Spatiotemporal patterns of GFAP upregulation in rat brain following acute intoxication with diisopropylfluorophosphate (DFP).

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Abstract

We previously demonstrated that the organophosphate diisopropylfluorophosphate (DFP) induced region-specific delayed neuronal injury in rat brain. The goal of this study was to examine the spatiotemporal pattern of the astroglial response after acute intoxication of rats with DFP. Rats were euthanized at varying times between 1 h to 7 days after DFP administration (9mg/kg, i.p.). The distribution of activated astrocytes in different brain regions was determined by immunostaining for glial fibrillary acidic acid (GFAP). GFAP immunoreactivity increased in the hippocampus, and piriform/entorhinal cortex at 1 h, peaked between 4-8 h then decreased from 16 to 24 h. GFAP labeling in the amygdala gradually increased over the 24 h period. A large increase in GFAP labeling was seen at 3-7 days following DFP administration in the hippocampus, piriform/entorhinal cortex and dorsolateral thalamus. At 3 days post-DFP exposure, GFAP immunoreactivity was totally absent in central areas of the hippocampus, piriform/entorhinal cortex and dorsolateral thalamus that contained numerous FluoroJade B (FJB) labeled neurons. By 7 days, GFAP expression was highly upregulated in all areas of each brain region. The mRNA expression of GFAP in hippocampus slowly increased at 24 h following DFP administration then dramatically increased at 3 days. This study suggests that activation of glial cells may contribute to the early neuropathological changes and later neuronal repair/plasticity following acute DFP intoxication.

Keywords: GFAP astrocytes organophosphate DFP intoxication seizure

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Introduction

Organophosphorus compounds (OPs) are widely used as pesticides in agriculture and as nerve agents in chemical warfare and there is extensive experimental and clinical data documenting their neurotoxic potential [1, 2]. Neuropathological alterations develop in the brain following acute intoxication with OP nerve agents, such as sarin, soman and diisopropylfluorophosphate (DFP), in both animal models and human patients [3, 4, 5, 6]. We recently showed that acute DFP intoxication resulted in regional and temporal specific patterns of delayed neuronal injury in the rat brain [6]. Although seizures were induced within minutes following DFP exposure, neuronal injury was not evident in most brain regions until 4-8 h later. Astrocytes have been shown to become activated early after intense neuronal activity and contribute to neuronal injury following seizure [7]. Astrocytes are a heterogeneous class of cells that play an essential role in maintaining homeostasis in the central nervous system and are involved in a number of functions including synaptic plasticity, maintenance of the blood-brain barrier, inflammation and neuronal repair [8]. Reactive astrogliosis is an astrocyte response to various types of mechanical or chemical brain injuries [9]. Reactive astrocytes are characterized by hypertrophy and the upregulation of glial fibrillary acidic protein (GFAP) [9, 10]. GFAP mRNA and protein are also upregulated in a number of species following exposure to OPs [4, 11, 12, 13, 14, 15, 16]. However, a detailed analysis of the spatiotemporal response of astrocytes in rat brain following DFP intoxication has not been previously reported.

In this study, we investigated the astroglial response from 1 h up to 7 days following a single exposure to a seizurogenic dose of DFP. Here, we demonstrated that DFP-induced neuronal injury was accompanied by an early, region-specific astroglial activation characterized by increased GFAP protein within 24 h. Three days after DFP, a large second wave of GFAP upregulation was seen that
Materials and Methods

