AD_____

Award Number: DAMD17-99-C-9090

TITLE: Comparison of Novel and Known Neuroprotectants for Treating Exposure to Different Types of Toxins

PRINCIPAL INVESTIGATOR: Ben A. Bahr, Ph.D.

CONTRACTING ORGANIZATION: University of Connecticut
Storrs, CT 06269-1133

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (16 Aug 2002 - 15 Aug 2003)

4. TITLE AND SUBTITLE
Comparison of Novel and Known Neuroprotectants for Treating Exposure to Different Types of Toxins

5. FUNDING NUMBERS
DAMD17-99-C-9090

6. AUTHOR(S)
Ben A. Bahr, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Connnecticut
Storrs, CT 06269-1133

E-Mail: bahr@uconn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Studies using cultured brain slices have found that compensatory signals are activated in response to different types of excitotoxicity/seizures related to environmental toxin or military threat agent exposure. Soman, an irreversible anticholinesterase and devastating weapon of mass destruction, produces convulsions, memory impairment, and cell loss in the brain, especially in the hippocampus. Soman-induced accumulation of acetylcholine initiates mechanisms responsible for the development of incapacitating seizures. However, another excitatory neurotransmitter, glutamate, also has been linked to the toxic action of the chemical agent. We found that after repeated exposures to sub-toxic levels of soman, long-term hippocampal slice cultures from rat exhibit enhanced vulnerability to brief excitotoxic episodes of glutamate receptor over-stimulation. In particular, the hippocampal tissue became sensitive to cytoskeletal damage and synaptic decline in response to secondary pathogenic events. Thus, seemingly innocuous soman exposures leave the brain vulnerable to excitotoxic insults that are implicated in traumatic brain injury and stroke. The findings also suggest that asyptomatic exposures to soman can lead to hippocampal damage and that early indicators of low-level soman contact are critical for the prevention of subsequent brain injury. Studies with receptor modulators are helping to identify key signal transduction pathways that lead to neuroprotection vs. those that enhance neuronal vulnerability. According, a neuroprotectant that acts against excitotoxicity through the MAP kinase pathway eliminates soman-induced neuronal vulnerability.

14. SUBJECT TERMS
Neurotoxin

15. NUER OF PAGES
196

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

3. TABLE OF CONTENTS

4. INTRODUCTION

Acute exposure to soman, a military-threat agent that is three times more potent than sarin, causes seizure activity and long-term damage in the central nervous system (Lemercier et al., 1983; Pazdernik et al., 1985; Taylor, 2001). Soman is a serious concern to soldiers as well as civilians during the threat of a chemical attack and the unpredictable movement of the airborne toxin. While an irreversible inhibitor of acetylcholinesterase, the nerve agent also promotes the release of excitatory amino acids such as glutamate that participate in the neurodegeneration (Wade et al., 1987; Lallement et al., 1991, 1992, 1993; Raveh et al., 2003). Evidence suggests that septo-hippocampal areas of the glutamatergic system are recruited early after soman exposure and the resultant accumulation of extracellular acetylcholine. Excess glutamatergic stimulation in turn causes distinct damage to brain tissue, and blocking specific glutamate receptors reduces neuropathogenic responses including soman toxicity (Choi, 1988; Sheardown et al., 1990; Sparenborg et al., 1992; Lallement et al., 1993; de Groot et al., 2001).

Excitotoxic levels of glutamate may be involved in the dendritic and synaptic damage following acute soman exposure that leads to neuronal dysfunction and memory impairment (Carpentier et al., 1991; Filliat et al., 1999; de Groot et al., 2001; Munirathinam and Bahr, 2002; Raveh et al., 2002, 2003). Over-stimulation of glutamate receptors indeed causes marked deterioration in the hippocampus (Siman et al., 1989; Bahr et al., 1995b, 2002), a brain region involved in information processing and one that exhibits early degeneration following ischemia and related brain trauma. The synaptic integrity required for stable neuronal connections is particularly vulnerable to damage. Hence, hippocampal circuitries that are important for memory encoding express a high susceptibility to both nerve agents and excitotoxicity.

Events of excitotoxicity in hippocampal neurons enhance the vulnerability to other types of neuropathogenesis (e.g., see Mattson, 1990; Bahr et al., 1994). The hippocampus is a brain region that has been extensively utilized to study the action of neurotoxins. Organotypic hippocampal slice cultures provide a sensitive model system that exhibits toxic responses comparable to those expected from *in vivo* studies (Bahr et al., 1994, 1995b, 2002; Munirathinam et al., 2002). The three-dimensional slice cultures possess features that are characteristic of the adult hippocampus in vivo, including circuitry, cellular interactions, morphological integrity, and organization of neuronal subfields (Bahr, 1995; Bahr et al., 1995a). Using this model, we report that the hippocampus becomes susceptible to injury after sub-toxic soman exposure. Selective deterioration of synapses is linked to the enhanced vulnerability, associated with proteolytic activation and resultant cytoskeletal damage.

5. BODY

Chemicals and antibodies. Soman (pinacolyl methylphosphonofluoridate) was synthesized and supplied by the U.S. Army. The glutamate receptor agonist AMPA and antagonists MK801 and CNQX were from TOCRIS (Ballwin, MO). Trimethyltin (TMT) was from Aldrich Chemicals (Milwaukee, WI). The monoclonal antibody against synaptophysin was obtained from Boehringer Mannheim (Indianapolis, IN), and anti-actin was from Sigma (St. Louis, MO). Affinity-purified antibodies to the AMPA receptor subunit GluR1 were prepared as described (Bahr et al., 1996). The spectrin breakdown product BDP_N was measured using affinity-purified antibodies against end residues (Gln-Gln-Glu-Val-Tyr) of the aminoterminal fragment produced by calpain I (Bahr et al.,

1995b). Cell culture supplies and protease inhibitors were obtained from Sigma and Boehringer Mannheim. Nitrocellulose paper was from Osmonics, Inc. (Westborough, MA). Alkaline phosphatase-conjugated and horseradish peroxidase-conjugated antibodies and substrate kits were from Bio-Rad Laboratories (Richmond, CA) and Vector Laboratories Inc. (Burlingame, CA).

