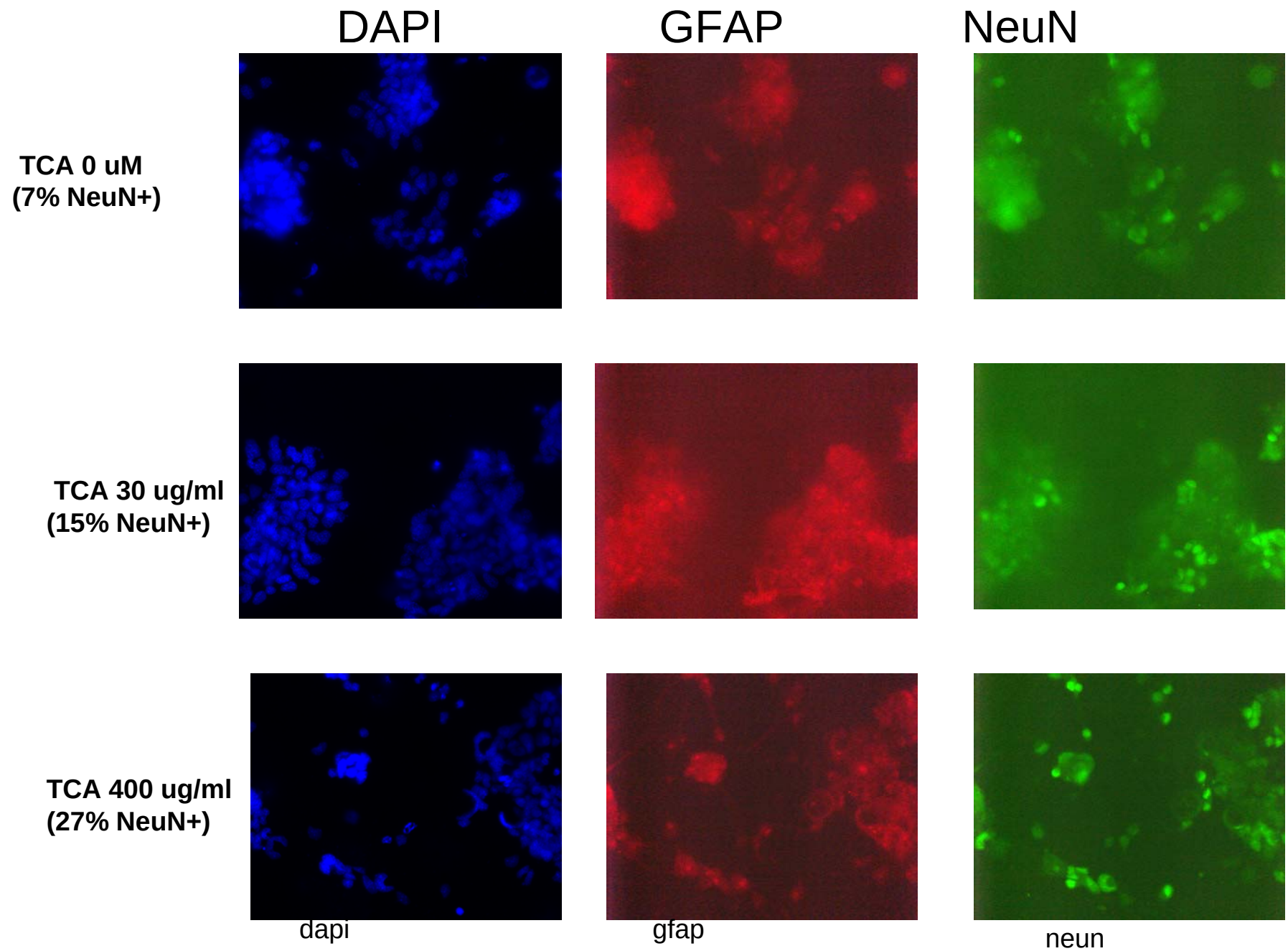
pre-neuralation

No

neural stem cell

Yes

Trichloroacetic Acid (TCA) 20 days Before RA



TCA Pretreatment Speeds Up RA-Induced Differentiation

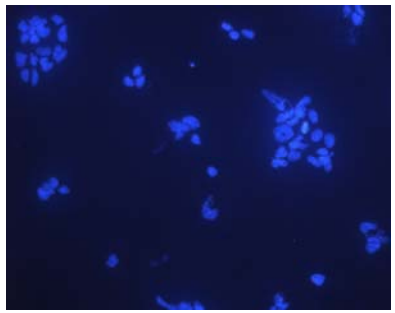
One Day after RA

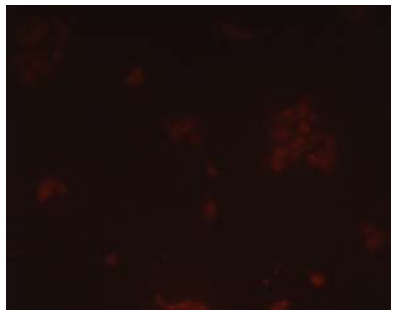
DAPI

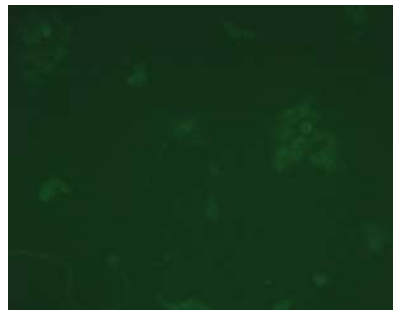
GFAP

NeuN