DFP Poisoning and brain collection
All animals used in these studies were treated humanely and with regard for alleviation of suffering and pain, and all protocols involving animals were approved by the Institutional Animal Care and Use Committee of Morehouse School of Medicine prior to the initiation of experimentation. Adult male Sprague-Dawley rats (280-320 g; Harlan Laboratories, USA) were used for this study. After anesthetization with 2% isoflurane (30% Oxygen, 70% Nitrous Oxide; NexAir, Suwanee, GA), animals were injected with pyridostigmine bromide (PB; 0.1 mg/kg in saline; TCI America, Portland, OR) and then 20 min later with atropine methyl nitrate (AMN; 20 mg/kg in saline; A0755, TCI America). Pretreatment with these drugs, which are centrally inactive, reduces mortality and eliminates peripheral symptoms of cholinergic toxicity thereby enabling clear demonstration of seizure activity [17]. Ten minutes after AMN injection, the rats were injected intraperitoneally with DFP (9 mg/kg diluted in water; D0879, Sigma Chemical Co., St. Louis, MO). DFP was always prepared fresh within 5 min before administration. The treatment groups included naïve control rats, antidote control rats (treated with PB, AMN and distilled water), and rats injected with DFP (with PB and AMN). Animals were euthanized 1 h, 4 h, 8 h, 16 h, 24 h, 72 h or 7 days post-injection (n = 5 for each condition). Brains were cut through the midline and the hippocampus was dissected from the left side for mRNA extraction while the entire right side of the brain was used for the immunostaining. All brains were snap frozen in dry ice and kept at −80°C until further use.

GFAP immunohistochemistry and FJB staining
Coronal sections of 20 µm thickness were cut with a cryostat and mounted on slides which were then stored at −80°C until further processed. Sections were dried at room temperature for 30 min and then incubated with 4% paraformaldehyde (Sigma Chemical Co.) for 15 min. After rinsing in 0.01 M phosphate buffered saline (PBS, pH=7.2), sections were blocked in buffer containing 5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.3% triton-x100 (Sigma Chemical Co.) for 1 h at 4°C and then incubated for 1 h at 37°C with a monoclonal mouse Cy3 conjugated anti-GFAP antibody (1:500, Cat. #C9205, Sigma). Sections were then washed with PBS and coverslipped with an anti-fade mounting medium (VectorLaboratories; Burlingame, CA). Negative controls were incubated with PBS instead of the primary antibodies. FluoroJade B (FJB; Millipore, Billerica, MA) labeling was performed as previously described [6]. Briefly, after drying for 30 min at 50°C, sections were post-fixed with 4% paraformaldehyde for 15 min, washed with distilled water and then directly incubated in 0.06% potassium permanganate (KMnO4) for 10 min on a shaker table followed by distilled water for 2 min. Sections were then incubated in a freshly prepared solution of 0.0004% FJB for 20 min, rinsed in distilled water and then dried at 50°C. Dried slides were cleared by immersion in xylene for 2 min before coverslipping with DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA). Five or more sections were labeled from each brain region of all animals (n = 5 animals per treatment group).

RNA Isolation and Real-time RT-PCR
Total RNA of hippocampus was isolated using TRIZOL reagent (Invitrogen, Carlsbad CA, USA) following sonication. RNA was treated with DNase I to remove any traces of genomic DNA. First-strand cDNA was synthesized from 1µg of each RNA sample using oligo (dT) and Omniscript reverse transcriptase (Qiagen, Valencia, CA), according to the manufacturer’s protocol. Real-time semi-quantitative RT-PCR was performed with SYBR Green (IQTm Sybr Green Supermix, Bio-Rad, Hercules, CA) using an iCycler (BioRad). To quantify GFAP mRNA expression, primers for rat GFAP (sense, 5'-CTGGTTGAGTTCTTGCGCGGCA-3'; antisense, 5'-TTGGGGCTTCTCCTCACAGCC-3') and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5'-ACCCAGAAGACTGTGGATGG-3'; antisense, 5'-ATGGGGGTAGGAACAC-3') were used. Cycling parameters were 95°C for 5 min, then 40 cycles of 95°C for 10 sec, 55°C for 45 sec, then 95°C for 1 min, 55°C for 1 min, and then melt following the manufacturer’s protocol. The fluorescence of the accumulating product was measured at the product melting temperature. To confirm the specificity of PCR products, melting curves were determined using iCycler software. The mRNA levels were normalized using GAPDH mRNA levels. GAPDH mRNA levels did not show any significant variation between treatment groups in our experiment.