Organotypic hippocampal slice cultures. Conventional methods were used to prepare hippocampal slices from rat pups (Bahr et al., 1995a). Sprague-Dawley rat litters (Charles River Laboratories, Wilmington, MA) were housed in accordance with guidelines from the National Institutes of Health. The brains from 11- to 12-day postnatal rats were rapidly removed and cooled in ice-cold buffer containing 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 4 mM $MgSO_4$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM D-glucose, 2 mM ascorbic acid and 75 μM adenosine (pH 7.2). Hippocampi were quickly dissected and cooled, and transverse slices of 400 μm were collected from the septal to the temporal end. Groups of 8-10 slices were distributed on Millicell-CM culture inserts (Millipore; Bedford, MA). The Biopore insert membrane was maintained in contact with culture media consisting of 50% Basal Medium Eagle, 25% Earl's salt solution, 25% horse serum, and supplemented to the following final concentrations: 136 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$, 4 mM $NaHCO_3$, 3 mM glutamine, 40 mM glucose, 0.4 mM ascorbic acid, 20 mM HEPES buffer (pH 7.3 at 23°C), 1 mg/L insulin (24 I.U. per mg), 5 units/ml penicillin, and 5 mg/l streptomycin. The slices were maintained for 14-18 days in culture before used, at 37°C in a humid incubator supplied with 5% CO_2, with the media being changed every 2-3 days.

For transient exposure to soman (nerve agent GD), a single treatment of freshly prepared agent was applied to both surfaces of the cultured slices, and this was repeated daily for up to one week. For induction of excitotoxic pathology, slices were subjected to a 3-min AMPA (100 μM) or 1-h TMT (100 μM) exposure. After removal of the excitotoxins, the cultures were subsequently quenched with media containing 40 μM CNQX and 20 μM MK-801 for 20 min to stop further glutamate receptor stimulation. Treatment groups (n=4-6 per time point) were staggered so that all slices were harvested on the same day, at which time they were washed thoroughly in serum-free media and prepared for subsequent analyses.

Immunoblot analysis. Cultured slices were gently removed with a soft brush and homogenized by sonication in groups of 6-8 slices each using ice-cold buffer containing 8 mM HEPES buffer (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3, 10 μg/l antipain, and 2 μg/l each of leupeptin, aprotinin, and pepstatin. Samples were assessed for protein content with a BSA standard, and equal protein aliquots (85 μg) were denatured in SDS and β-mercaptoethanol for 5 min at 100°C then separated by 4-16% SDS-PAGE and blotted to nitrocellulose. The nitrocellulose membrane was incubated with primary antibodies at 4°C overnight with gentle agitation. Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates, and color development used the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system. Development of immunoreactive bands was terminated before maximal intensity was reached in order to avoid saturation and to ensure a linear relationship with increasing amount of sample protein. Single blots were used for comparative studies between different antigens. Bands were scanned at high resolution and assessed for integrated density within single immunoblots with BIOQUANT software (R & M Biometrics, Nashville, TN).

Histology. Slices were rinsed with 0.1 M phosphate buffer, pH 7.4 (PB) and fixed for 2 h in PB plus 4% paraformaldehyde. Slices were then cryoprotected in 20% sucrose for 1 h and carefully removed

from the insert, and serial sections were prepared at 20-μm thickness and mounted on Superfrost plus coated slides (Fisher Scientific, Pittsburgh, PA). Sections were immunolabeled with anti-synaptophysin using the avidin-biotin-peroxidase technique and 3,3'-diaminobenzidine as the chromogen. Alternatively, separate sections were stained with cresyl violet. BIOQUANT image analysis included the use of a computerized deconvolution system with motorized Z focus drive.

Statistics. Immunoreactivity levels were expressed as means ± SEM and statistical significance was determined by unpaired t-tests and by one-way analyses of variance followed by the Tukey-Kramer multiple range tests. Differences were considered significant at $p<0.05$.

Results

Soman (nerve agent code GD) at 20 μM was administered to cultured hippocampal slices with single daily applications to produce transient exposure of the chemical agent before its rapid hydrolysis. After the first day, soman produced little to no effect on the synaptic vesicle component synaptophysin or the AMPA receptor subunit GluR1 (Fig. 1A, left lanes). Also absent was the amino-terminal spectrin breakdown product (BDP_N), a proteolytic marker that identifies toxic conditions and selective neurodegeneration (see Vanderklish and Bahr, 2000). Repeated applications of soman over 7 days, on the other hand, resulted in reduced levels of the pre- and postsynaptic proteins (Fig. 1A, right lanes; left and middle graphs in Fig. 1B). The dose-dependent decline in synaptophysin was evident throughout the hippocampal subfields, especially in the dendritic zones of the stratum radiatum (Fig. 2A, top panels) and the molecular layer of the dentate gyrus (lower panels).

Besides reduced concentrations of synaptic markers, repeated exposures to 20 μM soman also activated the protease calpain as indicated by spectrin fragmentation (Fig. 1A, right lanes). The cleavage product BDP_N was measured with antibodies directed against calpain's recognition site in the spectrin sequence (Bahr et al., 1995b). Calpain-mediated spectrin cleavage results from glutamatergic excitotoxicity and is thought to be a precursor to neuronal death (Siman et al., 1989; Bahr et al., 1995b, 2002). However, spectrin proteolysis caused by the transient soman exposures was not associated with extensive cell loss. Histological examination of soman-treated slice cultures revealed intact, densely packed neurons with no evident pyknotic changes in the major pyramidal fields of CA1 (Fig. 2B, top panels) and CA3 (Fig. 2B, lower panels) and in the hilus (Fig. 2C, upper panels). In the lower panels of Figure 2C, indications of pyknotic nuclei (arrows) were found among the granular neurons of the dentate gyrus, but their sparse distribution is an unlikely explanation of the marked synaptic decline in the molecular layer. Thus, soman toxicity produces synaptic decay before cell death.

Sub-toxic conditions were evident with low-level soman exposures. Slices treated with 0.15 μM soman exhibited no appreciable synaptic decline after 7 days of exposure (Fig. 1B). In addition, while slices exposed repeatedly to 20 μM soman generated pronounced levels of calpain-mediated proteolysis, daily applications of 0.15 μM soman caused only a small increase above background BDP_N levels (Fig. 1B, rightmost graph).

Surprisingly, hippocampal tissue that was treated with the sub-toxic soman concentration exhibited enhanced vulnerability to an excitotoxic insult. Excitotoxicity was induced by over-stimulating AMPA-type glutamate receptors for 3 min with 100 μM AMPA, a selective agonist known to cause neurodegeneration with longer treatment periods in the slice model (Bahr et al.,

1995b, 2002). The excitotoxic insult alone did not cause significant synaptic decline or calpain-mediated spectrin breakdown (Fig. 3A, rightmost lanes), nor did repeated exposures to the low-level soman (leftmost lanes). In contrast, the brief excitotoxic insult caused obvious pathogenic changes in slice cultures that were first subjected to a week of daily exposures to sub-toxic soman (Fig. 3B; see middle lanes of Fig. 3A). Similar findings of enhanced vulnerability resulted from a single application of soman. A 1-h treatment with trimethyltin (TMT), a neurotoxin that causes selective excitotoxic damage in the brain (Feldman et al., 1993; Ishida *et al.*, 1997; Munirathinam et al., 2002), was found to be more effective at causing synaptic and cytoskeletal deterioration when followed by a single exposure to low-level soman and assessed 24 h later (Fig. 3C). Therefore, we conclude that soman exposures either exacerbate common mechanisms of excitotoxicity or disrupt cellular repair signals that compensate for the excitotoxic consequences.

