

# Flupirtine Protects Against Permethrin/DEET-Induced Brain Injury in A Rat Model for Gulf War Illnesses

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## Gulf War Illnesses

**Approximately 250,000 of the 697,000 American military personnel who served in the Gulf War from August 1990 to June 1991, complained of symptoms known as Gulf War Illnesses (GWI)**

### Including:

- Chronic fatigue
- Headache
- Memory problems
- Forgetfulness
- Inability to concentrate
- Diarrhea
- Neurological deficits
- Gastrointestinal problems

○ (Institute of Medicine, 2012).

# CHEMICAL EXPOSURES

During the war, American military personnel were exposed to a combination of chemicals such as:

- The insect repellent DEET and the insecticide permethrin to protect against insect borne diseases.
- The nerve agent sarin
- Pyridostigmine bromide (PB) to protect against sarin attack.

## Flupirtine Maleate

### Action

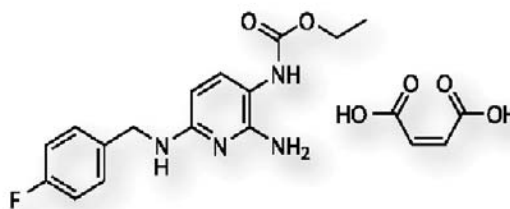
1. Analgesic drug that acts as antagonist of NMDA receptors.

2. Decreased glutamate-induced PC12 cells and Reactive Oxygen Species (ROS).

3. Anti-oxidant

4. Protect against Prion-induced neuronal cell death

### Chemical Formula



(MSDS Sigma-Aldrich)

# Flupirtine

Oral daily doses of flupirtine had beneficial effects on patients with:

1. Memory and sensorimotor deficits
2. Headache/migraine and abdominal spasms

In rats, it treated neuronal damage following global ischemia and reduced *neuronal damage in CA1 of the hippocampus*

In addition, flupirtine does not induce development of dependence or tolerance and does not have prominent side effects make it a promising and clinically safe drug for treatment of veterans with GW to improve their quality of life.

## HYPOTHESIS

**Flupirtine, a non-opiate analgesic that has been used in the treatment of:**

- memory deficits,
- muscular diseases in patients,

**could treat the Gulf War Veterans, whose major complaints include memory impairment and sensorimotor deficit.**

## FLUPIRTINE

### Protection/Treatment of GWI in a Rat-Model

#### Specific Aims

- 1. Test the ability of flupirtine to protect from the Gulf War Veteran's Illnesses in a rat-model
- Concurrent application with chemicals and flupirtine for 60 days
- 2. Test the ability of flupirtine to treat Gulf War Veteran's Illnesses in rat-model
- Treatment with chemicals for days to develop GWI, then treat with flupirtine for 60 days

## Rat-Model for Gulf War Illnesses

We used our previously developed rat model for the GWI<sup>\*</sup> :

1. Young adult Sprague-Dawley rats, weighing approximately 220-230 g.

2. Animals were treated with daily dermal doses for 60 days of:

a) 40 mg/kg DEET and

b) 0.13 mg/kg Permethrin

(Similar to exposure conditions during the Gulf War)

3. Vehicle: 70% ethanol.

4. Application site: A pre-clipped area (4 cm) on the back of the head

\* (Abdel-Rahman et. al., 2001; Abou-Donia et al., 2001,2004)

## Protection From DEET/Permethrin-Induced Neurotoxicity

### Treatment protocol:

Four groups (n = 20) of rats were treated as follows:

1. Control (vehicle, 70% ethanol), daily for 60 days
2. DEET/permethrin, daily for 60 days
3. Flupirtine (10 mg/kg) in water (1 ml/kg) daily for 60 days
4. DEET/permethrin + 10 mg/kg flupirtine in water (1 ml/kg) daily for 60 days

## EXPERIMENTAL

- Animal:** Male, Sprague Dawley Rats; 225-250 g
- Flupirtine:** 10 mg/kg IM daily dose
- Dose:** DEET: 40 mg/kg/day + Permethrin: 0.13 mg/kg /day
- Route of Administration:** Dermal
- Location:** 1 In 2 on pre-shaved area on the back of the neck
- Vehicle:** 70 % Ethanol
- Duration of treatment:** 60 days

