JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469

"JPET #83469"

Subcellular Alterations of Protein Kinase C Isozymes in the Rat Brain Following Organophosphate Poisoning

by

Eugenia Bloch-Shilderman, Tamar Kadar, Aharon Levy, Rita Sahar, Ishai Rabinovitz and Eran Gilat

Department of Pharmacology, Israel Institute for Biological Research, Ness Ziona, Israel

JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469
This article has not been copyedited and formatted. The final version may differ from this version.

"JPET #83469"

A) Running title: Alterations in PKC isozymes following sarin exposure.

B) Corresponding author: Dr. Shilderman Eugenia, Department of Pharmacology, Israel Institute for Biological Research, P.O.Box 19, Ness Ziona 74100, Israel. Tel: 972-8-9381447; Fax: 972-8-9381995; E-mail: ***shilderman@iibr.gov.il***

C) Number of text pages

Number of Tables: 1.

Number of figures: 6.

Number of references: 40.

Number of words in the abstract: 215.

Number of words in the introduction: 753.

Number of words in the discussion: 1222.

D) ABBREVIATIONS: cPKC, conventional protein kinase C; ChE, cholinesterase; CNS, central nervous system: DAG, diacylglycerol; KA, kainic acid; NMDA, N-metyl-D-aspartate; OD, optical density; OP, organophosphate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; SDS, sodium dodecyl sulfate;

E) Recommended section: Neuropharmacology

ABSTRACT

PKC signaling pathway has been associated with modulation of NMDA receptor activity, motor behavior, learning and memory, all of which are severely impaired in organophosphate (OP) intoxication. Nevertheless, the role of PKC in OP intoxication is largely unknown. The present study attempted to characterize alterations in the immunoreactivity levels of PKC isozymes expressed in different brain areas in the rat following exposure to the nerve agent sarin ($1xLD_{50}$). Furthermore, possible neuroprotective effect of selective PKC regulating peptide after such insult was evaluated. The results indicated that: 1) A significant reduction in the immunoreactivity level of the conventional betaII-PKC and the atypical zeta-PKC was observed in frontal cortex up to 24 hrs post sarin and in the striatum up to 5 days post sarin exposure. This reduction was in contrast to the increase in the immunoreactivity level of both isozymes seen in the hippocampus or thalamus. 2) Treatment with the anticonvulsant midazolam (0.5 mg/kg) 10 min post sarin exposure markedly reduced zeta-PKC immunoreactivity level, as well as betaII-PKC in the membrane fractions in the hippocampus. 3) betaII-PKC peptide (380 ng/kg), known to inhibit PKC translocation and activation, attenuated sarin-induced neuropathology. These observations suggest a role for both conventional and atypical PKC isozymes in OP-induced neuropathy in the rat and further support their involvement in cell death.

JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469
This article has not been copyedited and formatted. The final version may differ from this version.

The nerve agent sarin, a potent inhibitor of cholinesterase (ChE), is extremely toxic, mainly due to its interfering with the central and peripheral cholinergic nervous system (Taylor, 1985). The accumulation of acetylcholine at nerve terminals exhibits toxic manifestations of vital muscarinic and nicotinic sites such as salivation, muscle tremor, convulsions, respiratory center depression and peripheral respiratory arrest. Sarin-induced convulsions are accompanied by increased release of excitatory amino acids, leading to a massive activation of the glutamate receptors (Sloviter and Dempster, 1985; Olney *et al*., 1986; McDonough and Shih, 1997). Substantial induction of both cholinergic and glutamatergic systems leads eventually to brain damage, associated with a disruption of intracellular Ca^{2+} homeostasis. Some of the Ca^{2+} mobilization from intracellular and extracellular stores occurs through the cleavage of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to yield Inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) and triggers the activation of protein kinase C (PKC) to further continue a phosphorylating cascade. As emerging lately from the literature, PKC has been coupled with modulation of the NMDA receptor activity (Lan *et al*., 2001), appears to mediate the non-NMDA receptor Ca^{2+} mobilization (Connor *et al*., 1988; Alagarsamy *et al*., 2001) and also play different roles in epileptogenesis (Ono *et al*., 1994; Tang *et al*., 2004). Neurotoxic doses of glutamate induced a persistent subcellular redistribution of PKC isozymes in vitro (Favaron *et al.*, 1990; Durkin *et al*., 1996), while its rigid structural analogue, kainic acid (KA), was also shown to affect expression of PKC isozymes in vivo (Guglielmetti *et al*., 1997; McNamara *et al.*, 1999). PKC inhibitors were found to delay the increase in intracellular Ca^{2+} and the neurotoxicity induced by glutamate receptor hyperstimulation (Favaron *et al.*, 1990; Felipo *et al*., 1993).

The PKC family members consist of at least 12 serine/threonine kinases, activated indirectly by G-protein-coupled receptor stimulation. They differ in their distribution in the CNS, subcellular target receptors, substrate affinities and second messenger activators (Tanaka and Nishizuka, 1994; Newton, 2001). Each PKC isozyme is expressed in a specific manner within different cells, and multiple PKC isozymes are expressed in a single cell (Mochly-Rosen, 1995). The PKC isozyme are grouped into three classes: conventional (α,β,γ) which are Ca^{2+} and DAG-dependent, novel (δ, ε, θ, η) which are DAG-dependent and atypical (ζ, ι/λ) which are insensitive to both Ca^{2+} and DAG. The accepted mechanism has been that these isozymes are cytosolic in the inactive state and translocate to the inner leaflet of the cellular membrane, as part of their activation process (Sakai *et al*., 1997). Thus, classical as well as novel PKC isozymes become activated as a result of the association of the cytosolic enzyme with membranes containing phospholipids and elevations in intracellular Ca^{2+}, both which are considered to be elicited by hyperactivity of the CNS.

Several brain regions are vulnerable to OP intoxication. It has been found that in surviving rats following exposure to sub lethal doses of OP, there was an enlargement of brain ventricles on the expense of other brain tissues and mainly the piriform cortex, thalamus and hippocampus are damaged, e.g. a significant decrease in CA1 and CA3 hippocampal cells was detected (Lemercier *et al*., 1983; Kadar *et al*., 1995). Pretreatment with carbamate ChE inhibitors, such as pyridostigmine, followed by atropine sulfate and oxime therapy (Sidell, 1974) could increase significantly the survival rate in rats. However, this treatment regimen does not eliminate sarin-induced seizures and convulsions (Gordon *et al*., 1978; Shih *et al*., 1999). Furthermore, the seizure activity may progress into *status epilepticus* and thus extends neuronal cell pathology (Lemercier *et al*., 1983). Anticonvulsant compounds such as

benzodiazepine decrease seizure activity although incompletely, depending mostly on the administration-time post exposure (Lallement *et al*., 1991; McDonough *et al*., 1995). The compounds included in the treatment regimen are characterized as receptor up-stream effectors. Nevertheless receptor-mediated downstream effectors were scarcely studied. Also, the molecular and cellular events responsible for the brain pathology following OP poisoning, including the role of PKC were scantily investigated (Bodjarian *et al*., 1992). Therefore, the present study aimed to find a link between PKC activation and sarin-induced brain damage by characterization of the alterations in brain PKC isozymes expression in the rat. The immunoreactivity level in membrane and cytosolic fractions of individual PKC isozymes in different brain regions was examined during sarin-induced seizure activity. In addition, a possible neuroprotective effect of selective peptide regulators for PKC isozymes (Ron *et al*., 1995; Koponen *et al*., 2003) was evaluated following OP insult.

The results suggested that up-regulation of PKC isozymes, e.g. beta-II and zeta-PKC, might contribute to the histopathological sequelae produced by sarin.