To test whether low-level soman exposures disrupt repair signaling, we attempted to offset the enhanced vulnerability by positive modulating endogenous survival signals. This was done with 1-(quinoxalin-6-ylcarbonyl)piperidine, a neuroprotectant that promotes basal glutamatergic responses and their connection to repair systems including the mitogen-activated protein kinase (MAPK) pathway (Wang et al., 1997; Hayashi et al., 1999; McKinney et al., 1999; Bahr et al., 2002; Limatola et al., 2002). The compound was introduced into the daily applications of sub-toxic soman, and a week later the hippocampal slices were assessed for excitotoxic vulnerability. As shown in Figure 4A, the repeated soman exposures allowed a normally undamaging AMPA treatment to produce marked synaptic decline and cytoskeletal breakdown (lane 2, compared to control slices in lane 1). The addition of 1-(quinoxalin-6-ylcarbonyl)piperidine completely prevented the AMPA-induced pathogenic responses (Fig. 4A, lane 3, and Fig. 4B). Pre- and postsynaptic markers remained at levels similar to those in control slices, and calpain activation was not evident. These data indicate that soman-induced neuronal vulnerability does not involve disruption of endogenous repair responses.

This project has identified repair mechanisms that are able to counteract the effects of neurotoxin exposure. We have shown that AMPA-type glutamate receptors are linked to appropriate signaling events in order to prevent neuronal injury as well as enhance recovery. As reported in Bahr et al. (2002), AMPA stimulation in hippocampal slice cultures caused the selective activation of MAPK through the upstream activator MAPK kinase (MEK). Excessive glutamatergic activity through AMPA receptors is no doubt a critical feature of excitotoxic damage (Buchan et al., 1991; Sheardown et al., 1993). On the other hand, enhancing basal stimulation of AMPA receptors and associated MAPK signaling with the positive modulator 1-(quinoxalin-6-ylcarbonyl)piperidine (also known as Ampakine CX516), was found to promote neuronal survival after toxic exposure (Bahr et al., 2002; Munirathinam et al., 2002). As with AMPA receptors, we recently showed that stimulation of signals through cannabinoid receptors and proteoglycan-binding adhesion receptors also mediate neuroprotection in the slice model.

AMPA receptors are known to participate in higher cognitive functions in the mammalian brain. Positive modulation of AMPA receptors by the Ampakine class of compounds selectively improves channel function by making the receptors more responsive to endogenous ligand and specific agonists (Tang et al., 1991; Arai et al., 1996; Suppiramaniam et al., 2001). The resultant increase in glutamatergic transmission has been shown to be associated with enhanced synaptic plasticity and memory retention (Staubli et al., 1992, 1994; Granger et al., 1996; Hampson et al., 1998; Lebrun et al., 2000). Thus, AMPA receptors are part of a cellular mechanism(s) that are necessary for both information processing and repair systems.

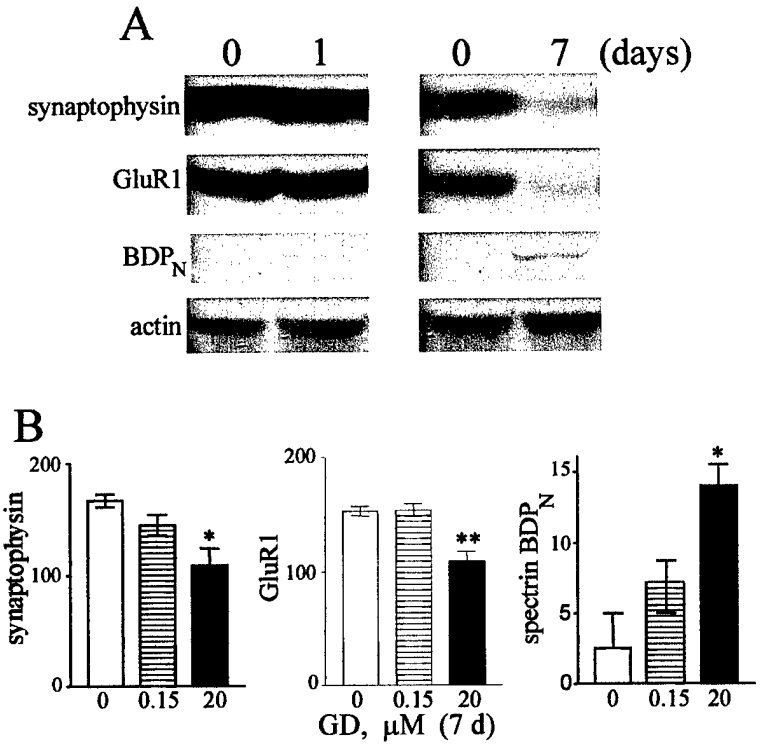


Fig. 1. Synaptic decline and cytoskeletal breakdown after repeated soman exposures. (A) Hippocampal slice cultures were prepared from postnatal day 12 Sprague-Dawley rats and maintained in culture for 3 weeks (16), after which a single daily treatment of 20 μM soman was applied for the noted number of days. Equal protein aliquots from slice homogenates were used to determine amounts of synaptophysin, AMPA receptor subunit GluR1, and calpain-mediated spectrin breakdown product BDP_N by immunoblotting (13). Blots were reprobed with an antibody to β-actin to indicate the relative amount of protein loaded in each lane. (B) Integrated optical density levels of the immunoreactive bands were determined for slices subjected to repeated exposure to 0, 0.15, or 20 μM soman over 7 days (mean ± SEM; n = 5-9 groups of 6-8 slices each). Post-hoc tests compared to non-treated control: *$p<0.01$, **$p<0.001$.