# Animal Protocol

Four groups of 20 rats:

1. **Vehicle**, 0.1 ml/kg; IM injection for 60 days
2. **DEET**, 40 mg/kg/day + **Permethrin**, 0.13mg/kg for for 60 days
3. **Flupirtine**, 10 mg/kg im daily dose for 60 days
4. **DEET**, 40 mg/kg/day + **Permethrin**, 0.13mg/kg + **Flupirtine**, 10 mg/kg IM daily dose for 60 days

# Animal Evaluation

Animals were evaluated for neurological deficits by determining:

1. Clinical signs
2. Sensorimotor functions
3. Learning and cognition function
4. pathological alterations

# Assays

- The animals were weighed weekly and their clinical condition was monitored daily.
- Twenty four hours after the 60-day treatment period, the animals were evaluated for neurobehavioral and pathological parameters:
  - A group of 10 rats from each group will be evaluated for cognition using Morris Water Maze test.
  - The other group of 10 animals will be tested for sensorimotor performance.
  - Neuropathological alterations were carried out in 10 rats; 5 from each behavioral sub-group.

# RESULTS

## Body Weight

No Statistical difference was observed in the weights of animals treated with:

- Vehicle,
- Flupirtine,
- DEET/permethrin or
- DEET/permethrin and flupirtine.

# RESULTS

## Clinical Condition

The clinical condition of animals treated with daily dermal application of DEET and permethrin or combination of these pesticides and flupirtine was not different from that of vehicle control or flupirtine groups.

## Learning and Memory: Morris Water Maze (MWM)

- Spatial learning and memory were assessed using the Morris Water Maze
  
- Spatial memory acquisition in this test is known to be associated with:
  - dysfunction of hippocampus,
  - striatum,
  - basal forebrain,
  - cerebellum, and neocortex
  - can adversely affect other aspects of MWM performance



## **RESULTS of MWM**

- **Vehicle, Flupirtine had no effect**
- **DEET/Permethrin treatment caused memory deficits**
- **Flupirtine improved memory deficits induced by DEET/permethrin treatment.**

## **Sensorimotor performance**

**Sensorimotor performance of control and treated groups were assessed by a battery of behavioral tests that included:**

- 1. Beam walk and Beam Score**
- 2. Incline plane performance, and**
- 3. Forepaw grip time**

## Beam Walking and Beam Score

### Description:

- The apparatus consists of an elevated wooden beam, a goal box with light and an opening located at the end of the beam.
- ***Beam Walking Time:*** The time till the animal's nose enters the box (up to 90 seconds)
- ***Beam Walking Score:*** A 7-point scoring system for the use of the hind paw to aid locomotion

### Results of Beam Walking and Beam Score

- Animals treated with DEET and permethrin showed significant deficit in beam walk score and beam walk time, compared to vehicle control and flupirtine.
- Flupirtine reversed the effects of DEET and permethrin.

## Incline Plane Performance

### Description:

- Rats are placed on a flat plane in the horizontal position, with their heads facing the side of the board to be raised.
- The angle at which the rat begins to slip is recorded.

### Results:

1. Animals treated with DEET and permethrin exhibited significant impairment in incline plane testing compared to vehicle control and flupirtine.
2. Flupirtine reversed the effects of DEET and permethrin.

## Grip Time

### Description

This test is to assess forepaw grip strength.

1. Have the rat grip a 6-mm diameter wooden dowel.
2. The time to release grip is recorded.

### Results

1. Animals treated with DEET and permethrin caused impairment in forepaw grip strength testing compared to vehicle control and flupirtine.
2. Flupirtine reversed the effects of DEET and permethrin.