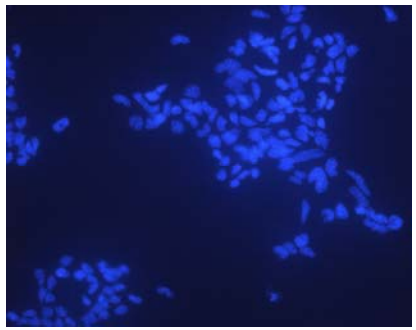
No TCA

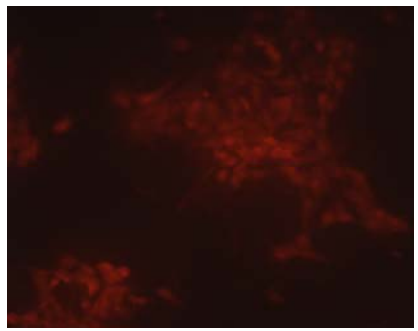


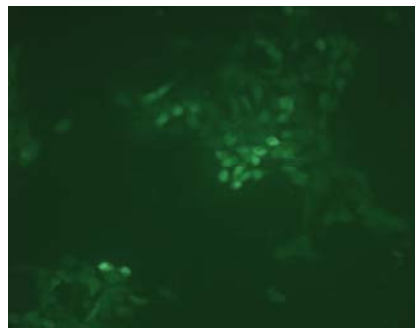




TCA

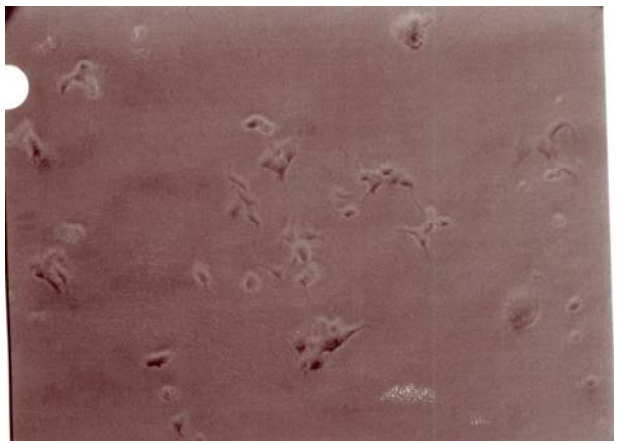




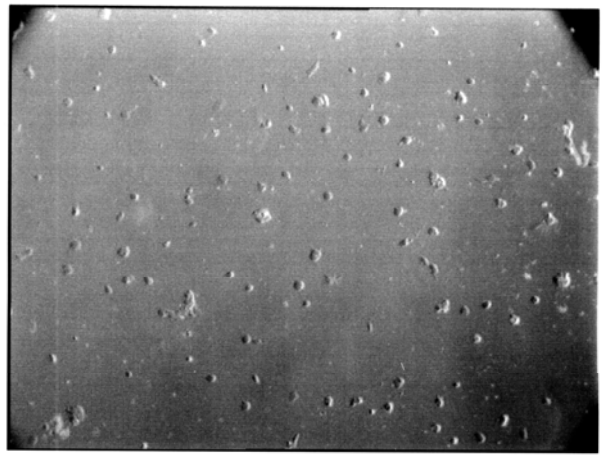


TCA Is Effective Only During Pre-Neuralation

Before RA



24 hr after RA



pre-neuralation

Yes

neural stem cell

No

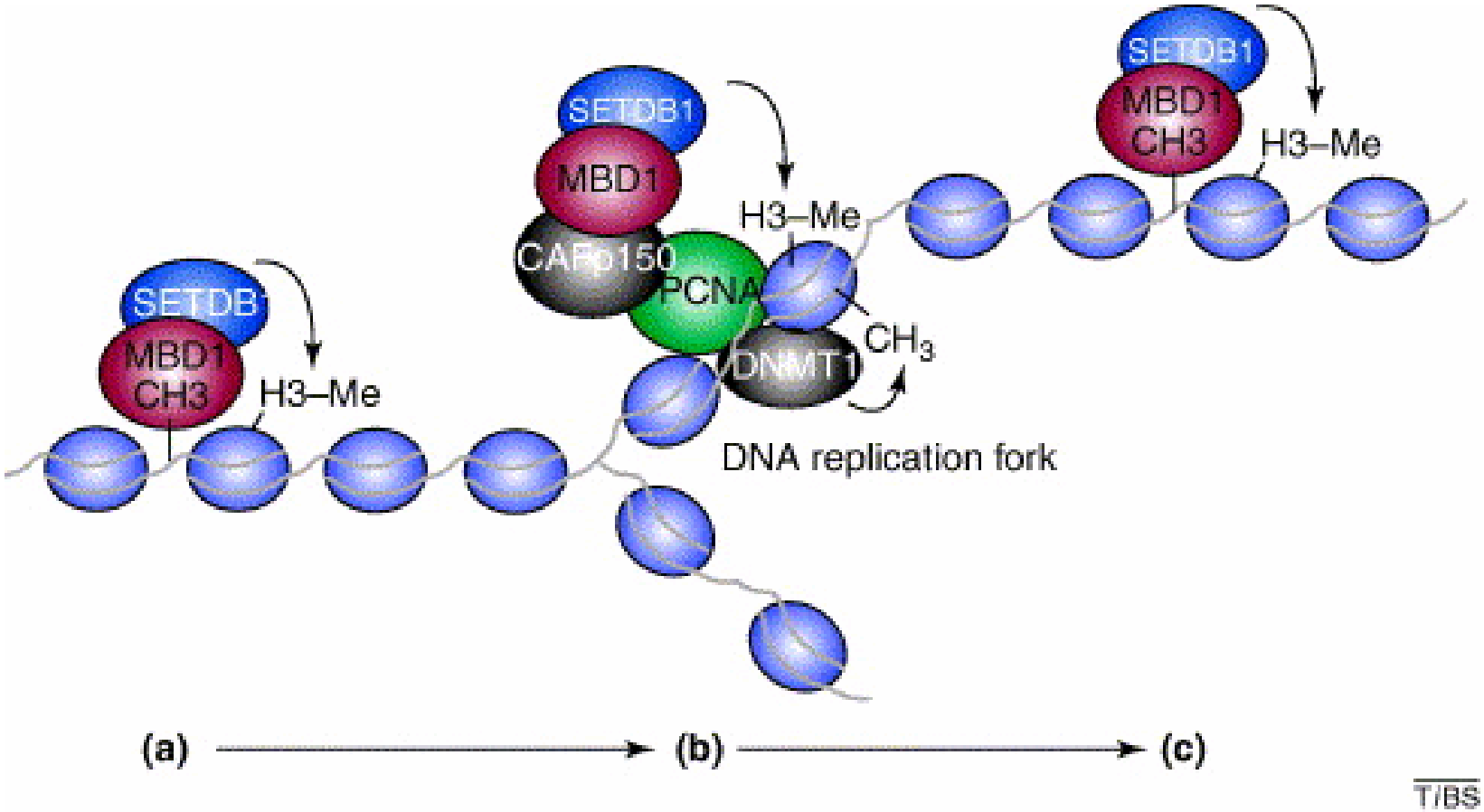
POSSIBLE MECHANISM UNDERLYING THE EFFECTS OF TCA

- INHIBITS DNA METHYLATION PROMOTING GENE (NEURONAL) EXPRESSION

  Direct proof of less methylation

  Methylation is inherited

METHYLATION IS INHERITED



Effects of TCA Are Inherited

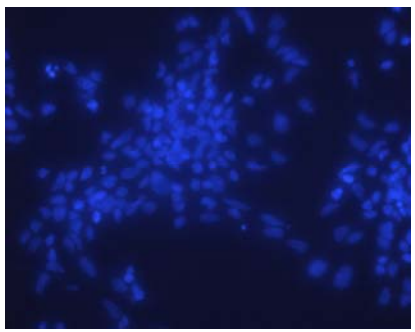
Treat cells with TCA (0.4 mg/ml) for 20 days (No RA) then clone without TCA