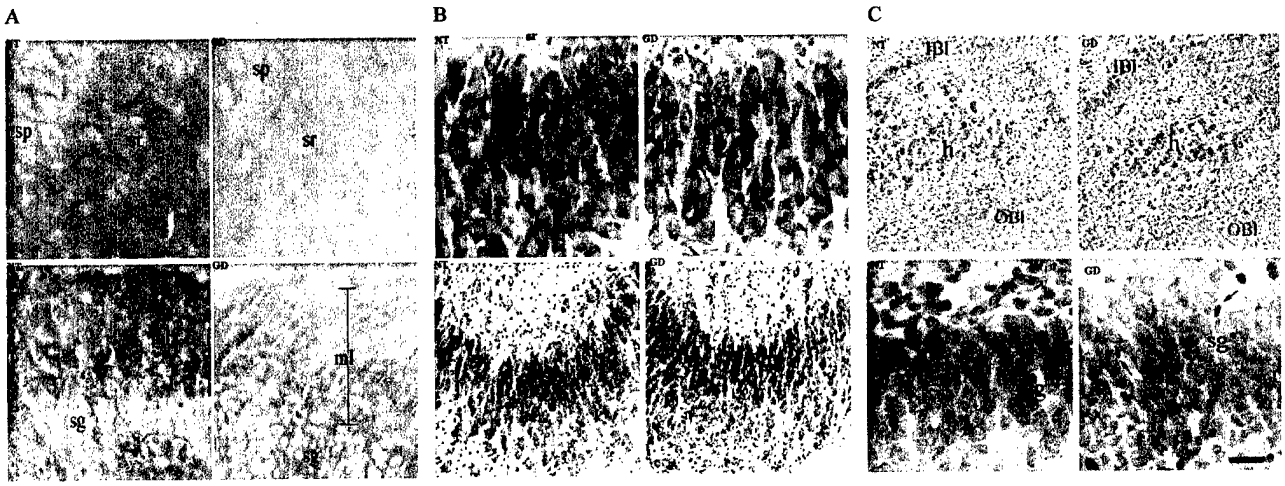


Fig. 2. Synaptic decline before neuronal loss in soman-treated hippocampus. (A) Hippocampal slice cultures were repeatedly exposed to 20 μM soman (nerve agent code GD) for 7 days, after which they were fixed, sectioned, and stained for synaptophysin (16); non-treated control slices were analyzed in parallel (NT). The immunoreactivity in the hippocampal subfield CA1 (upper panels) and dentate gyrus (lower panels) is shown. (B, C) Similarly treated slice cultures were stained with cresyl violet. Photomicrographs show pyramidal neurons in CA1 and CA3 (B; upper and lower panels, respectively), as well as the dentate gyrus zone and granular neurons from the inner blade (C; upper and lower panels, respectively). Pyknotic nuclei are noted with arrows. h, hilus; IBl, inner blade; ml, molecular layer; OBl, outer blade; sg, stratum granulosum; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: upper panels of A-C, 35, 35, and 150 μm, respectively; lower panels of A-C, 50, 110, and 30 μm, respectively.

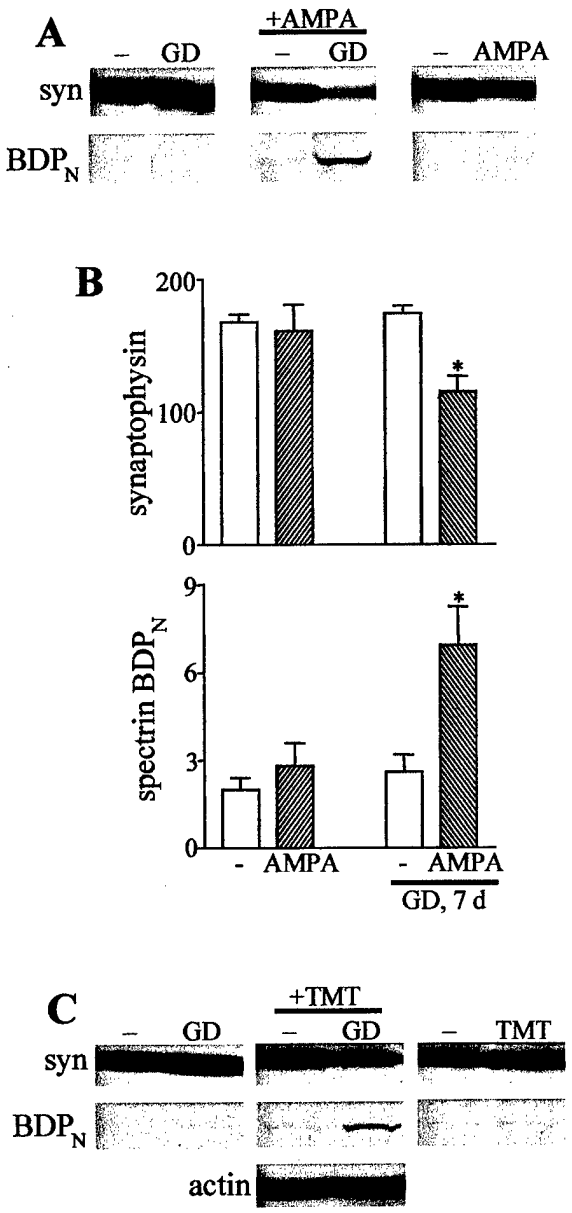


Fig. 3. Enhanced vulnerability in hippocampal slices exposed to low-level soman. (A) Slice cultures were treated *i*) with or without 0.15 μM soman (GD) for a week of daily exposures (left lanes), *ii*) with or without a 3-min excitotoxic insult using 100 μM AMPA 24 h before harvesting slices (right lanes), and *iii*) with or without the repeated soman exposures in conjunction with the AMPA insult on day 6 (middle lanes). Slices were harvested on the same day and assessed for synaptophysin (syn) and the spectrin breakdown product BDP_N by immunoblotting (13). (B) Integrated optical density levels were determined for synaptophysin and BDP_N in the different treatment groups (mean ± SEM; n = 7-15 groups of 6-8 slices each). Unpaired, two-tailed t-test: *$p<0.01$. (C) Slices were treated once with or without 0.15 μM soman (left lanes), with or without 100 μM TMT for 1 h (right lanes), and with or without the TMT exposure followed by the single soman application (middle lanes). Immunoblotting was conducted 24 h post-insult. Blots were reprobed with an antibody to β-actin to indicate the relative amount of protein loaded in each lane.