Statistical analysis
PCR results are from 45 animals (n = 5 per treatment group) including naïve rats, antidote control rats and rats at various time points after DFP intoxication. Intensity values are presented as the mean ± SEM. Statistical analysis was carried out using ANOVA. Differences were considered significant at the level of p<0.05.

Results

GFAP immunostaining in the hippocampus following acute DFP intoxication
GFAP immunoreactivity was present to various degrees in all brain regions of naïve and antidote control brain....
In the hippocampus of DFP-treated animals, GFAP immunoreactivity was increased in the hilus of the dentate gyrus and the lacunosum moleculare compared to naive animals 1 h after DFP intoxication (Figs. 1A, B). In contrast, there was a dramatic decrease of GFAP immunostaining in the striatum radiatum layer of hippocampus 1-8 h after DFP treatment (Figs. 1B-D). In the hilus of the dentate gyrus and lacunosum moleculare layer, GFAP immunoreactivity remained high at 4 h and decreased to near baseline levels at 8-24 h (Figs. 1C-F). At 3 days after DFP administration, there was increased GFAP immunolabeling in all regions except for a distinct area that traversed the striatum radiatum and moleculare layers of hippocampus where GFAP expression was completely absent (Fig. 1G; arrows). There was a dramatic increase of GFAP expression in the hippocampus 7 days after DFP administration compared to day 3. At 7 days post-DFP exposure, GFAP expression was still absent in the striatum radiatum and moleculare layers (Fig. 1H).

Figure 1. Acute DFP intoxication upregulated GFAP expression in a regional and temporal dependent manner. Representative photomicrographs of GFAP immunoreactivity in the hippocampus and cortex of naïve controls (A) and DFP-exposed rats (B-H) at varying times post-exposure. Relative to control rats (A), GFAP immunoreactivity was noticeably increased in the hilus of the dentate gyrus and lacunosum moleculare layer at 1h (B) and 4h (C) post-DFP exposure. In contrast, GFAP decreased in the striatum radiatum layer at 1h (B), 4h (C) and 8h (D) post-DFP exposure. Levels of GFAP immunoreactivity returned to control levels from 16h (E) to 24h (F). GFAP immunostaining was noticeably absent in a distinct area (arrows) that traversed the striatum radiatum and moleculare layers of the hippocampus at 3 days post-DFP administration (G). At 7 days post-DFP exposure (H), GFAP expression in hippocampus increased dramatically and was more intense relative to any of the earlier time points examined. Interestingly, the area not immunostained by GFAP at 3 days was still evident at 7 days (H, arrows). GFAP staining in the parietal cortex did not change relative to control levels at 1-24h post-DFP exposure (B-F), but increased at 3 days(G) and peaked at 7 days (H) post-DFP exposure. Hi=hilus of dentate gyrus; LM=lacunosum moleculare layer; SM=striatum radiatum layer; PC=parietal cortex; Scale bar= 100 µm.

Figure 2. Acute DFP intoxication increased GFAP expression in the amygdala . Representative photomicrographs show that relative to naïve controls, GFAP immunoreactivity in the amygdala increased from 1h to 8h post-DFP exposure (B-D) and was still high at 16h (E) and 24h (F). The increase persisted at 3 days (G) and peaked at 7 days (H) post-DFP exposure. AMG= amygdala; Pir C= piriform cortex; Scale bar=100 µm.
Figure 3. GFAP expression is increased in the piriform cortex after DFP intoxication.
Increased GFAP immunoreactivity was apparent in layer II and III of the piriform cortex at 1h post-DFP exposure (B) compared to naïve controls (A) in these representative photomicrographs. GFAP immunostaining peaked at 4h (C) and 8h (D) and then returned to baseline levels at 24h (F). Downregulation occurred through 1-8h (B, C & D) in the layer I and returned to baseline at 24h (F). There was a GFAP loss in the piriform cortex at 3 days following DFP treatment (G) compared to naïve controls. However GFAP was significantly increased across all layers of the piriform cortex at 7 days post-DFP exposure (H). Scale bar= 100 µm.