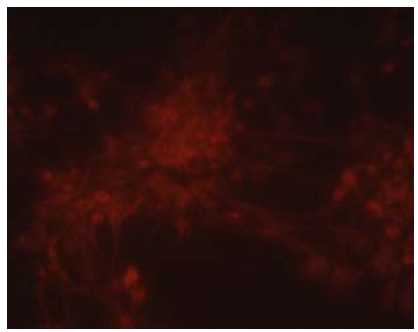
Controls also cloned

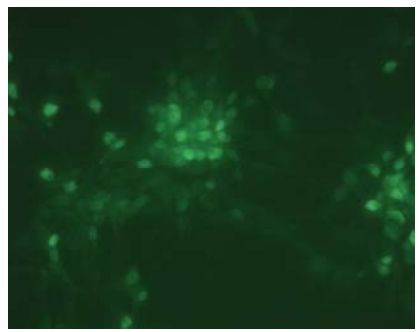
“

DAPI GFAP NeuN

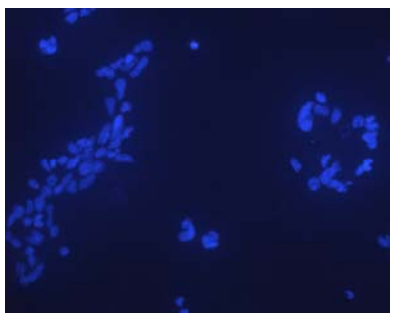
TCA 2/12

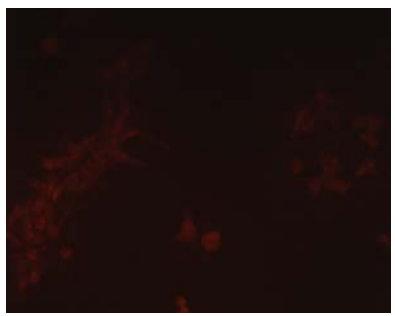


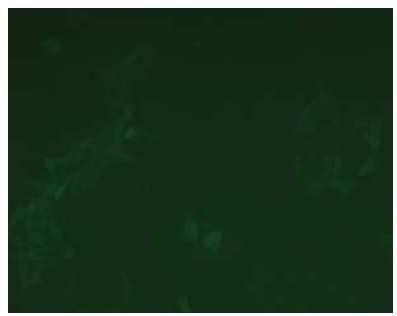




Control 0/15

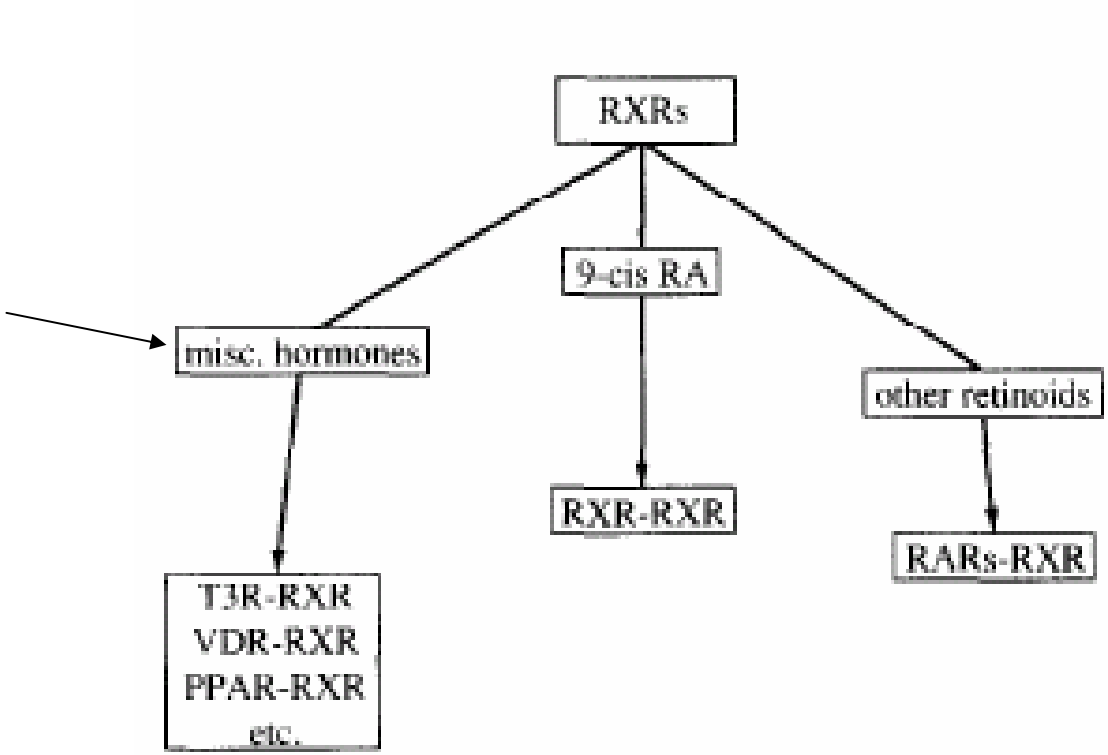






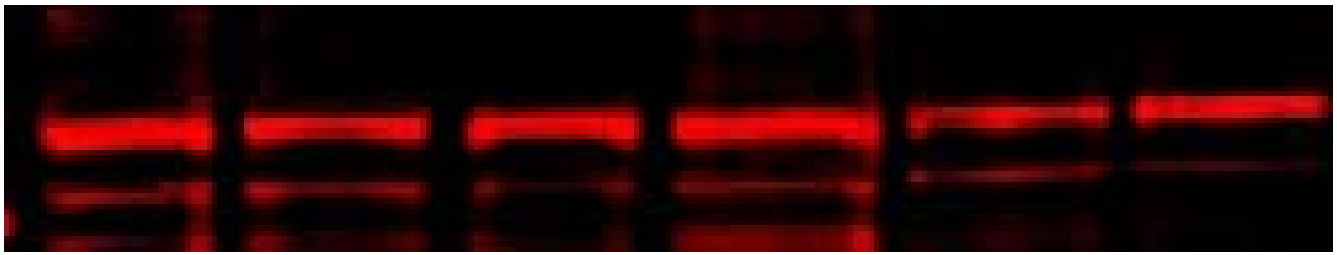
Alternatively TCA Activates Peroxisome Proliferator Receptor

Peroxisome proliferator (TCA)

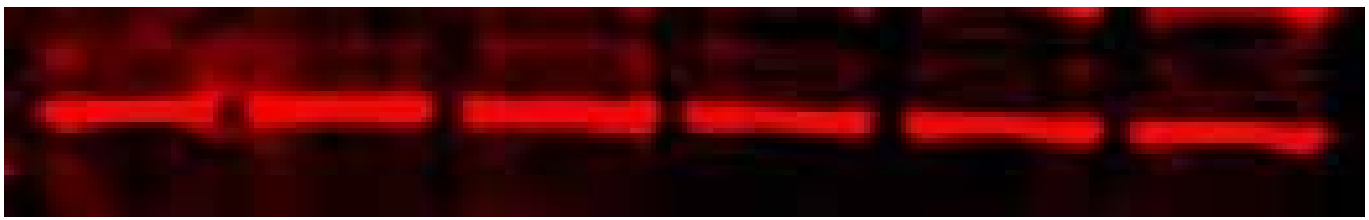


No Increase In Peroxisome Markers

CONTOL TCA ARSENITE



PEX-13



Catalase

GAPDH

Probable Mechanism

TCA’s possible mechanism is through DNA methylation

Arsenite through a different mechanism

Summary Of Effects Of Pollutants On Neural Differentiation

Pollutant	Lowest concentration without killing	NeuN (%)	GFAP (%)
control	none	2.9±0.5	4.5±0.9
cadmium	0.5 µM	3.5±0.6	6.3±1.0
nickel	<400 µM	2.7±0.8	5.5±1.1
Methyl Hg	0.5 µM	4.2±1.1	7.1±0.5
Sodium arsenite	0.2 µM	0.5±0.9[a]	4.9±1.1[a]
Trichloroacetic acid	30 ng/ml	9.3±1.2[a]	5.1±0.6

a. $p < 0.05$, determined by ANOVA using Tukey's posthoc test

Summary Slide (Public Health)

Environmental pollutants, TCA and arsenite, are carcinogens and will disrupt neural differentiation. Maxiumum tolerant levels of TCA, 50 ug/L and we found effects (so far) at 3,000 ug/L.

Arsenite not regulated in U.S.

Mouse embryonic stem cell lines are a good model for screening chemicals for neurotoxicity.

In future studies we hope to develop new cell lines with reporter gene constructs for determining neural lineages in a high-input screening system.

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