# RESULTS

## Histopathological Assessments

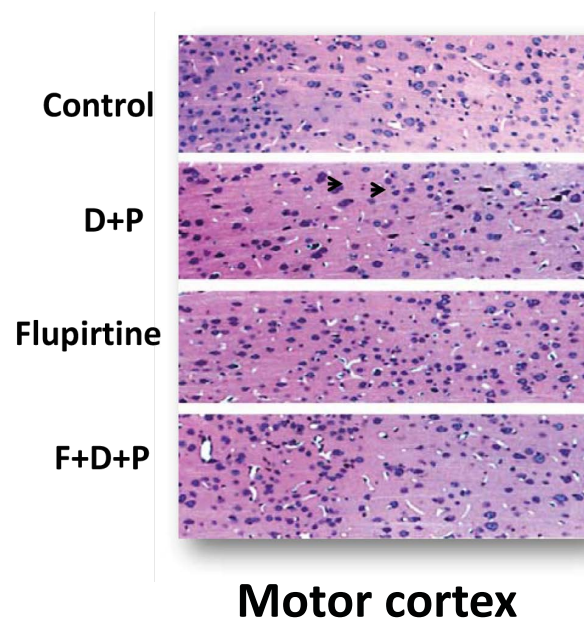
Brain neuropathological alterations were diffuse neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and the hippocampus, and Purkinje neuron loss in the cerebellum.

### Hematoxylin Eosin (H&E)

- 1. H&E stain revealed neuronal degeneration in rats treated with DEET + permethrin in comparison to the three other groups.**
- 2. Degenerated neurons were characterized by dark eosinophilic staining of both cell body and proximal dendrites.**
- 3. In contrast, the healthy neurons in the same section, as well as in the other groups, exhibited hematoxylin-stained nuclei (with clear nucleoli) and eosin-stained prenuclear cytoplasm.**

## Hematoxylin Eosin (H&E)

1. The most obvious neurodegeneration was present in the:
  - motor cerebral cortex,
  - hippocampus, and
  - Purkinje cell layer of the cerebellum.
2. Brains from DEET/permethrin + flupirtine treated animals, showed:
  - occasional dying (eosinophilic) neurons in some animals;
  - the overall cytoarchitecture remained comparable to that of animals from vehicle control of flupirtine groups.

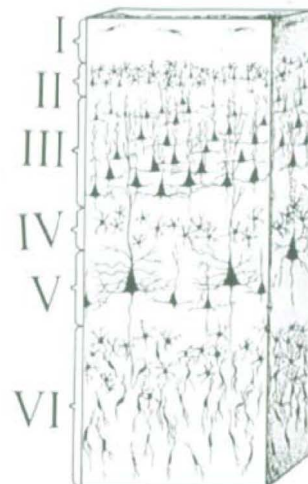


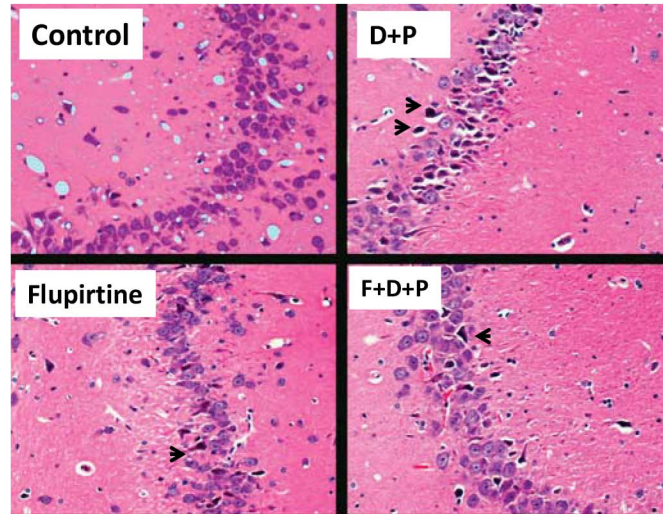
# Cerebral Cortex

1. **H&E-Stained sections exhibited degeneration both in the superficial (layers I-III) and deeper (layers IV – VI) regions of motor cortex.**
2. **The majority of degenerating neurons were were of the pyramidal type**
3. **Flupirtine greatly reduced the number of dead neurons.**

## TYPES OF NEURONS IN CEREBRAL CORTEX

- I. Molecular layer
- II. External granular layer
- III. External Pyramidal layer
- IV. Internal granular layer
- V. Internal pyramidal layer
- VI. Multiform