Methods

Materials. Atropine sulfate, pyridostigmine hydrobromide and other chemicals were purchased from Sigma Chemical Co., UK. Sarin (isopropyl methylphosphono-fluoridate) was synthesized by the Department of Organic Chemistry, IIBR, Israel and used in freshly prepared saline solutions. Sodium pentobarbitone (Nembutal) for anesthesia was purchased from C.T.S Ltd., Israel. The oxime 1-(4-aminocarbonylpyridinium) 1'-(2"-pyridiniumaldoxime) dimethylether dichloride (HI-6) was received as a gift from the German Ministry Of Defense; Midazolam (Dormicum, 5 mg/ml solution) was purchased from Hoffman La Roche, Nutley, NJ, USA; Rabbit anti cPKC (1:100), betaII (1:2000) and aPKC zeta (1:1000) polyclonal antibodies, including their blocking peptides for control analysis and Western blotting luminol reagent (ECL) were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. Second antibodies, anti-rabbit or -mouse IgG horseradish peroxidase conjugate were purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA. Fuji Super HRG film was purchased from FUJI PHOTO FILM CO., LTD., Tokyo, Japan. Peptides regulators of PKC isozymes conjugated to antennapedia (translocation inhibitor of βII-PKC (βII-V5-3 (QEVIRN)) and Antennapedia carrier monomer as a control, Stebbins and Mochly-Rosen, 2001) were purchased from Stanford Protein and Nucleic Acid Facility, SF, USA. Bradford regent for protein determination and other reagents for Western-blotting were purchased from Bio-Rad Laboratories Gmbh, Munchen, Germany.

Animal model. The following experimental procedures were approved by the Institutional Animal Care and Use Committee at the Israel Institute for Biological Research and are in accordance with the principles enunciated in the *Guide for the*

Care and Use of Laboratory Animals, National Academy Press, Washington DC, 1996. Adult male Sprague-Dawley rats (weighing 290-340 g; Charles River-Labs) were exposed to sarin in five experimental groups:

1. Sarin exposure (90 mg/kg~1LD_{50}, i.m.).
2. Sarin exposure (108 mg/kg~1.2LD_{50} i.m.) with a supportive treatment (oxime (HI-6, 5 mg/kg i.m.) and ChE inhibitor, pyridostigmine (0.1 mg/kg, i.m.) 20 min prior to sarin exposure and muscarinic antagonist, atropine (3 mg/kg i.m.) 1 min after exposure.
3. Sarin exposure (108 mg/kg~1.2LD_{50} i.m.) with supportive treatment as in group number 2, including treatment with midazolam (5 mg/kg) 10 min post initiation of seizure activity.
4. Sarin exposure (108 mg/kg~1.2LD_{50} i.m.) with supportive treatment as in group number 2, including peptide regulators for PKC isozymes injected (380 ng/kg, i.c.) 10 min post sarin-induced convulsive activity.
5. Naive or sham operated rats served as a control group.

Subcellular fractionation. Rats from groups 1 and 3 were sacrificed by decapitation at the following time points: 10, 30 min, 2 & 24 hr and 5 & 10 days post sarin exposure. Brains were rapidly removed and further microdissected into frontal cortex, striatum, hippocampus and thalamus components. Tissues were then homogenized with a freshly prepared homogenization buffer [20 mM HEPES (pH 7.5), 2 mM EGTA, 0.3 mg/ml dithioerythritol, 0.16 mg/ml phenylmethylsulfonyl fluoride, 0.02 mg/ml aprotinin] and prepared in accordance with subcellular fractionation procedures (McNamara *et al*., 1999). Briefly, homogenates were centrifuged at 100,000×g for 1 h at 4ºC. The resulting supernatant represented cytosolic proteins. Membrane proteins were collected by solubilizing the remaining

pellet in homogenization buffer containing 0.1% Triton X-100 on ice for 45 min and centrifuging at 50,000×g for 30 min at 4ºC. Both fractions were normalized to contain a final concentration of 0.05% Triton. Protein concentration was determined by the method of Bradford (1972). Samples were immediately boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 3 min and stored at –80ºC until used.

Western blot analysis. PKC isozyme-specific antibodies, βII- and ζ–PKC were used to determine PKC isozyme expression. Western blot analysis was performed by loading 40 μg of protein on 10% SDS-PAGE and electrophoresing the samples for ~ 1.45 hr at 100 V. The gels were transferred to a nitrocellulose membrane (0.45 μm) in a Trans-Blot Semi-Dry electrophoresis apparatus (Bio-Rad) at 10 V for 25 min. Following transfer, the blots were rinsed in TS buffer [20 mM Tris (pH 7.5) and 500 mM NaCl], and nonspecific binding sites were blocked by incubating the blots in 5% dry milk in TS buffer for 1 hr at room temperature. The primary PKC isozyme-specific antisera were diluted in TS buffer and titrated to yield optimal specific binding. Blots were incubated over night with the primary antibodies at 4ºC, washed three times for 15 min in TS buffer containing 0.2% Tween 20, and subsequently incubated with the second antibody (anti-mouse or –rabbit IgG horseradish peroxidase conjugate) for 1 hr at room temperature. The blots were washed, incubated with the enhanced chemoluminescence reagent for 1 min, exposed to film and developed. For graphing purposes, potential changes in PKC isozyme levels following sarin exposure were expressed as a percentage of control values that were electrophoresed on the same gels.

Surgery-Procedure. Rats anesthetized with pentobarbitone (25mg/kg) were implented a cannula to the right carotid artery. 72 h post surgical processes rats were

exposed to sarin (as described in animal model section, group 2). Peptide regulators for PKC isozymes were injected (380 ng/kg, i.c.) 10 min post the initiation of sarin-induced convulsions.

Histology. Rats from groups 4 and 5 were sacrificed by decapitation 48 hr post sarin exposure. Brains were fixed in 4% neutral buffered paraformaldehyde and processed routinely for paraffin embedding. Six microns sections were cut and stained with hematoxylin and eosin staining (H&E) and further evaluated by light microscopy analysis.

Statistics. Densitometry analyses for Western blotting were performed using the Scion Image software. Statistical differences between band intensities were determined by ANOVA ($p \leq 0.05$). Significant statistical differences between animal groups were also analyzed by ANOVA ($p \leq 0.05$).

RESULTS

Expression of PKC isozymes. In the present study the expression of members of the PKC isozyme family was examined in cytosolic and membrane fractions derived from rat frontal cortex, striatum, hippocampus and thalamus. The involvement of conventional PKC isozymes in sarin-induced brain damage was first detected by using a non-selective antibody for the α, β, and γ isotypes (data not shown). Since each conventional isotype contribution could not be evaluated with the non-specific c-PKC antibody, a specific antibody for the βII-PKC isotype was used. As can be seen in Fig. 1A, there was hardly a significant change in the immunoreactivity level either in the cytosolic or in the membrane fractions in the frontal cortex, except for a 30% reduction 2 hrs post sarin exposure. On the contrary, βII-PKC isotype was highly expressed (3-4 folds vs. control) in both cytosolic and membrane fractions in the hippocampus (Fig. 1B) and thalamus (Fig. 1C), accompanied by a remarkable down regulation (50%) in the striatum (Fig. 1D). The expression of the atypical protein member, ζ–PKC isozyme, also changed significantly following sarin exposure. A dramatic increase (3-5 folds) of ζ–PKC immunoreactivity was observed 5 and up to 10 days post sarin exposure in the hippocampus (Fig. 2B) and thalamus (Fig. 2C), respectively. Quite the opposite was seen in the frontal cortex (Fig. 2A) whereas a decrease (30-50% of control values) in both fractions was found. No changes were seen in the striatum (Fig. 2D). Treatment with the anticonvulsant midazolam (0.5 mg/kg) 10 min post sarin exposure attenuated seizure activity, causing rats an immediate sedation-like state. Rats administered midazolam and sacrificed 2 hrs post sarin-exposure showed a markedly reduction in βII- and ζ-PKC expression in the membrane fraction in the hippocampus (Fig. 3A and B, respectively).

JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469
This article has not been copyedited and formatted. The final version may differ from this version.

Clinical and histopathological observations. Manifestation of symptoms shown in Table 1 in sarin-treated rats appeared within 2-4 min after injection. Animal follow-up included clinical observations for 24 hrs post sarin-exposure, weight monitoring and histopathology evaluation 48 hrs following poisoning. Weight loss reached its maximal level 48 hrs post intoxication (15%, $p<0.05$, in 90% of exposed animals). Table 1 presents both clinical and histopathological scores graded from 0 to 5 according to the severity condition of each parameter.

The influence of βII-PKC inhibitor peptide on sarin neurotoxicity. Since both βII- and ζ–PKC isotypes were over expressed post sarin exposure, the effect of certain peptide regulator of these kinases has been determined. βII-PKC inhibitor peptide (380 ng/kg), known to inhibit PKC translocation and activation, was chosen as an example for such challenge. As can be seen in Fig. 4-6, βII-PKC inhibitor peptide attenuated sarin-induced neuropathology. Notice the viability of hippocampal CA1 cells in the histological brain sections stained with hematoxylin and eosin (Fig. 4C) in contrast to the neuronal damage post sarin exposure (Fig. 4B). By counting the intact cells in the CA1 layer, the neuroprotective effect of βII-PKC inhibitor peptide could be quantitated (Fig. 5). Also, as can be seen in Fig. 6, a fine correlation between clinical score and histological findings was observed.

DISCUSSION

OP nerve agent studies were conducted during the past several years to define mechanisms of OP-induced convulsions in order to improve the currently utilized nerve agent antidotal regimen. Since subcellular and molecular events were scarcely studied the purpose of the present study was to characterize specific messengers that might participate in OP-induced brain damage. In this study we have shown that PKC isozymes, which have been implicated in different neuronal signaling processes, namely the conventional βII-PKC and the atypical ζ-PKC were involved in sarin-induced brain damage. This was demonstrated by the following findings: 1) Significant elevations (2-4 folds) in βII-PKC immunoreactivity were observed up to 10 days post sarin-exposure in the membrane fractions in the hippocampus and thalamus, but barely in the frontal cortex, or as opposed to the striatum (50% decrease). 2) A dramatic elevation (3-5 fold) in ζ-PKC immunoreactivity was found in the hippocampus and thalamus, 5 and to a lesser extend, 10 days post sarin-exposure. ζ-PKC isozyme showed also alterations in the frontal cortex, characterized by remarkable down regulation immediately post sarin intoxication and lasted up to 10 days, mostly in both the cytosol and membrane fractions. 3) Anticonvulsive treatment following administration of the conventional antidotal regimen reduced, almost to baseline, the immunoreactivity of ζ-PKC as well as βII-PKC isozymes in the hippocampal membrane fractions post sarin poisoning. 4) Injection of βII-PKC inhibitor peptide into the rat carotid artery attenuated sarin-induced neuropathology. These observations support accumulating evidences that βII- and ζ-PKC isozymes play a role in promoting neuronal cell death (McNamara *et al.*, 1999; Koponen *et al*., 2003; Tang *et al*., 2004).

In the present study different patterns of protein expression for βII- and ζ-PKC isozymes were detected, emphasizing the diversity in the regulation of both PKC isozymes in different brain areas. Although not in the exact pattern in the hippocampus and thalamus, a significant up-regulation of both βII- (Fig. 1B and C) and ζ-PKC isozymes (Fig. 2B and C) has occurred. The high expression in both the cytosolic and membrane fractions is likely not due to translocation, which is characterized by an increase in content in the membrane, accompanied by a reduction of a similar magnitude in the cytosol. Instead, such increase could be attributed to a reduction in proteolitic degradation or a rapid induction in synthesis. A combination of both processes could explain our observations. Young *et al*., (1987) reported that synthesis of PKC takes minutes following phorbol ester treatment, verifying the possibility of de-novo synthesis. In the other hand, the reduction in the PKC isozymes observed minutes after sarin exposure (Fig. 1D, 2A and D), in the absence of apparent translocation from the other fraction, indicates a modulation in proteolitic degradation processes. Our data are in accord with the findings of McNamara *et al*., (1999) which demonstrated a redistribution of PKC isozymes following KA administration in the rat hippocampus. No translocation was observed, whereas remarkable changes in isozymes expression were found. Also, a dramatic up-regulation of ζ-PKC occurred 5 days post KA administration, similar to the present data (Fig. 2B and C). Albeit different mechanism of action, similarity between KA and OP in seizure-induced neuropathology can now be presented in the subcellular level, involving the modulation of PKC isozymes, alone with histopathological studies (O'Shaughnessy and Gerber, 1986; Kadar *et al*., personal communication). Our results described an up regulation of PKC isozyme immunoreactivity in the hippocampus and thalamus, the brain regions that are known to be vulnerable to OP intoxication (Lemercier *et al*.,

1983; Kadar *et al*., 1995). On the contrary, no significant changes or down regulation had occurred in the fontal cortex or striatum (Fig. 1A, D and 2A and D). This regional correlation between isozyme high expression and vulnerability to OP intoxication might underscore the contribution of βII- and ζ-PKC isozymes to neuronal cell death. However, the precise mechanism of action and how the decrease in isozyme expression might be related to cell survival remains to be elucidated.

Protein kinase C has been implicated in the delayed neurotoxic effects of glutamate that is known to participate in OP intoxication. Previous studies have suggested a role for PKC isozymes in brain damage and outlined a correlation between sustained glutamate release, PKC activity and downstream neuronal damage (Nakane *et al*., 1998: Skeberdis *et al*., 2001; Alagarsamy *et al*., 2001). Although conflicting results concerning its activation or inhibition, PKC plays a role in excitotoxic neuronal death (Favaron *et al*., 1990; Felipo *et al*., 1993; Durkin *et al*., 1996; Wagey *et al*., 2001). For example, Felipo *et al*., (1993) claimed that inhibitors of PKC are able to protect cultured cerebellar neurons from excitotoxic death. In contrast, Wagey *et al*., (2001) showed that pre-exposure to PMA significantly augmented death of NMDA-treated transfected HEK cells. Lately, Koponen *et al*., (2003) have suggested a role for ζ-PKC in NMDA excitotoxicity demonstrated by confocal mycroscopic photographs. They showed that inhibition of ζ-PKC, but not δ-PKC, confers protection to neuron-astrocyte cultures exposed to a level of NMDA, which produced excitotoxic cell death. Taking an advantage of commercially available specific antibodies for PKC isotypes and previously characterized peptides, which inhibit different PKC isozymes translocation (Ron *et al*., 1995), it was possible to study their involvement in sarin neurotoxicity in vivo. In the present study, inhibition of βII-PKC translocation, by a specific peptide, not only attenuated the clinical manifestations following sarin-

intoxication (Fig. 5), but also increased neuronal survival as was detected by histopathological studies, supported by morphometric analysis (Fig. 4). These rationally designed peptides were found to selectively inhibit PKC isotype translocation and induce cardioprotection (Chen *et al.*, 2001; Inagaki *et al.*, 2003) and neuroprotection (Koponen *et al*., 2003; Bright *et al*., 2004). Even though the above studies described PKC involvement in the development of ischemia and apoptotic neuronal death, which are not the primary mechanisms related to OP exposure, it is reasonable to assume that both iscemia and OP-induced excitotoxicity share related downstream processes. All together, these findings support the hypothesis of a modulation role for PKC in NMDA-receptor mediated neuropathology and its possible clinical value. Completing the study with ζ-PKC inhibitor and others will verify PKC involvement in OP poisoning and provide a potentially new target for drug development and treatment.