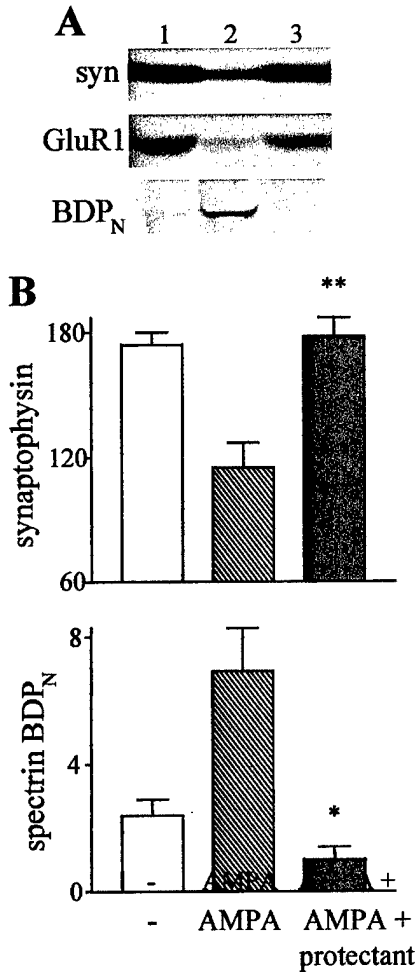


Fig. 4. Active cellular repair signaling against soman-induced neuronal vulnerability. (A) Hippocampal slice cultures were repeatedly exposed to 0.15 μM soman in the absence (lane 1) or presence of the neuroprotectant 1-(quinoxalin-6-ylcarbonyl)piperidine (100 μM; lane 3). Six days later, portions of each slice group were exposed for 3 min to a normally undamaging AMPA insult (see Figure 3). After an additional 24 h, the slices were harvested and samples assessed for synaptophysin (syn), GluR1, and the spectrin breakdown product BDP_N by immunoblotting. In slices subjected to the AMPA insult (lanes 2 and 3), only those without the protectant exhibited synaptic decline and spectrin proteolysis. (B) Integrated optical density levels were determined for synaptophysin and BDP_N in the different treatment groups (mean ± SEM; n = 7-17 groups of 6-8 slices each). Post-hoc tests of the protectant effect: *$p<0.01$, **$p<0.001$.

Gene Regulation Events: Exposure to the excitotoxin NMDA

Gene Array Probe Preparation and Hybridization. Total RNA was isolated form treated and control hippocampal slices using Trizol® (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Total RNA (15μg) was used as a template for cDNA library construction using SuperScript Double Stranded cDNA synthesis kit (Invitrogen). First-strand synthesis was performed using a T7-$(dT)_{24}$ primer (IDT, Coralville, IA). The cDNA was extracted with phenol/chloroform, ethanol-precipitation, and used as a template for *in vitro* transcription to synthesize biotin-labeled cRNA using the ENZO BioArray HighYield RNA transcript Labeling Kit (Affymetrix, Santa Clara, CA). After determining the concentration at $O.D._{260}/O.D._{280}$, the cRNA was fragmented (40mM Tris, pH 8.1, 100mM potassium acetate, and 30mM magnesium acetate) by heating to 94°C for 35 min. Fifteen micrograms of the prepared cRNA was used to inoculate the Rat Neurobiology U34 Array (Affymetrix) and the hybridization, staining, scan and analysis were conducted as recommended by the manufacturer. Alterations in RNA transcript levels were analyzed using the Affymetrix Microarray Analysis V5 software. Gene induction or repression was tabulated when the positive or negative change in intensity was above 50 percent and was found to be significant as determined by statistical tests in the Affymetrix software.

We utilized neuron-specific gene array analyses to characterize the apparent two phases of the cellular response to neurotoxin exposure. To examine gene changes in the early and late phases of the cellular response, RNA samples were prepared from slices 1 and 24 h after a 20-min exposure to the excitotoxin NMDA.

Using three separate groups of slices per condition (including non-treated control groups for comparision), only those genes whose expression consistently changed 50% were marked as an increase or decrease. Among the 1323 genes represented on the array, 105 had expression levels that met the confidence criteria. Of the 105 genes whose expression changed, 36 genes were altered at 1 h post-insult and 79 at 24 h post-insult, and 10 gene regulation events were shared by both time points. The genes were identified using the GenBank accession numbers that were provided for each probe. Genes were grouped according to their proposed functions and collected under broad titles consisting of cell cycle proteins, cytokines and inflammatory agents, transcription factors, chaperons and heat shock proteins, receptors and channels, kinases and signal transduction proteins, growth hormones, transporters and neurotransmitters, adhesion molecules, and calcium homeostasis proteins (see Table 1). Clones that could not be placed under any of the above headings were designated as other. Brief NMDA exposure resulted in the upregulation of 31 genes and down-regulation of 5 gene transcripts 1 h following the insult. At 24 h post-insult, 22 gene transcripts were upregulated, while 57 genes were down-regulated according to the confidence criteria.

Of the 105 genes whose expressions changed due to the excitotoxic insult, many are for normal cellular functions involving homeostasis, metabolism, and maintaining cellular integrity, along with proteins for receptors and channels. In order to further address the idea of early protection and delayed pathogenic pathways, survival and pro-apoptotic genes were classified according to the alteration in their response time. For the purpose of addressing the idea that early phase is involved in the promotion of protection while the late phase is pathogenic, we grouped early survival and early protection genes.

The genes were subdivided according to their roles in either contributing to or preventing cellular injury/death (Table 2). Protective changes occurring in the early phase of the cellular response are the upregulation of survival genes IL-6, pJunB, EGR-1, SOCS-3, HES-1, cpg21, as well as the down-regulation of a pathogenic gene, ICE. These are different from the protective changes in the delayed phase which involve the upregulation of IGF II, HSP27, HSP10, JAK-2, IGF II BP, and HO-1 as the protective genes, along with the down-regulation of such pathogenic genes as CX3C ligand 1, and nNOS (Table 2). Interestingly, NOR-1, a well-established pathogenic gene, is increased early in the response, but is down-regulated in the delayed phase as part of protective changes.

The pathogenic changes occurring early consist of the down-regulation of the protective IL-15 transcript and the upregulation of the pathogenic genes such as, TNF-α, IL-1β, NGFI-B, NOR-1, fra-1, IRF-I, ICAM-1, MIP1α, and C/EBP (Table 3). Note that the latter two genes remain upregulated as part of the pathogenic events in the delayed phase as were calpain II and ICE transcripts, while survival genes BDNF, Egr-1, trkB, neuroD4, and neuronatin-α were down regulated.

Independent message validation utilizing real-time PCR, indeed provided data in accordance with the changes observed by microarray analyses. Other investigators have validated similar changes in a number of transcripts highlighted in our study.

When listing only those pathogenic gene changes that are unique to the delayed phase of the cellular response (i.e., not found as part of the early phase), the delayed pathogenic pathway thought to be responsible for long-term neurodegeneration appears to consist of five survival-related genes that are decreased in expression, while the pathogenesis-linked genes calpain and ICE are increased (see Fig. 5).

Corresponding with an early protective and a delayed pathogenic phase, we previously reported increased message of an anti-apoptotic gene in the early phase, and increased message of a pro-apoptotic gene in the delayed phase (Caba and Bahr, 2003). Gene array analyses were vital to further explore such different gene regulation events as part of characterization of the distinct phases of the cellular response to neurotoxin exposure. It is noteworthy to mention that gene array analyses confirmed synaptopathogenesis associated with such exposure that is known to cause dramatic decreases in synaptic protein and message levels (Bahr et al., 2002). Gene array tests identified SNAP25B as a synaptic component whose expression decreased very early, as early as 1 h post-insult. Later, other synaptic markers also exhibited down-regulation, including SNAP25A, synapsin 2, synaptophysin and SVOP.