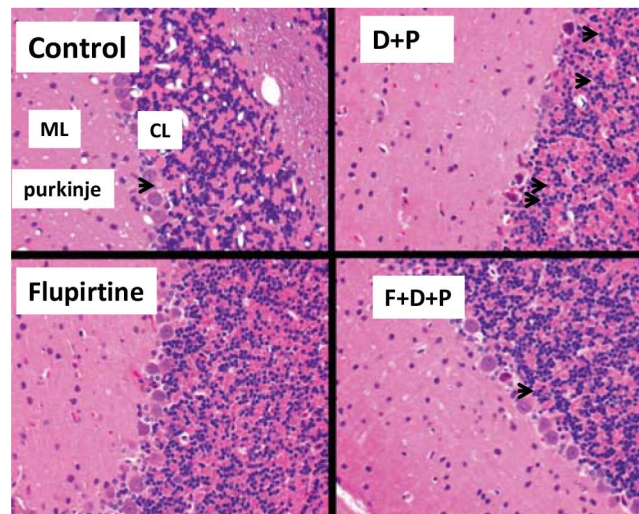




## Hippocampus

### Hippocampal Formation

1. Brains from animals treated with DEET and permethrin exhibited neuronal degeneration in the dentate gyrus and CA1 and CA3 subfields of the hippocampal formation.
2. Flupirtine greatly reduced the number of dead neurons.



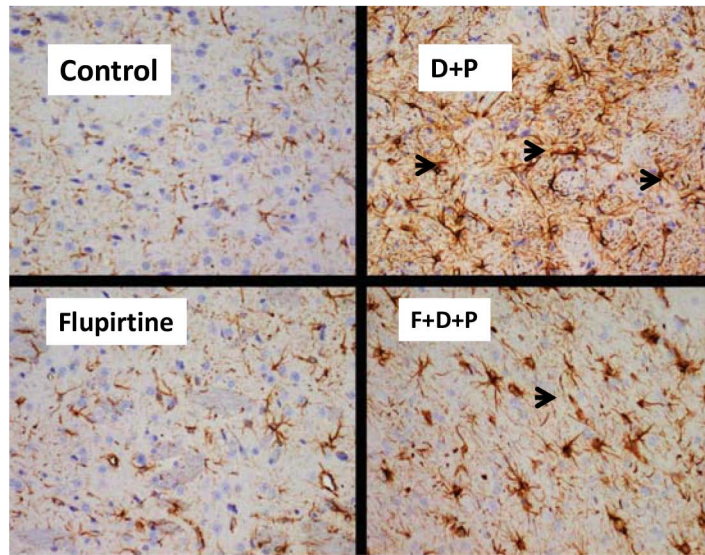
## Cerebellum

## Cerebellum

### DEET/Permethrin:

- Increased numbers of dead cells in the
  - Purkinje layer and
  - granular layer with increased staining intensity
- Flupirtine greatly reduced the number of dead neurons.