According to the results presented in this study, and others (Weissman and Raveh, 2003; Gilat *et al*., 2004) we propose a possible sequence of neurochemical events following OP-induced seizures: The inhibition of ChE by OP causes a remarkable elevation of acetylcholine, which is responsible for the initiation of crucial events (McDonough and Shih, 1997). The earlier cholinergic phase, which is started as a result of massive accumulation of ACh and it's binding to the muscarinic and nicotinic receptors probably induces PLC activation and PKC isozymes mobilization and activation in different brain areas. This PKC activation is not necessarily the one that is related to the degenerative processes. However, due to the progressive events that include the disruption of catecholamine and excitatory amino acid neurotransmitters following the cholinergic phase, there is a maintenance of the vast seizure activity leading to brain damage. The excitatory amino acid phase post OP

exposure may involve PKC isotypes in a Ca^{2+}-dependent and independent manner. Paralleled or sequenced activation of different pathways including phospholipases (A and D) and their metabolites (such as cyclooxygenase), reactive oxygen and nitrogen species, kinases, proteases and cytokines may further account for the neurodegenerative processes.

References

Alagarsamy S, Sorensen SD and Conn PJ (2001) Coordinate regulation of metabotropic glutamate receptors. *Curr Opin Neurobiol* **11**:357-362.

Bodjarian N, Lallement G, Carpentier P, Baubichon D and Blanchet G (1992) Soman-induced phosphoinositide hydrolysis in rat hippocampal slices: biochemical characterization. *Neurotoxicol* **13**:715-721.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.

Bright R, Raval AP, Dembner JM, Perez-Pinzon MA, Steinberg GK, Yenari MA and Mochly-Rosen D (2004) Protein kinase C delta mediates cerebral reperfusion injury in vivo. *J Neurosci* **24**:6880-6888.

Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GWII and Mochly-Rosen D (2001) Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc Natl Acad Sci USA* **98**:11114-11119.

Connor JA, Wadman WJ, Hockberger PE and Wong RKS (1988) Sustained dendritic gradients of Ca^{2+} induced by excitatory amino acids in CA1 hippocampal neurons. *Science* **240**:649-653.

Durkin JP, Tremblay R, Buchan A, Blosser J, Chakravarthy B, Mealing G, Morely P and Song D (1996) An early loss in membrane protein kinase C activity precedes the excitatory amino acid-induced death of primary cortical neurons. *J Neurochem* **66**:951-962.

Favaron M, Manev H, Siman R, Bertolino M, Szekely AM, DeErausquin G, Guidotti A and Costa E (1990) Down-regulation of protein kinase C protects cerebellar

granule neurons in primary culture from glutamate-induced neuronal death. *Proc Natl Acad Sci USA* **87**:1983-1987.

Felipo V, Minana MD and Grisolia S (1993) Inhibitors of protein kinase C prevent the toxicity of glutamate in primary neuronal cultures. *Brain Res* **604**:192-196.

Gilat E, Chapman S, Grauer E, Kapon J and Kadar T (2004) Midazolam treatment against sarin-induced seizures: The effect on the neuroinflammatory cascade in the rat brain. *Bioscience Review* 172.

Gordon, JJ, Leadbeater L and Maidment MP (1978) The protection of animals against organophosphate poisoning by pretreatment with carbamate. *Toxicol Appl Pharmacol* **43**:207-216.

Guglielmetti F, Rattray M, Baldessari S, Butelli E, Samanin R, and Bendotti C (1997) Selective up-regulation of protein kinase C epsilon in granule cells after kainic acid-induced seizures in rat. *Brain Res Mol Brain Res* **49**:188-196.

Inagaki K, Chen L, Ikeno F, Lee FH, Imahashi K, Bouley DM, Rezaee M, Yock PG, Murphy E and Mochly-Rosen D (2003) Inhibition of delta-protein kinase C protects against reperfusion injury of the ischemic heart in vivo. *Circulation* **108**:2304-2307.

Kadar T, Shapira S, Cohen G, Sahar R, Alkalay D and Raveh L (1995) Sarin-induced neuropathology in rats. *Hum Exp Toxicol* **14**: *252-259.*

Koponen S, Kurkinen K, Akerman KE, Mochly-Rosen D, Chan PH and Koistinaho J (2003) Prevention of NMDA-induced death of cortical neurons by inhibition of protein kinase Czeta. *J Neurochem* **86**:442-450.

Lallement G, Carpentier P, Collet A, Pernot-Marino I, Baubichon D, Sentenac-Roumanou H and Blanchet G (1991) Involvement of glutamic system of

amygdala in generalized seizures induced by soman: comparison with the hippocampus. *CRAcad SciIII* **313**:421-426.

Lemercier G, Carpentier P, Sentenac-Roumanou H and Morelis P (1983) Histological and histochemical changes in the central nervous system of the rat poisoned by an irreversible anticholinesterase organophosphorus compound. *Acta Neuropathol* **61**:123-129.

Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MV and Zukin RS (2001) Protein kinase C modulates NMDA receptor trafficking and gating. *Nat Neurosci* **4**:382-390.

McDonough JH, Dochterman LW, Smith CD and Shih TM (1995) Protection against nerve agent-induced neuropathology, but not cardiac pathology, is accociated with the anticonvulsant action of drug treatment. *NeuroToxicology* **16**: 123-132.

McDonough JH and Shih TM (1997) Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology. *Neurosci Biobehav Rev* **21**:559-579.

McNamara RK, Wees EA and Lenox RH (1999) Differential subcellular redistribution of protein kinase C isozymes in the rat hippocampus induced by kainic acid. *J Neurochem* **72**:1735-1743.

Mochly-Rosen D (1995) Localization of protein kinases by anchoring Proteins: A theme in signal transduction. *Science* **268**:247-251.

Nakane H, Yao H, Ibayashi S, Kitazono T, Ooboshi H, Uchimura H and Fujishima M (1998) Protein kinase C modulates ischemia-induced amino acids release in the striatum of hypertensive rats. *Brain Res* **782**:290-296.

Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors and macromolecular interactions. *Chem Rev* **101**:2353-2364.

Olney JW, Collins RC and Sloviter RS (1986) Excitotoxic mechanisms of epileptic brain damage. *Adv Neurol* **44**: 857-877.

Ono M, Akiyama K, Tsutsui K and Kuroda S (1994) Differential changes in the activities of multiple protein kinase C subspecies in the hippocampal-kindled rat. *Brain Res* **660**:27-33.

O'Shaughnessy D and Gerber GJ (1986) Damage induced by systemic kainic acid in rats is dependent upon seizure activity - a behavioral and morphological study. *Neurotoxicology* **7**: 187-202.

Ron D, Luo J and Mochly-Rosen D (1995) C2 region-derived peptides inhibit translocation and function of β protein kinase C in vivo. *J Biol Chem* **270**: 24180-24187.

Sakai N, Sasaki K, Ikegaki N, Shirai Y, Ono Y and Saito N (1997) Direct visualization of the translocation of the γ-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein. *J Cell Biol* **139**:1465-1476.

Shih TM, McDonough JH and Koplovitz I (1999) Anticonvulsants for soman-induced seizure activity. *J Biomed Sci* **6**:86-96.

Sidell FR (1974) Soman and sarin: clinical manifestations and treatment of accidental poisoning by organophosphates. *Clin Toxicol* **7**:1-17.

Skeberdis VA, Lan J, Opitz T, Zheng X, Bennett MV and Zukin RS (2001) mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. *Neuropharmacology* **40**:856-865.

Sloviter RS and Dempster DW (1985) "Epileptic" brain damage is replicated qualitatively in the rat hippocampus by central injection of glutamate or aspartate but not by GABA or acetylcholine. *Brain Res Bull* **15**: 39-60.