Figure 4. Acute DFP intoxication decreased GFAP expression in the dorsolateral thalamus.
Relative to naïve controls (A) GFAP expression remained unchanged in the dorsolateral thalamus at 1h (B), 4h (C) and 8h (D) post-DFP exposure in these representative photomicrographs. GFAP immunostaining slightly decreased at 16h (E) and 24h (F) after DFP administration. There was a loss of GFAP immunoreactivity in the center of neuronal injury at 3 days after DFP treatment (G) with GFAP upregulated in the bordering area. At 7 days post-DFP exposure (H), there was a noticeable increase of GFAP immunoreactivity in the dorsolateral thalamus that included much of the region where GFAP was lost at day 3. Scale bar= 100 µm.

Figure 5. Spatial relationship of neuronal injury and reactive astroglisis differs regionally in brains of rats acutely intoxicated with DFP.
Representative photomicrographs of FJB labeling (green, B, D, F, H) and GFAP immunostaining (red, A, C, E, G) were taken from adjacent sections of the same brain region at 3 days post-DFP exposure. In the amygdala (A, B) and dentate gyrus (G, H) regions of FJB labeling are also immunostained by GFAP. In contrast, in the piriform cortex (E, F) and dorsolateral thalamus (C, D) GFAP staining is not apparent in the center of the area of significant neuronal injury. AMG= amygdala; Pir C= piriform cortex; DG= dentate gyrus. Scale bar= 100 µm.

Spatiotemporal patterns of GFAP upregulation in rat brain.....
GFAP immunostaining in the amygdala following acute DFP intoxication

In the amygdala, GFAP expression similarly increased 1 h post-DFP exposure compared to naïve controls (Figs 2A, B). GFAP immunostaining in the amygdala further increased between 4 and 24 h following DFP exposure (Figs 2C-F). GFAP immunoreactivity continued to increase 3 days after DFP exposure (Fig. 2G). At 7 days post-DFP exposure, there was a dramatic and widespread increase in GFAP expression throughout the amygdala, and the intensity of GFAP immunoreactivity was higher than observed at earlier time points (Fig. 2H). Unlike the hippocampus, there was no loss of GFAP expression at any time after DFP administration. In the adjacent piriform cortex, there was a slight increase in GFAP labeling 1-24 h after DFP administration; however, GFAP labeling disappeared by 3 days post-DFP exposure (Fig. 2G). As observed in the amygdala, GFAP immunoreactivity in the piriform cortex noticeably increased again at 7 days after DFP treatment (Fig. 2H).

GFAP immunostaining in the cortex following acute DFP intoxication

GFAP immunoreactivity in the cortex was increased in layers II and III of the piriform cortex compared to naïve control animals from 1-8 h following DFP administration (Figs. 3A-D), but then returned to baseline levels by 16-24 h post-DFP exposure (Figs. 3E, F). A similar pattern was observed in the entorhinal cortex (data not shown). However, in layer I of the piriform cortex, GFAP staining decreased 1-8 h after DFP exposure before returning to control levels at 16-24 h. At 4-8 h, GFAP upregulation peaked in layers II and III of the piriform and entorhinal cortices but staining was still reduced in layer I of the cortex at these two time points. No apparent change in GFAP expression was noted in other cortical brain regions up to 3 days post-DFP exposure (data not shown). At 3 days, there was a loss of GFAP immunoreactivity in the piriform (Fig. 3G) and entorhinal cortices. GFAP expression at 7 days (Fig. 3H) was increased in all cortical layers compared to control levels (Fig. 3A).