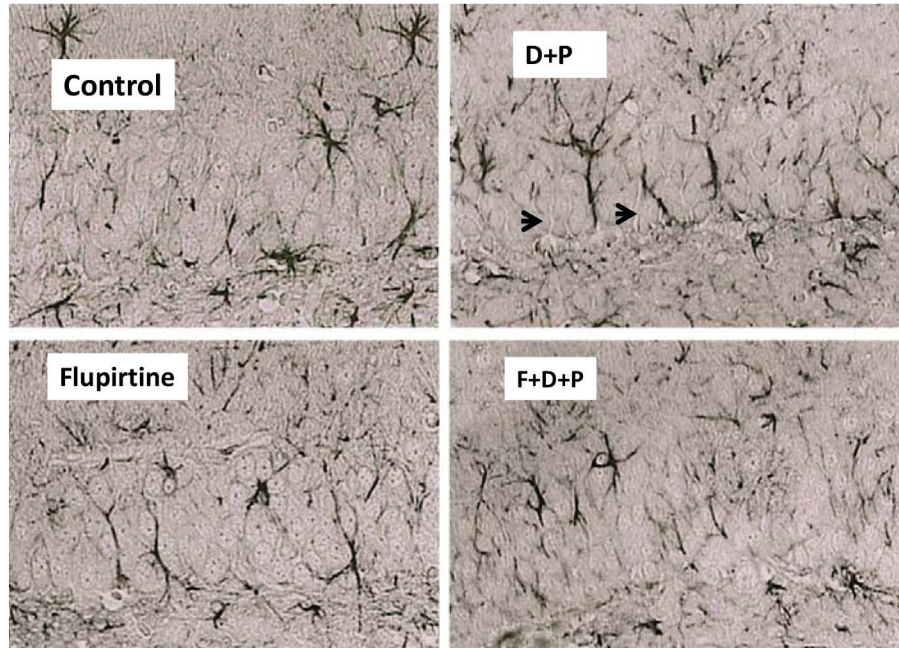




## Cortex-GFAP

### Glial Fibrillary Acidic Protein (GFAP)

1. DEET/Permethrin: Immunostaining with GFAP demonstrated hypertrophy of astrocytes with increased GFAP expression
2. Flupirtine greatly reduced reactive GFAP



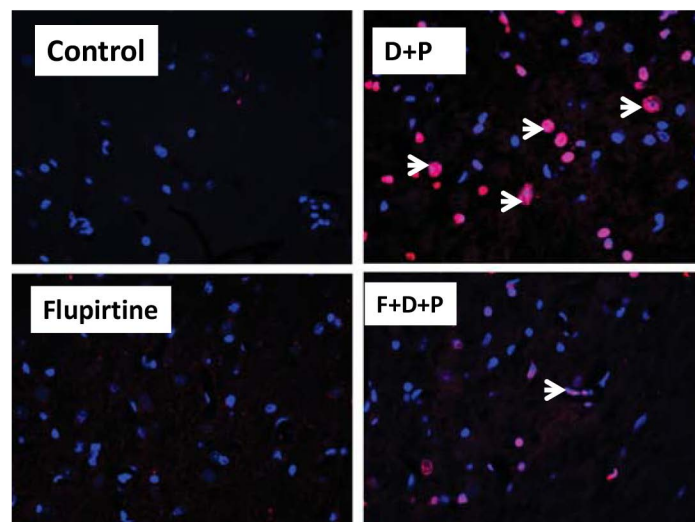
## Hippocampus-GFAP

### Hippocampus (GFAP)

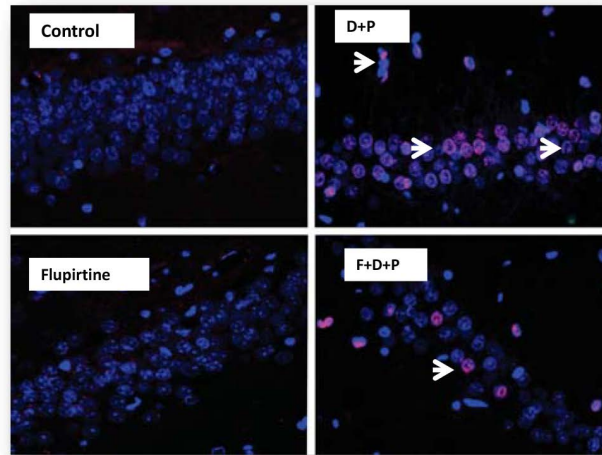
1. DEET/Permethrin: Immunostaining with GFAP demonstrated hypertrophy of astrocytes with increased GFAP expression in:
  - a) The molecular layer
  - a) The CA3 subfield of the hippocampus.
2. DEET/Permethrin + flupirtine treated animals did not show any damaged cells.

# TUNEL STAINING

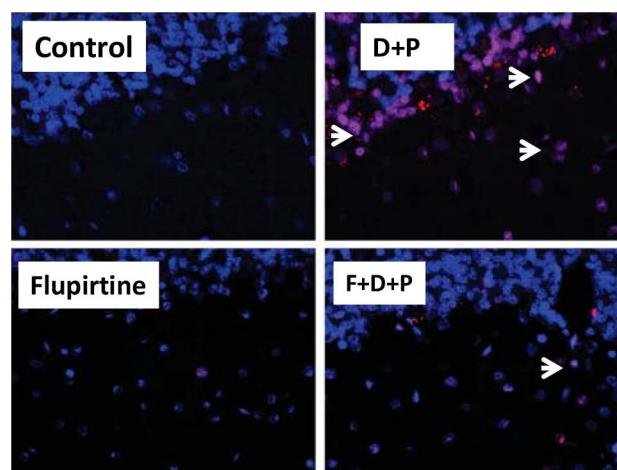
- To determine the involvement of apoptosis in the mechanism of cell death, the brain sections were immunostained with TUNEL.
- Dying neuronal cells were found in the cortex, hippocampus and cerebellum, both the molecular layer and Purkinje cells
- Concurrent treatment of animals with Flupirtine and DEET and Permethrin significantly reduced the number of dying cells in both layers .
- The total number of cell death decreased by 78% from 45% to 10% when Flupirtine was used concurrently with DEET and permethrin in the brain.