Stebbins EG and Mochly-Rosen D (2001) Binding specificity for RACK1 resides in the V5 region of βII protein kinase C. *J Biol Chem* **276**:29644-29650.

Tanaka C and Nishizuka Y (1994) The protein kinase C family for neuronal signaling. *Annu Rev Neurosci* **17**:551-567.

Tang FR, Lee WL, Gao H, Chen Y, Loh YT and Chia SC (2004) Expression of different isoforms of protein kinase C in the rat hippocampus after pilocarpin-induced status epilepticus with special reference to CA1 area and the dentate gyrus. *Hippocampus* **14**:87-98.

Taylor P (1985) Anticholinesterase agents, in *The Pharmacological Basis of Therapeutics* (Gilman AG, Goodman LS, Rall TW and Murad F eds), 6th Edn, pp 110-129, Macmillan, New York.

Wagey R, Hu J, Pelech SL, Raymond LA and Krieger C (2001) Modulation of NMDA-mediated excitotoxicity by protein kinase C. *J Neurochem* **78**: 715-726.

Weissman BA and Raveh L (2003) Peripheral benzodiazepine receptors: on mice and human brain imaging. *J Neurochem* **84**: 432-437.

Young S, Parker PJ, Ullrich A and Stabel S (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. *Biochem J* **244**: 775-779.

Footnotes:

a) Part of this work had been presented in the following meetings:

2003 - 13th Annual Meetings of the American Summer Neuropeptide Conference & the European Neuropeptide Club, NY, USA.

2003 – 11th annual Meeting of the Israel Society for Neurosciences, Eilat, Israel.

2004 – Bioscience 2004 US Army Medical Defense Meeting, Maryland, USA.

b) **Send reprint request to:** Dr. Shilderman Eugenia, Department of Pharmacology, Israel Institute for Biological Research, P. O. Box 19, Ness Ziona 74100, Israel. E-mail: shilderman@iibr.gov.il

JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures:

Fig. 1. Immunoreactivity of conventional βII-PKC isozyme following different time points post sarin exposure. Rats exposed to sarin (90 mg/kg~1LD_{50}, i.m.) were sacrificed by decapitation at the following time points: 10, 30 min, 2 & 24 hr and 5 & 10 days post sarin exposure. Hippocampal, frontal cortex, thalamus and striatum tissues were further micro dissected, homogenized, separated to cytosolic and membrane fractions and subjected to Western blot analysis. PKC isozyme-specific βII-PKC antibody was used to determine PKC isozyme expression. A) βII-PKC immunoreactivity in the rat frontal cortex. B) βII-PKC immunoreactivity in the rat hippocampus. C) βII-PKC immunoreactivity in the rat thalamus. D) βII-PKC immunoreactivity in the rat striatum. Autoradiograph from a representative Western blot illustrates membrane (M) and cytosolic (C) fractions from hippocampus and thalamus probed with anti-βII-PKC antibody from rats decapitated in different times indicated above. Data are mean ±S.E.M. (bars) values (n=6 per time point), expressed as percentage of control (saline-treated) values (100%, dotted line). *$p<0.05$ compared with control (OD per microgram protein) values.

Fig. 2. Immunoreactivity of atypical ζ-PKC isozyme following different time points post sarin exposure. Rats exposed to sarin (90 mg/kg~1LD_{50}, i.m.) were sacrificed by decapitation at the following time points: 10, 30 min, 2 & 24 hr and 5 & 10 days post sarin exposure. Hippocampal, frontal cortex, thalamus and striatum tissues were further micro dissected, homogenized, separated to cytosolic and membrane fractions and subjected to Western blot analysis. PKC isozyme-specific ζ-PKC antibody was used to determine PKC isozyme expression. A) ζ-PKC immunoreactivity in the rat frontal cortex. B) ζ-PKC immunoreactivity in the rat hippocampus. C) ζ-PKC

immunoreactivity in the rat thalamus. D) ζ-PKC immunoreactivity in the rat striatum. Autoradiograph from a representative Western blot illustrates membrane (M) and cytosolic (C) fractions from thalamus probed with anti-ζ-PKC antibody from rats decapitated in different times indicated above. Data are mean ±S.E.M. (bars) values (n=6 per time point), expressed as percentage of control (saline-treated) values (100%, dotted line). *$p<0.05$ compared with control (OD per microgram protein) values.

Fig. 3. The effect of midazolam (Mid) on βII – and ζ–PKC immunoreactivity in the rat hippocampus post sarin exposure. Rats from group 3 (as described in *Methods*) were exposed to sarin and 10 min later were administered midazolam. 2 hrs post exposure rats were sacrificed by decapitation. Hippocampus tissues were further micro dissected, homogenized, separated to cytosolic and membrane fractions and subjected to Western blot analysis. Data are mean ±S.E.M. (bars) values (n=6 per time point), expressed as percentage of control (saline-treated) values (100%). *$p<0.05$ compared with control (OD per microgram protein) values.

Fig. 4. Reduction of sarin-induced convulsion-related neuropathology by βII-PKC inhibitor as shown in histological brain sections stained with hematoxylin and eosin. Rats were exposed to sarin (group 2). βII-PKC inhibitor was injected (380 ng/kg i.c.) 10 min post seizure activity initiation. Rats were sacrificed by decapitation 48 hrs later. Brains were fixed in 4% neutral buffered paraformaldehyde and processed routinely for paraffin embedding. Six microns sections were cut and stained for light microscopy analysis. A) Normal rat hippocampus. B) Sarin-exposed rat hippocampus. C) Sarin-exposed and βII-PKC inhibitor peptide-treated rat hippocampus. Notice

severe neuronal degenaration in CA1 hippocampal region (B) following sarin exposure, and a significant reduction in brain damage following βII-PKC peptide treatment (C). n=6 per group.

Fig. 5. Morphometric analysis of survival CA1 cells 48 hrs treated with βII-PKC inhibitor peptide post sarin exposure. CA1 cells were counted in brain sections at the hippocampal level, corresponding to Figure 32 in Paxinos and Watson's Rat Brain Atlas. The analysis included counting of intact cells, which apparently had the same phenotype as normal CA1 pyramidal cells and had the typical staining characteristics of normal neurons in hematoxylin and eosin stained sections, i.e. large, clear nucleus stained brightly. Damaged cells, in contrast, had pyknotic nucleus, stained intensely dark by hematoxylin and had also acidophilic cytoplasm (stained by eosin). The number of cells was counted in two frames of 280x400 μm^2 each, in both hemispheres, in 2-3 serial sections of each animal (6-10 animals per group), using a X40 microscopic objective (Nikon).

Fig.6. A correlation plot between histopathological and clinical scores post sarin exposure. Severity scores of both histopathological and clinical evaluations were: 0 = no lesion/signs; 1 = minimal; 2 = mild; 3 = moderate; 4 = moderate to severe; 5 = severe, as described in Table 1.

JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Description of clinical and histopathological scores post sarin exposure

Score	Clinical symptoms	Histopathological evaluation
0	Normal behavior	Normal morphology
1 (minimal)	Grooming and piloerection	Enlargement of ventricles, few pyknotic cells
2 (mild)	Salivation and tremor	Typical OP damage (loss of cells and vacuolization) in one out of the three vulnerable regions (piriform cortex, hippocampus, thalamus)
3 (moderate)	Impairments in motor activity and weight loss	Typical OP damage in two out of the three vulnerable regions (see above)
4 (moderate to severe)	Dyspnea	Typical OP damage in the three vulnerable regions
5 (severe)	Convulsions	Degeneration over 50% of cells in the three vulnerable regions including additional brain regions such as septum and amygdala

Rats were exposed to sarin (108 mg/kg~1.2LD_{50} i.m. with a supportive treatment) as described in *Methods*, and observed for clinical symptoms and weight monitoring. 48 hrs post exposure rats were sacrificed, brains were fixed and processed routinely for paraffin embedding and further evaluated by light microscopy analysis. Both clinical and histological observations were scored using a scoring scale that is based on the well-characterized symptoms and brain damage induced by OP. Each score includes all the above symptoms plus the added one.