GFAP immunostaining in the dorsal lateral thalamus following acute DFP intoxication

Relative to naïve controls (Fig. 4A), GFAP immunolabeling was virtually unchanged in the dorsolateral thalamus 1-8 h after DFP administration (Fig. 4B-D), but appeared to be slightly decreased at 16-24 h (Figs. 4E, F). At 3 days post-DFP exposure, there was a massive loss of GFAP expression in the dorsolateral thalamus with increased GFAP immunoreactivity in the bordering area (Fig. 4G). At 7 days following DFP exposure, there was a significant increase of GFAP immunoreactivity in the thalamus that included much of the region where GFAP was lost at day 3 (Fig. 4H).

**Figure 6. Relative levels of GFAP mRNA in the hippocampus at different time points post-DFP exposure.**

Total RNA was collected from hippocampi of naïve controls, antidote controls 24 h following treatment with PB and AMN (A+P) and from DFP-exposed animals at varying times up to 3 days post-DFP exposure. GFAP mRNA significantly increased at 3 days compared to naïve control animals (* denotes p<0.001). Data are presented as the mean ± SEM (n = 5 per treatment group).

Spatial relationship of GFAP and FJB labeling following DFP intoxication

To determine the relationship between neuronal injury and GFAP up- and down-regulation, we labeled adjacent sections of brains obtained at 3 days post-DFP exposure with FJB, a marker of injured neurons, and the anti-GFAP antibody. Expression patterns were examined in multiple brain regions, including the amygdala (Figs. 5A, B), thalamus (Figs. 5C, D), piriform cortex (Figs. 5E, F) and hippocampal dentate gyrus (Figs. 5G, H). As illustrated in Figure 5, there were numerous FJB positive cells in the piriform cortex and dorsolateral thalamus 3 days after DFP intoxication. In these areas, there was negligible GFAP immunoreactivity at the focus of the neuronal injury. In contrast, GFAP immunoreactivity was evident within the same areas containing numerous FJB positive cells, in the amygdala and dentate gyrus.

Delayed induction of GFAP mRNA in the hippocampus following acute DFP intoxication

We measured GFAP mRNA in the hippocampus to determine whether the upregulation of GFAP immunolabeling was due to transcriptional mechanisms. Figure 6 shows that GFAP mRNA was not significantly altered over the first 16 h following DFP intoxication. However, GFAP mRNA was marginally upregulated at 24 h then significantly increased another 3-fold at 3 days following DFP intoxication.
Discussion

The goal of this study was to characterize the spatiotemporal expression of GFAP protein and mRNA expression from 1 h to 7 days following acute intoxication with DFP at seizurogenic doses. In the brain regions we examined, there was an initial peak of increased GFAP immunoreactivity within 4-16 h after DFP exposure. These findings are consistent with studies demonstrating by immunocytochemistry that soman exposure induced rapid astroglial activation that peaked at 8 h [12]. We observed an initial up-regulation of GFAP immunolabeling at 1 h in the layer II of piriform/entorhinal cortices and amygdala, consistent with findings examining GFAP protein from brains of soman poisoned rats [11, 12]. However, we also found a significant increase of GFAP immunoreactivity in the hilus of dentate gyrus, lacunosum molecular layer of hippocampus at this early time point. We have previously demonstrated that there is no evidence of neuronal death in any brain region at this early time point [6], indicating that this rapid regulation of GFAP precedes neuronal degeneration [6, 11, 12]. Given that seizures are elicited within minutes of DFP administration in our model [6], our findings are consistent with studies demonstrating that astrocytes become rapidly activated after intense neuronal activity and seizures [7].