Perhaps the down-regulation seen in the synaptic proteins is linked to the delayed decrease of genes that regulate Ca^{2+} dependent proteins or are calcium sensor proteins (neural visinin-like calcium binding protein (NVP), neurogranin (RC3), hippocalcin, and PEP-19). PEP-19 and RC3 have been shown to regulate calcium/calmodulin-dependent enzymes possibly to decrease calcium-mediated toxicity (Slemmon et al., 2000). Since the regulation of calcium levels within the cell plays an integral role in signaling, it is of utmost importance to maintain ionic homeostasis inside the cell. Thus, it is not surprising that calcium binding proteins and those proteins involved maintaining homeostasis are down regulated in the delayed phase.

Table 1. Functional classification (reported or suggested function) of genes whose expressions are altered following NMDA exposure.

In each case, the fold change is of comparisons between treated versus control (n=3 chips per group) using the Affymetrix Genechip software V5.0.

GENES	GenBank Accession Number	Unigene Accession Number	Hours post-inult	
			1 h	24 h
Cell Cycle proteins				
cyclin L	AF030091	12962	2.32	
Cytokines and Inflammatory Agents				
IL-1 beta	M98820	9869	21.89	
Macrophage inflammatory protein-2 precursor	U45965		5.94	
Monocyte chemotactic protein	X17053		5.89	2.46
Chemokine (C-X3-C motif) ligand 1	AA800602	4106		0.14
cAMP phosphodiesterase (PDE4)	M25350		1.42	
cAMP phosphodiesterase 4B	J04563	2485	1.75	
Trancripton factors				
c-fos	X06769		4.46	
c-jun	X17163		1.80	
CREM	S66024		1.75	
IL-6 C/EBP-related transcription factor	S77528		2.84	2.00
C/EBP	X60769	6479	3.16	2.04
IRF-1	M34253	6396	1.94	
ANA-1	AF030086		2.02	
ANA-4	AF030089	40517		1.63
Brain finger protein (BFP) zinc finger protein 179	AF054586	7544		0.16
Chaperons and Heat Shock Proteins				
Hsp27	AI176658	3841		2.39
HSP10 (chaperonin 10)	AI170613	1540		1.69
Receptors & Channels				
GluR-A	M36418			0.28
GluR-B	M38061	11364		0.29
GluR1	X17184	29971		0.27
GluR-K3 (74049)	X54656	74049		0.06
Homer 1	AF030088	37500	6.03	
Narp (neuronal activity-regulated pentraxin)	S82649			0.52
GABA-B receptor gb2	AF058795	30039		0.34
GABA-A receptor alpha-1	L08490			0.43
Peripheral-type benzodiazepine receptor (PKBS)	J05122	1820		1.52
Degenerin channel MDEG	U53211	37523		0.23
Na+ channel I	M22253	32079		0.37
Na+ channel II	M22254	10136		0.28
K+ channel Kv4.2	M59980	10754		0.08
Ca2+ channel alpha-1 subunit	U14005		0.54	
Pore-forming Ca2+ channel alpha-1B	AF055477	85880		0.45
K+-dependent Na+ /Ca2+ exchanger (NCKX2)	AF021923	74242		0.35
ATPase, Na+K+ transporting, beta polypeptide 3	rc_AA943304	5041	1.37	1.69
K+ channel protein (RHK1)	M32867	9884		0.25
Dihydropyridine-sesitive L-type Ca2+ channel alpha-2 subunit (CCHL2A)	M86621	11276		0.30
Thyroid hormone receptor	M31174	11307		0.57
IP3 receptor	J05510	3841		0.39

Kinases and Transduction Molecules				
nNOS	AF037071	9903		**0.19**
MAPK phosphatase (cpg21)	AF013144	10877	**2.47**	
Ca2+/calmodulin-dependent PK I beta	AB004267	11178		**0.13**
Ca2+/calmodulin-dependent PK II alpha	M16960			**0.25**
Ca2+/calmodulin-dependent PK II beta	M16112	9743		**0.05**
Integrin-linked kinase	AI102079	37725		**1.63**
JAK2	U13396	18909		**1.87**
IP3 3-kinase	X74227	44632		**1.52**
IP3-kinase	X56917	9877		**0.05**
PKC gamma	M13707	9747		**0.28**
PKC beta	K03486	9745		**0.48**
GS-alpha	L10326	31		**0.48**
Guanine nucleotide-binding protein, beta-1 subunit	AI227660	7106	**1.58**	
Plasma membrane calcium ATPase isoform 1	L04739			
Calpain II 80 kDa	L09120	6822		**1.70**
Calcineurin A alpha	D90035	6866		**0.43**
Ca2+ ATPase-isoform 2	J03754	11280		**0.33**
(Na+, K+)-ATPase-beta-2 subunit	J04629	10624		**1.63**
Ca2+-dependent tyrosine kinase 2 beta	AF063890	11025		**0.48**
PLC-1	M20636			**0.34**
c-kit receptor tyrosine kinase	D12524	54004		**0.43**
Growth Hormones				
VGF	M74223	9704		**1.91**
IGFII	X17012			**1.52**
insulin-like growth factor-binding protein (IGF-BP3)	M31837	26369		**1.81**
Transport Proteins and Neurotransmitters				
Neurofilament, heavy polypeptide	AA818677	1429		**0.31**
Neurofilament protein middle (NF-M)	Z12152	10971		**0.30**
Smallest neurofilament protein (NF-L)	M25638			**0.10**
Class I beta-tubulin	AI229707	2458		**2.01**
SNAP-25A	AB003991			**0.23**
SNAP-25B	AB003992		**0.51**	**0.08**
Synaptophysin	X06655	11067		**0.28**
Synapsin 2	AI145494	506		**0.09**
Ras-related rab3	X06889	44409		**0.32**
Microtubule-associated protein 2	X53455			**0.37**
SV2 related protein (SVOP)	AF060173	30057		**0.36**
Solute carrier family 1, member 3	AI101255	34134		**0.38**
Cholecystokinin (CCK)	X01032	9781		**0.33**
Na+ dependent neurotransmitter transporter	S56141			**0.46**
Adhesion Molecules				
Neurocan	M97161	10177		**1.76**
C-CAM4	U23056	2382		**1.75**
Calcium Sensor Proteins				
PEP-19	M24852	9736		**0.27**
Neurogranin (RC3)	L09119	11236		**0.10**
Hippocalcin	D12573	11019		**0.29**
Neural visinin-like Ca2+-binding protein type 2 (NVP-2)	D13125	34529		**0.26**
Ca2+ binding protein P23k beta	D14819		**0.64**	
Other				
Synuclein 1 alpha	AF007758	1827		**0.38**
Phosphoneuroprotein 14 (beta-synuclein)	D17764			**0.43**
t-PA	M23697	1002	**1.62**	
Neuronatin alpha	U08290	5785		**0.42**
EST				
EST	AI228113			**0.30**

Table 1. ANIA, activity and neurotransmitter induced early gene; CREM, cAMP responsive element modulator; IRF-1, interferon-γ regulatory factor-1; IL, interleukin; PTBR, peripheral-type benzodiazepine receptor; HO-1, heme oxygenase-1; rNFIL-6/C-EBP-related TF, CCAAT/enhancer-binding protein-related transcription factor; SNAP, synaptosomal-associated protein; SOCS, suppressor of cytokine signaling;; HSP, heat shock protein; ICAM, intercellular adhesion molecule; ICE, interleukin-1β converting enzyme; IGF, insulin-like growth factor; IP-3, inositol triphosphate; IGIF, interferon-γ inducible factor; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; MT, metallothionein; NGFI, nerve growth factor induced; TNF, tumor necrosis factor; t-PA, tissue plasminogen activator.