Fig. 1

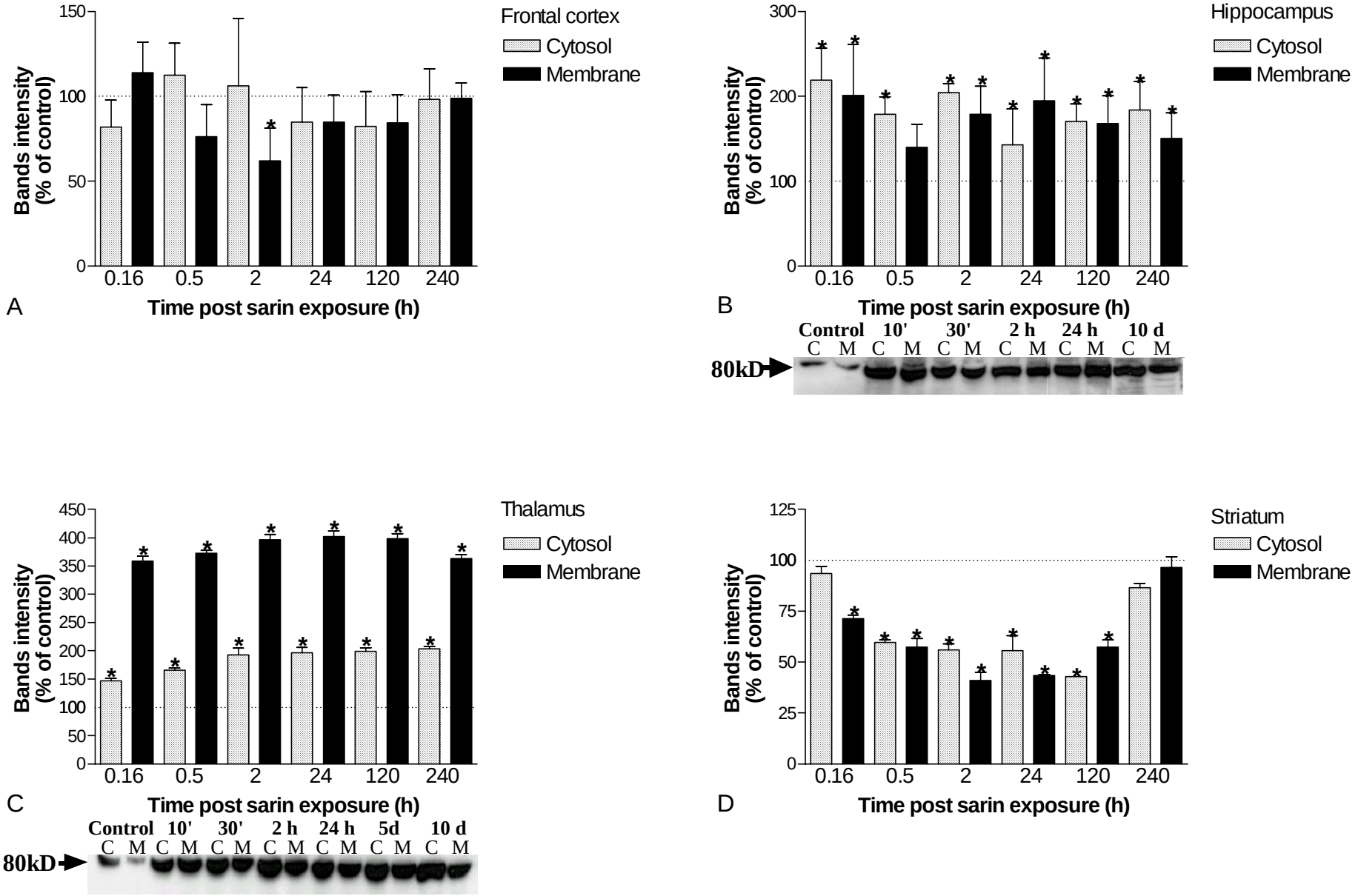
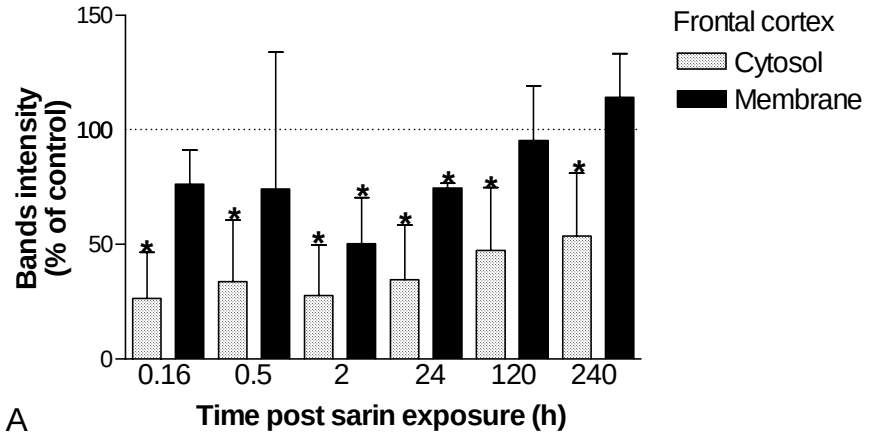
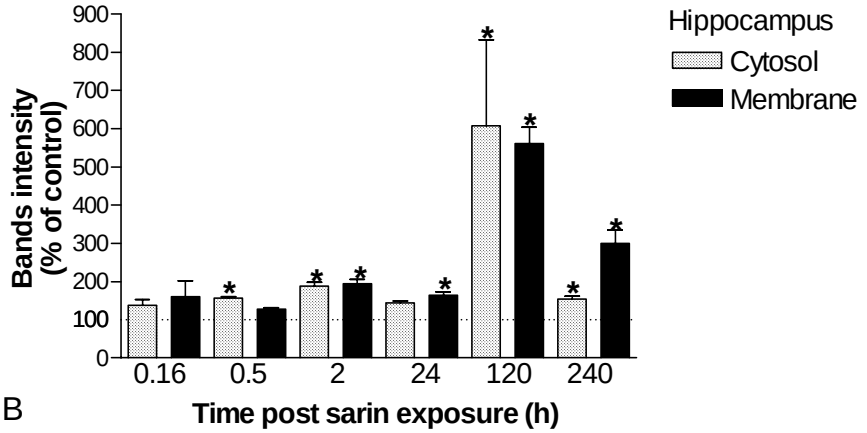
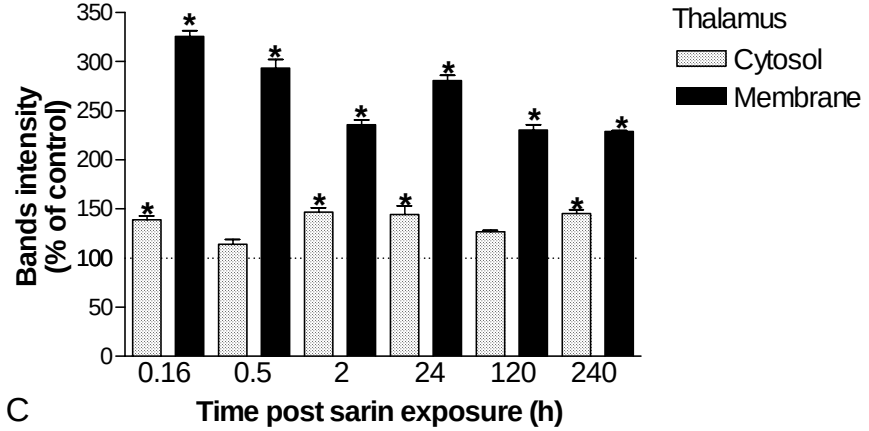
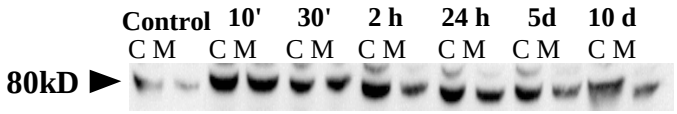