In contrast to previous studies of astrocyte activation following acute soman intoxication that terminated at 24 h [12], we carried out our studies to 7 days post-exposure, and observed a second massive wave of GFAP upregulation that occurred 3-7 days after DFP administration. Similarly, in a study of mice exposed to soman, long-term delayed astroglial activation was shown to peak at 3 days and persist for 90 days and this was associated with glial scar formation [15]. The rapid astroglial activation and late astroglial activation was also demonstrated in rats intoxicated with kainic acid [18, 19]. The role(s) of activated astrocytes in DFP-induced neurotoxicity are not clear. Inflammatory responses involving astrocytes and microglial cells have been shown to occur both early and late in the injured brain following OP intoxication [12, 15, 20, 21, 22, 23, 24, 25]. Microglial activation follows the early increase of GFAP immunoreactivity and precedes neuronal injury suggesting that astrogliosis induced by seizures results in an inflammatory response that is involved with neuronal injury [11, 12]. However, there is evidence suggesting that delayed astrogliosis could mediate synaptic plasticity and neuronal repair [5]. This possibility is also suggested by studies in GFAP knockout mice in which deficiencies in GFAP were associated with blood brain barrier impairment, impaired maintenance of white matter integrity and myelination and altered neuronal function. GFAP has also been shown to be necessary for proper glial scar formation after CNS trauma [26, 27], and GFAP knockout mice displayed increased neuronal damage in the hippocampus following brain trauma and kainate excitotoxicity [28]. Conversely, GFAP knockout mice were reported to have increased levels of GDNF resulting in protection of neurons from quinolinic acid-induced excitotoxicity [29].

An intriguing finding of our study was the total loss of GFAP immunoreactivity at the focus of neuronal injury in the thalamus, hippocampus and piriform/entorhinal cortex at 3 and 7 days after DFP while the staining dramatically increased in the areas bordering the injury. Decreased GFAP could result from astrocyte loss or cytoskeletal destabilization/degradation and loss of GFAP antigenicity. Alternatively, the early loss of GFAP could be due to astrocytes migrating from layer I to layer II in the piriform/entorhinal cortex or from the striatum radiatum layer to the lacunosum molecular layer in the hippocampus. Comparable results were reported in a study measuring GFAP levels in the brains of hens 1-20 days after exposure to DFP. Using Northern blot hybridization, cerebral tissue from the brains of hens exposed to DFP exhibited down regulated GFAP mRNA level at day 1 compared to controls and upregulated GFAP mRNA at 10 and 20 days [13]. The investigators suggested that GFAP levels were reduced in areas most susceptible to OP-induced damage. This is consistent with our data indicating that GFAP is reduced in areas with high numbers of FJB positive cells in some regions. Decreased GFAP expression did not occur in the amygdala, dentate gyrus molecular layer and other cortical regions at 3 and 7 days after DFP exposure; however, we had previously shown that neuronal injury peaks very early (8 h post-DFP exposure) in these brain regions [6]. GFAP reactivity is significantly upregulated in these injured regions. This suggests the possibility that the function(s) of astrocyte activation following DFP exposure may vary across different brain regions with regards it role in neuronal injury and function [5].

It is unlikely that increased GFAP labeling at early time points (< 16-24 h post-DFP exposure) was due to increased GFAP transcription and translation. It has been suggested that the rapid increase in GFAP immunoreactivity after OP exposure or seizures results from astrocytic swelling leading to cytoskeletal changes that expose GFAP antigenic sites [10, 12]. In support of this proposal, inhibition of protein synthesis by actinomycin D did not prevent OP-induced upregulation of GFAP [11, 12]. The late astroglial activation is likely due to gliosis leading to over-expression of GFAP protein [15]. Indeed, our RT-PCR data showed GFAP mRNA was induced at 3 days following DFP administration, but not at earlier time points. Collectively, these data suggest that there is an early wave of GFAP upregulation that does not involve transcriptional/translation mechanisms and a delayed up-regulation of GFAP mediated by transcriptional mecha-
isms and/or astrocyte proliferation.

In conclusion, our studies suggest an important role for astroglial activation during OP-induced neuronal injury. The spatiotemporal patterns of GFAP expression following acute DFP intoxication indicates that GFAP may have multiple and distinct roles during the initiation and progression of OP-induced neurotoxicity. Understanding how GFAP is altered following OP intoxication will give insight into the roles for GFAP in mediating neuronal damage and tissue repair following CNS injuries.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Spatiotemporal patterns of GFAP upregulation in rat brain…..


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