Neural Stem Cell Dysfunction & Its Implications on Memory and Mood in a Rat Model of Gulf War Illness

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Gulf War illness (GWI)

Affected population
Veterans who served in the 1991 Persian Gulf War-1 (PGW-1)
[~25% of 697,000 US Servicemen & women]

Symptoms
A set of non-specific concurrent symptoms with an emphasis on CNS impairments

- Memory and Concentration Problems
- Depression, Anxiety, and Chronic headaches
- Dizziness & Alterations in Sleep, widespread pain etc.
Possible Causes of Gulf War Illness

Exposure to a Mixture of Biological and Chemical Environments during PGW-1

(1) Intake of Pyridostigmine bromide (PB)  
   *As a Prophylactic measure against nerve gas attack*

(2) Exposure to N, N-diethyl-m-toluamide (DEET) & Permethrin  
   *To Combat insects and rodents in the region*

Other suspected factors:
Low-level exposure to nerve gas agents, proximity to oil-well fires, receipt of multiple vaccines, and effects of combinations of Gulf war exposures etc..

Based on the report of “The Research Advisory Committee on Gulf War Veterans' Illnesses”

The symptoms exhibited by Gulf war veterans are likely owed to a synergistic interaction of chemicals PB, and pesticides (such as DEET and permethrin).

Rat model of Gulf War Syndrome

Exposure of rats for prolonged periods (e.g. 28 days) to low doses of PB, DEET & Permethrin

PB: 1.3 mg/kg/day, oral  
DEET: 40 mg/kg/day, dermal  
Permethrin: 0.13 mg/kg/day, dermal
Experiment #1

Immediate Effects of 28-Day Exposure to Chemicals PB, DEET, and Permethrin on Hippocampal Neurogenesis

Neurogenic Regions in the Adult Brain

Dentate Gyrus & Subventricular Zone

Neurogenesis in Non-Neurogenic Regions
Cerebral Cortex, Striatum, Substantia Nigra etc.
Dentate Neurogenesis in the Adult Hippocampus

- Production of new neurons in the DG occurs throughout life.
- Newly generated neurons mature into functional neurons.
- Extent of dentate neurogenesis in the adult depends on multiple positive and negative regulators.

- Cell death
- Concentration of stem/progenitor cell proliferation factors (FGF-2, IGF-1, VEGF, EGF, BDNF)
- Serotonin
- Enriched environment, exercise, learning & memory training
- Vascular niche
- Glucocorticoids (Stress)
- Hippocampal inflammation
- Aging
- Drugs of abuse (e.g. alcohol)

Functions of Dentate Neurogenesis

- DG neurogenesis and hippocampal-dependent learning and memory.
- New neurons incorporate into dentate gyrus circuits supporting spatial memory (Kee et al., 2007).
- Genetic Ablation of Newly Formed Neurons leads to impairments in spatial memory (Imayoshi et al., 2008) and recognition memory (Jessberger et al., 2009).
- Positive behavioral effects of chronic antidepressants are associated with increased DG neurogenesis.
Analyses of Dentate Neurogenesis

Different Stages of DG Neurogenesis

Identification of newly born cells and neurons

Vehicle Group

Chemical Group

[DDW and 70% Ethanol] 28 days DEET 40mg/kg, PB 1.3mg/kg & Permethrin 0.13 mg/kg

Four BrdU injections Over 18-hr Period

BrdU Immunostaining Doublecortin Immunostaining BrdU-DCX dual Immunofluorescence & Confocal Microscopy

Optical Fractionator and cell Counting Optical Fractionator and Cell Counting Measurement of the Percentage of Newly Born Cells that Differentiated into Neurons

Quantification of Net Neurogenesis

Research Design (Expt. #1)
Short-term Effects of PB, DEET & Permethrin Exposure on Production of New Cells/Day in the Neurogenic Region (SGZ-GCL) of the Hippocampus

BrdU labeling Study (one injection every 6 hrs over an 18-hr period)

Short-term Effects of PB, DEET & Permethrin Exposure on Neuronal Fate-Choice Decision of Newly Born Cells in the Neurogenic Region of the Hippocampus
Short-term Effects of PB, DEET & Permethrin Exposure on Net Neurogenesis per Day in the Hippocampus

![Graph showing the number of newly born neurons per SGZ & GCL for Control, Vehicle, and Chemical groups, with statistical significance indicated by p < 0.001.]

Short-term Effects of PB, DEET & Permethrin Exposure On The Status of Hippocampal Neurogenesis, as revealed by Doublecortin Immunostaining

![Images of control, vehicle, and chemical groups showing DCX+ neurons in DH, ML, and GCL layers, with a bar graph showing statistical significance by p < 0.0001.]
Conclusion (Expt. #1)

28-day exposure to a combination of GWI-related chemicals diminishes hippocampal neurogenesis in the immediate post-exposure period

Expt. #2

Does the decline in hippocampal neurogenesis affect functions such as learning, memory and mood?