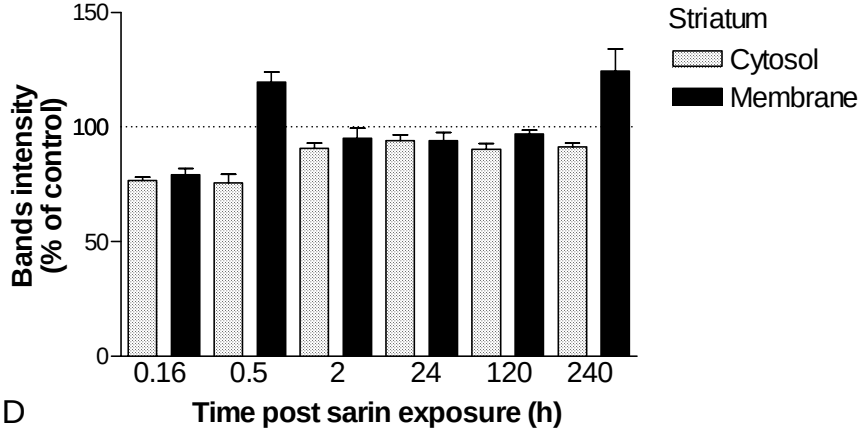
Fig. 2





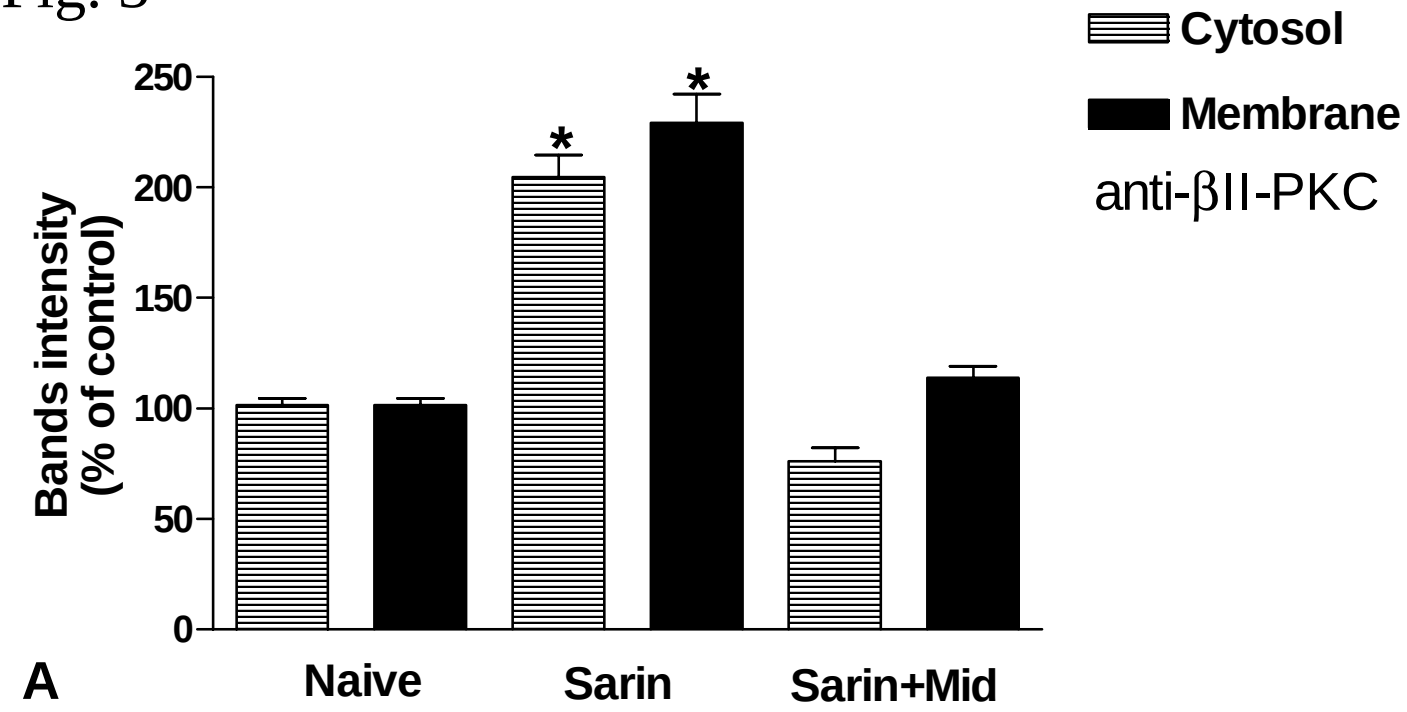


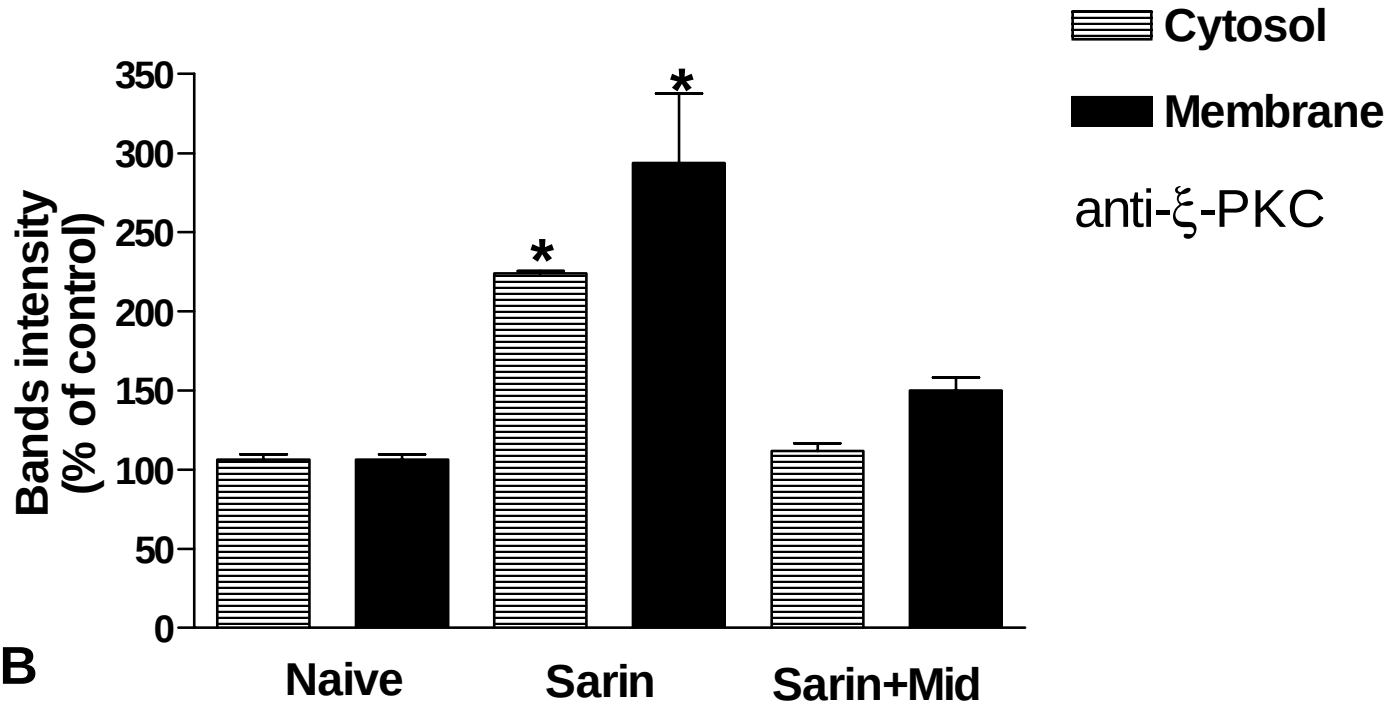




JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 3





JPET Fast Forward. Published on February 16, 2005 as DOI: 10.1124/jpet.105.083469
This article has not been copyedited and formatted. The final version may differ from this version.