Table 2. Array analyses shows distinct pattern of survival gene regulation in early vs. the late phase of the cellular response.

Early Protective Changes		Delayed Protective Changes	
IL-6	↑	HO-1	↑
junB	↑	HSP27	↑
Egr-1	↑	HSP10	↑
HES-1	↑	JAK 2	↑
SOCS-3	↑	VGF	↑
HO-1	↑	IGF II	↑
CREM	↑	IGF II BP3	↑
ICE	↓	NOR-1	↓
		nNOS	↓
		CX3C	↓

Table 3. Array analyses shows distinct pattern of expression regulation of genes involved in pathogenesis in early vs. the late phase of the cellular response.
Genes linked to pathogenesis are designated as red.).

Early Pathogenic Changes			Delayed Pathogenic Changes	
IL-15	↓		neuroD4	↓
TNF-alpha	↑		Egr-1	↓
IL-1 beta	↑		BDNF	↓
NGFI-B	↑		trkB	↓
NOR-1	↑		neuronatin a	↓
fra-1	↑		Calpain	↑
C/EBP	↑		C/EBP	↑
MIP-1alpha	↑		MIP-1alpha	↑
IRF I	↑		ICE	↑
ICAM-1	↑			

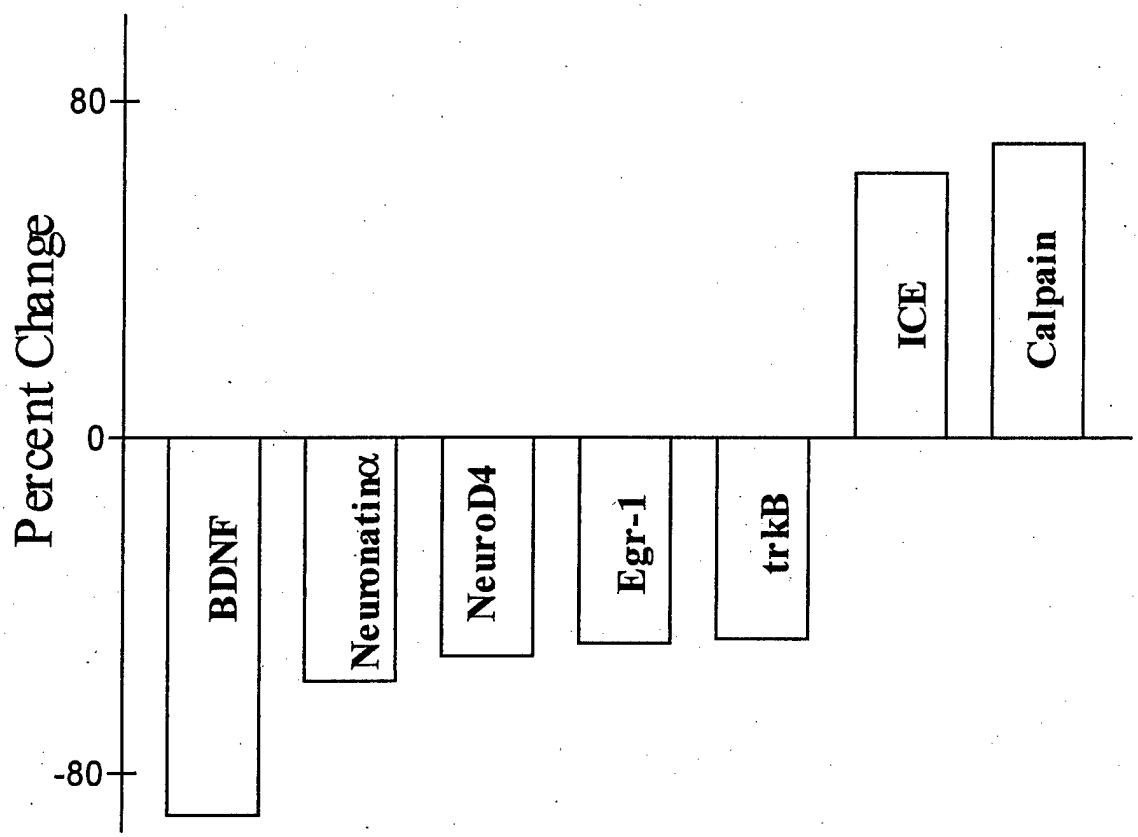


Figure 5. Changes unique to the delayed phase of the cellular response in transcripts may be contributing to the pathogenic nature of this phase.

Tissue treated with excitotoxin were used for gene array analyses. Percent change was plotted of the seven genes whose expression were uniquely altered in the delayed phase.

Gene Regulation Events: Repeated exposures to low-level soman

Neuron-specific Affymetrix microarrays were used to assess which genes are altered in cultured hippocampal slices that were subjected to six daily soman applications to produce transient exposure of the chemical agent. Four separate groups of slices were used in this study. Gene induction or repression was tabulated in Table 4 when the positive or negative change in average intensity was above 50 percent, and was found to be significantly different from the non-treated control groups as determined by statistical tests in the Affymetrix software.

There is a striking difference in the number of gene regulation events that occur after low-level soman exposure vs. after the 20-min excitotoxic insult via NMDA. The excitotoxin NMDA was used at a concentration that causes intense excitatory responses and burst activity, and the stimulation was accompanyed by 105 genes whose expression changed (see Table 1). Low-level soman exposures, on the other hand, were intentionally subtle in order to identify candidate gene regulation events that may explain how asymptomatic soman exposure increases vulnerability to damage. Perhaps then it should not be surprising that only 11 genes exhibited consistent alteration across soman-treated slice samples (Table 4). Most noteable, BDNF and heme oxygenase exhibited marked declines in expression, which could very well lead to enhanced neuronal vulnerability. BDNF reduction, in fact, is one of the pathogenic changes associated with NMDA exposure. Also of interest is that distinct compensatory events occur in response to the different toxic exposures. NMDA exposure leads to increases in the protective HSP27 and heme oxygenase, whereas these genes are reduced in soman-treated slices. Low-level soman causes a robust increase in the potentially protective gene interleukin-15 (IL-15), while IL-15 is reduced in the first phase of the NMDA response. Thus, select compensatory pathways identified through microarray experiments may provide ideal strategies with which to offset soman-induced enhanced vulnerability.