Research Design (Expt. #2)

Vehicle Group  Chemical Group

DDW and 70% Ethanol  DEET 40mg/kg, PB 1.3mg/kg & Permethrin 0.13 mg/kg

28 days  Six weeks of waiting period

Analyzed Learning and Memory, and Depression like Activity
Effects of PB, DEET & Permethrin Exposure on Spatial Learning Function, as Examined by a Water Maze Test

Effects of PB, DEET & Permethrin Exposure on Memory Function, as Examined by a Memory Retrieval Test
**Effects of PB, DEET & Permethrin Exposure On Depression, as Examined by a Forced Swim Test (FST)**

![Graph showing time spent in floating (seconds)](image)

**Conclusions (Expt. #2)**

1. 28-day exposure to a combination of GWI-related chemicals leads to impairments in Functions such as learning, memory, and mood.

2. As learning, memory, and mood functions are linked to the extent of hippocampal neurogenesis, it is likely that declined hippocampal neurogenesis underlies cognitive dysfunction and depression in this model.
Does the decline in hippocampal neurogenesis induced by the chemicals persists for prolonged periods after the exposure?

**Expt. #3**

Analyses of hippocampal neurogenesis at 3-months after the exposure regimen.

**Research Design (Expt. #3)**

- **Vehicle Group**
  - [DDW and 70% Ethanol]
  - 28 days
  - 3 Months
  - 12 BrdU Injections
  - 1 Month
  - BrdU Immunostaining
  - Optical Fractionator and cell Counting
  - Quantification of Net Neurogenesis

- **Chemical Group**
  - DEET 40mg/kg, PB 1.3mg/kg & Permethrin 0.13 mg/kg
  - 28 days
  - 3 Months
  - 12 BrdU Injections
  - 1 Month
  - Doublecortin Immunostaining
  - Optical Fractionator and Cell Counting
  - Measurement of the Percentage of Newly Born Cells that Differentiated into Neurons
  - BrdU-NeuN dual Immunofluorescence & Confocal Microscopy
Long-Term Effects of PB, DEET & Permethrin Exposure on the
Addition of New Cells to the Granule Cell Layer of the Hippocampus

Long-Term Effects of PB, DEET & Permethrin Exposure on the
Differentiation of Newly Born Cells into NeuN+ Mature Neurons in the Granule Cell Layer
Long-Term Effects of PB, DEET & Permethrin Exposure on Net Neurogenesis in the Hippocampus

![Graph showing the comparison between Vehicle Group and Chemical Group in terms of absolute number of NeuN+ Neurons. The graph indicates a significant difference (p < 0.01).]

Long-Term Effects of PB, DEET & Permethrin Exposure on the Status of Hippocampal Neurogenesis, as examined by Doublecortin Immunostaining

![Images showing the status of hippocampal neurogenesis under different conditions. The bar graph compares the number of DCX+ newly born neurons per SGZ & GCL between Vehicle Group and Chemical Group, highlighting a significant difference (p < 0.001).]
Conclusions (Experiments 1-3)

- 28-day exposure to a combination of GWI-related chemicals greatly diminishes hippocampal neurogenesis for prolonged periods.

- Reduced hippocampal neurogenesis is linked with impaired learning, memory and mood functions.

- Reduced hippocampal neurogenesis persists at four months after the exposure.

What happens if stress is added during the chemical exposure?

- Unpredictable Chronic Stress (UCS)
  
  *Well known to greatly increase stress hormones and decrease hippocampal neurogenesis and cause learning & memory impairments and depression.*

- Predictable Chronic Mild Stress (PCMS)
  
  *5 minutes of restraint stress per day for 28 days*
Effects of PCMS alone on Depression, and Hippocampal Neurogenesis (Expt. #4)

Experimental Design

**Naive Group**
- Handling for 28 days
- Rats Subjected to PCMS for 28 Days

**Stress Group**
- Rats Subjected to PCMS for 28 Days

Analyses of Depressive-like behavior using a Forced Swim Test (FST)
- BrdU Treatment Over 18-hr Period
  - Perfusion at 6 hrs After the Last BrdU Injection
  - Tissue Processing

BrdU Immunostaining
Doublecortin Immunostaining
BrdU-DCX dual Immunofluorescence & Confocal Microscopy

Optical Fractionator Cell Counting
Morphometric Analysis of Relatively Mature Neurons
Measurement of the Percentage of Newly Born Cells that Differentiate into Neurons

Effects of PCMS on Depressive-like Behavior in a Forced Swim Test (FST)

A. FST at One-day after PCMS Regimen

B. FST at Two-months after PCMS Regimen

Parihar et al., Molecular Psychiatry, 2010
## Effects of PCMS on the Extent of Hippocampal Neurogenesis

**Parihar et al., Molecular Psychiatry, 2010**

<table>
<thead>
<tr>
<th>Number of BrdU+ Cells per 100μM2</th>
<th>p = 0.001</th>
</tr>
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Red = Control Rats
Green = Rats that underwent PCMS

## Effects of PCMS on Spatial Learning and Memory Function

### Experimental Design

**Naive Group**
- Handling for 28 days
- Rats Subjected to PCMS for 28 Days

**Stress Group**
- Novel Object Recognition Test (NORT)

Spatial Learning & Memory Analyses at 1.5 months after PCMS Regimen
PCMS Enhances Spatial Memory Retention Ability

Comparison of Learning Index between the Control and PCMS Groups

Comparison of Performance in Memory Test between the Control and PCMS Groups

PCMS Enhances Ability for Recognition Memory

Exploration Phase

Testing Phase

Object Exploration in Memory Testing Phase of NORT
Conclusion (Expt. #4)

PCMS has beneficial effects, which include enhancements in hippocampal neurogenesis, mood and memory function.