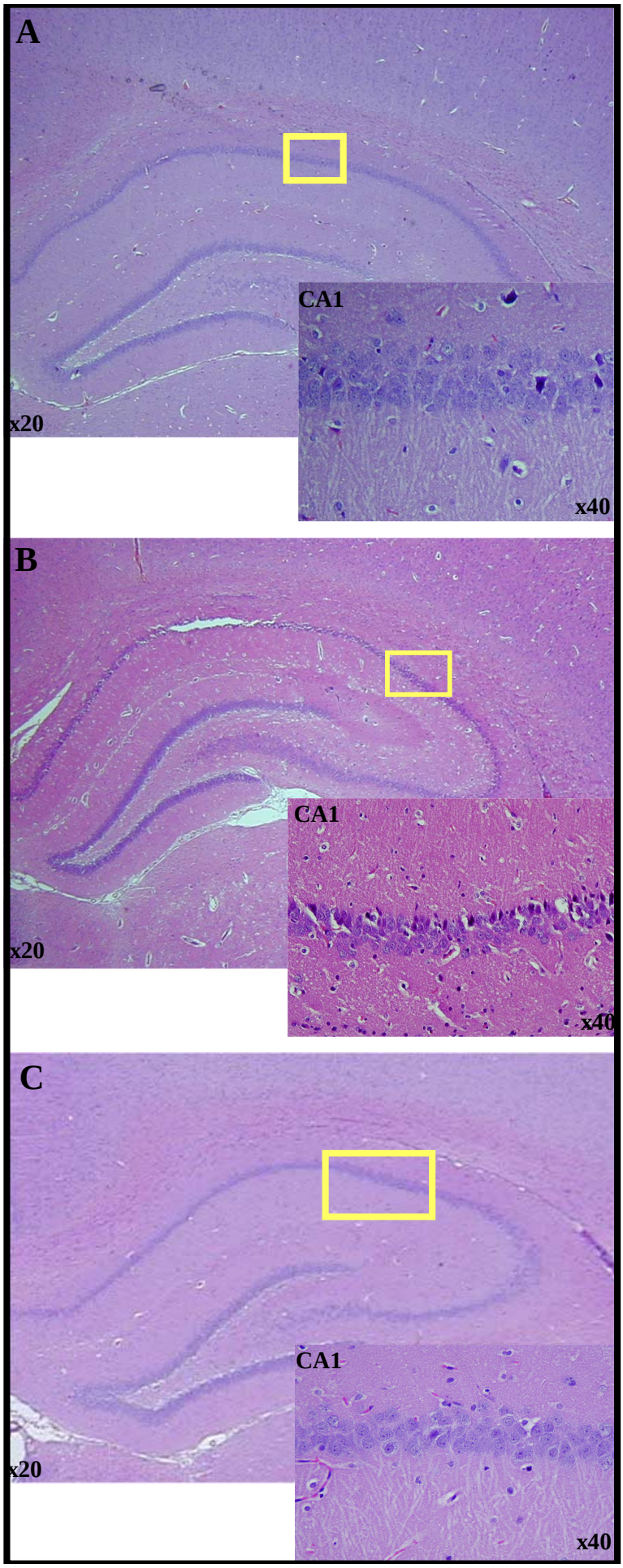


Fig 4

Fig 5

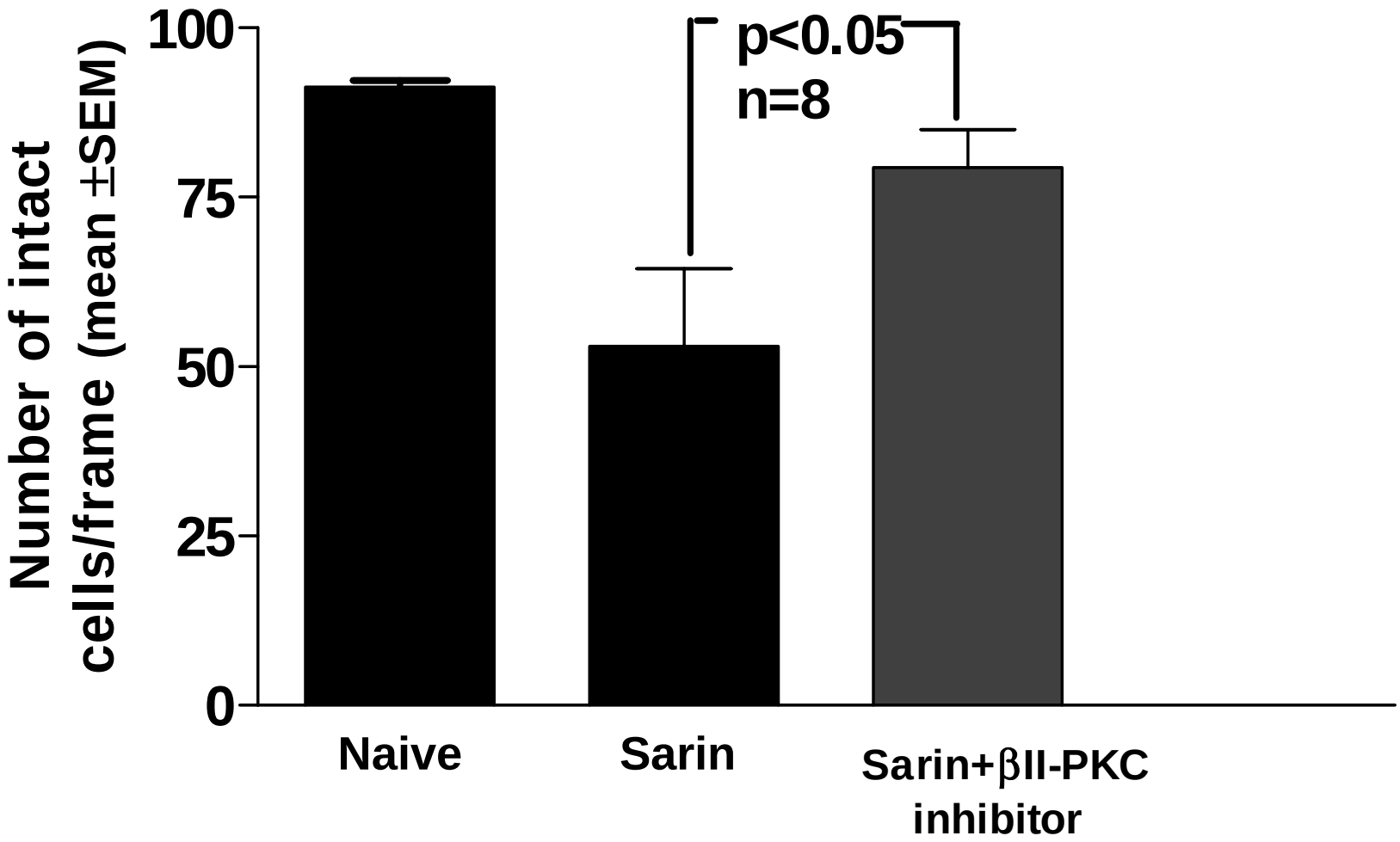


Fig 6