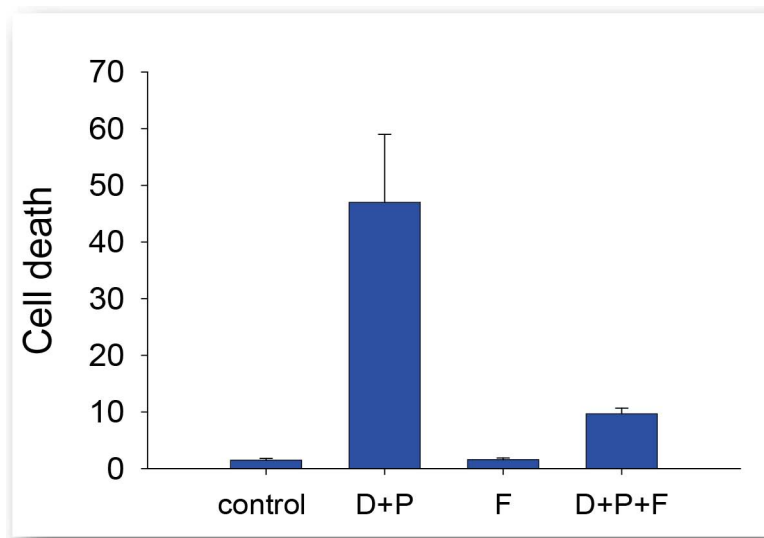
**Cortex-TUNEL**



## Hippocampus-TUNEL



## Crebellum-TUNEL



**Total cell death (Tunel positive cells) in the three areas of the brain**

## **Neuronal Cell Death Are consistent with symptoms og GWI**

1. Significant death of cerebral cortex neurons results in muscular weakness and loss of strength.
2. A loss of significant amount of hippocampal neurons leads to progressive loss of memory and results in learning disabilities.
3. Loss of Purkinje cells in the cerebellum may cause:
  - a. Ataxia
  - b. Terminal tremor at the end of the movement.
  - c. Disorders in the spatial coordination of hand and finger muscle.

# SUMMARY

1. **Body weight and clinical condition of rats treated with DEET and permethrin for 60 days at levels and route of exposure comparable to the Gulf War environment were same as vehicle controls.**
2. **Sensorimotor deficits and Water Maze poor performance by animals exposed to DEET/Permethrin is consistent with brain neuronal cell death in specific regions in the brain.**
3. **Treatment of rats with DEET and permethrin combined with a daily dose of flupirtine for 60 days protected the animals from the action of DEET/Permethrin as follows:**
  - a. **Reversed sensorimotor deficits**
  - b **Showed normal Memory functions**
  - c. **Greatly protected bran cells from injury caused by DEET/Permethrin-induced oxidative stress.**

# CONCLUSIONS

1. **The results and suggest that flupirtine could be used as a safe prophylaxes to protect from chemical-induced nervous system injury, such as in military missions that may result in chemical exposure of military personnel.**
2. **The results also suggest that flupirtine may be useful in treating veterans with mild or moderate symptoms resulting in improvement of the patients' quality of life.**

# CONCLUSIONS

- 1. The results are promising.**
- 2. Other scenarios with varying periods of exposure to DEET/ permethrin and treatment with flupirtine should be tried to find the optimum condition for successful treatment with flupirtine.**
- 3. This conclusion is supported by the findings that flupirtine**
  - does not induce development of dependence or tolerance and**
  - does not have prominent side effects**