Expt. #5

What happens if PCMS component is added during the exposure to three chemicals (PB, DEET, Permethrin)?

Short-term Effects of Combined Exposure to Chemicals and PCMS on the Production of New Cells/day in the Hippocampus
Short-term Effects of Combined Exposure to Chemicals & PCMS on Neuronal Differentiation of Newly Born Cells & Net Neurogenesis

Neuronal differentiation of newly born cells

- Control
- Vehicle alone
- Stress alone
- Chemicals alone
- Chemicals + Stress

Net Neurogenesis

Number of newly born neurons in the SGZ/GCL

Short-term Effects of Combined Exposure to Chemicals and PCMS on the Status of Hippocampal Neurogenesis

Number of DCX+ Neurons per SGZ/GCL

- Control
- Vehicle alone
- Stress alone
- Chemicals alone
- Chemicals + Stress
Conclusion (Expt. #5)

Addition of mild stress (PCMS) exacerbates the effects of GWI-related chemicals on hippocampal Neurogenesis.

Expt. #6

Long-term effects of combined exposure to GWI-related chemicals & PCMS on learning, memory and mood functions and hippocampal neurogenesis.

Effects of combined exposure to GWI chemicals & PCMS on spatial learning function, as examined by a water maze test.
Effects of combined exposure to GWI chemicals & PCMS on memory, as examined by a memory retrieval test

A Combined Exposure to Chemicals & PCMS Impairs Recognition Memory

Exploration Phase

Testing Phase

Time spent with Novel Object (Seconds)

- Vehicle Group
- Chemical Group
- Chemicals + Stress group

Latency to Reach the Target Area (Seconds)

Number of Target Area Crossings (Seconds)

Time Spent in Target Area (Seconds)
Effects of combined exposure to GWI chemicals & PCMS on depression, as examined by a forced swim test (FST)

Conclusions (Expt. #6)

Addition of mild stress (PCMS) increases the overall adverse effects of GWI-related chemicals on functions such as learning, memory & mood.

As learning, memory, and mood functions are linked to the extent of hippocampal Neurogenesis, it is likely that decline in hippocampal neurogenesis underlies cognitive dysfunction and depression in this model.
Long-term effects of combined exposure to GWI-related chemicals & PCMS on the addition of new cells to the hippocampus

Long-term effects of combined exposure to GWI-related chemicals & PCMS on the differentiation of newly born cells into NeuN+ mature neurons
Long-term effects of Combined Exposure to GWI-related chemicals & PCMS on Net Neurogenesis in the Hippocampus

Addition of mild stress (PCMS) during the exposure to GWI-related chemicals enhances the adverse long-term effects of these chemicals on hippocampal neurogenesis.

Enhanced adverse effect on neurogenesis is associated with worsening of functions such as learning, memory and mood.

Thus, stem cell dysfunction in the hippocampus likely underlies the cognitive and mood impairments observed in this GWI model.

Conclusions (Expt. #6)
Is the adverse effect of GWI-related chemicals specific to hippocampal stem cells?

(1) Possible loss of neurons in different regions of the hippocampus.  

*Distribution of NeuN+ neurons in the DG, CA1 & CA3 subfields at 4 months after the exposure.*

(2) Inflammation in the hippocampus.  

*Analyses of activated microglia using ED-1 immunostaining.*

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Exposure to GWI-related chemicals or GWI chemicals & stress does not induce widespread hippocampal neurodegeneration.
Exposure to GWI-related chemicals or GWI chemicals & stress does induce some inflammation in the hippocampus

Activated microglia – ED1 Immunohistochemistry

Exposure to a single GWI-related chemical (PB, permethrin or DEET) does not decrease hippocampal neurogenesis
Overall Conclusions

1. A combined exposure to GWI-related chemicals impairs hippocampal neurogenesis as well as hippocampal-dependent functions such as learning, memory and mood.

2. The adverse effects appear to be due to an interaction of the three chemicals, as exposure to any of these chemicals alone has no significant effect on neurogenesis.

3. Exposure to a combination of the GWI-related chemicals appears to have a specific effect on hippocampal stem cell function, as this exposure did not induce widespread hippocampal neurodegeneration or inflammation.

4. Presence of even a mild stress during the exposure exacerbates the various adverse effects of GWI–related chemicals.

ACKNOWLEDGMENTS

FUNDING

This work was supported by a Gulf War Research Grant from the Department of Veterans Affairs (04/01/07 to 03/31/10)

CONTRIBUTORS

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