Table 4. Genes whose expression is altered following 6 daily exposures to low-level soman. The fold change is from comparing treated and control slices using Affymetrix software.

Low-level soman exposure

GENES	GenBank Accession Number	Unigene Accession Number	fold change
BDNF	D10938	11307	**0.48**
Heme oxygenase	AI179610	3160	**0.41**
MAP-kinase phosphatase (cpg21)	AF013144	10877	**0.44**
Cbp/p300-interacting transactivator	AA900476	31765	**0.41**
HSP27	AA998683	3841	**0.38**
VGF	M74223	9704	**0.47**
Monocyte chemotactic protein	X17053	4772	**0.29**
P-selectin	L23088	10012	**5.10**
hsp60	X54793	221	**2.15**
interleukin-15	U69272	2490	**11.34**
thyroid hormone receptor	M31174	11307	**9.28**

6. KEY RESEARCH ACCOMPLISHMENTS

- The extension of Specific Aim I led to the identification of multiple classes of receptors that elicit compensatory repair signals similar to those linked to the pro-survival MAPK pathway. Common signaling elements are potentially involved in such endogenous repair mechanisms through different receptors.

- We established that enhancing the signaling through AMPA receptors as well as cannabinoid receptors and adhesion-type receptors leads to neuroprotection in a tissue model of neurotoxin exposure. For this model, organotypic hippocampal slice cultures are used to provide a sensitive experimental system that exhibits toxic responses comparable to those expected from *in vivo* studies. The hippocampus is a brain region that has been extensively utilized to study the action of neurotoxins. The three-dimensional slice cultures possess features that are characteristic of the adult hippocampus *in vivo*, including circuitry, cellular interactions, morphological integrity, and organization of neuronal subfields. Interestingly, there is evidence of additive effects between distinct avenues of neuroprotection, e.g., enhancing MAPK compensatory responses vs. anti-inflammatory action.

- As reported in Bahr et al., 2002, up-regulation of AMPA receptor-MAPK repair signaling facilitated long-term cell survival and synaptic maintenance assessed 7-10 days post-insult *in vitro* and *in vivo*.

- Experiments of Specific Aim II further established that endogenous repair signals activated through different receptor systems protect against the effects of neurotoxin exposure. To be thorough, we added a mitochondrial toxin (3NP) to the list of toxic agents tested. Mitochondrial dysfunction has been implicated in stroke and age-related diseases such as Parkinsons, and may be involved in enhancing neuronal vulnerability after low-level toxin exposure. Recent work shows the same neuroprotectants that act against TMT and GD also protect against the action of the mitochondrial toxin (paper in preparation).

- Work from Specific Aim II also established that neuroprotectant can be applied to brain tissue after the toxic exposure and still elicit pronounced levels of cellular and synaptic repair. More importantly, we discovered that neurons become acutely vulnerable to damage after low-level chronic exposure to toxic agents such as TMT and GD. While pathogenic markers were not evident in brain tissue following the low-level exposure, the resultant increased vulnerability allowed an inconsequential minor insult to produce marked levels of cellular damage and synaptic decline (paper recently submitted to *Science*).

- Specific Aim III found that endogenous repair responses involving MAPK signaling are still effective protection pathways when stimulated 3-6 hours after neurotoxin exposure. Thus, there appears to be a sizeable window of opportunity to activate recovery systems.

- As shown is last year's progress report, signaling experiments found that glutamate receptors as well as cannabinoid receptors and proteoglycan-binding adhesion receptors activate the MAPK pathway and focal adhesion kinase (FAK), the primary mediator of integrin signaling. Blocking integrin signaling disrupted MAPK and FAK activation and, correspondingly, enhanced neuronal vulnerability. These results indicate that adhesion responses contribute to endogenous signaling pathways underlying cellular recovery.

- Work under Specific Aim III utilized microarray and gene regulation analyses to study the cellular response to neurotoxin exposure. The cellular response is biphasic. Evidence suggests that the initial phase consists of neuroprotective changes, while the second phase consists of degenerative pathways and gene changes. Neuroprotectant-induced signaling overlaps with the second phase, offsetting the neurodegenerative chemistries with distinctive transcription factor activation and the upregulated expression of anti-apoptotic genes. The original idea that neuroprotection is mediated through blockage of the transcription factor NF-κB is not true. The first phase of NF-κB activation after a toxic insult appears to represent a compensatory repair response, while the second phase of the NF-κB response is consistent with delayed pathogenesis that may explain the increased vulnerability following chronic exposure to mild toxin levels. Indeed, genes activated during the delayed phase appear to be those that enhance neuronal vulnerability.

7. REPORTABLE OUTCOMES

- MANUSCRIPTS (new information since 2002 report)

1. Munirathinam S and Bahr BA (2003) Enhanced vulnerability in hippocampus following repeated exposures to low-level soman. Submitted to *Science*.

2. Caba E and Bahr BA (2003) Biphasic activation of NF-κB in the excitotoxic hippocampus. Submitted to *J Neuropathol Exp Neurol.*

3. Chicoine LM and Bahr BA (2003) Polysaccharide modulation of AMPA receptors results in protection against excitotoxicity. Submitted to *J Neuropathol Exp Neurol.*

4. Vaithianathan T, Matthias K, Bahr BA, Schachner M, Suppiramaniam V, Dityatev A, and Steinhäuser C (2003) Neural cell adhesion molecule-associated polysialic acid potentiates AMPA receptor currents. Submitted to *J Neuroscience*.

5. Chicoine LM, Suppiramaniam V, Vaithianathan T, Gianutsos G, and Bahr BA (2003) Sulfate- and size-dependent polysaccharide modulation of AMPA receptor properties. *J Neurosci Res*, in press.

6. Ekdahl CT, Zhu C, Bonde S, Bahr BA, Blomgren K, and Lindvall O (2003) Death mechanisms in status epilepticus-generated neurons and effects of additional seizures on their survival. *Neurobiol Disease*, in press.

7. Emgard M, Hallin U, Karlsson J, Bahr BA, Brundin P, and Blomgren K (2003) Both apoptosis and necrosis occur early after intracerebral grafting of ventral mesencephalic tissue: A role for protease activation. *J Neurochem* **86**:1223-1232.

8. Ferrand-Drake M, Zhu C, Gidö G, Hansen A, Karlsson J-O, Bahr BA, Zamzami N, Kroemer G, Chan P, Wieloch T, and Blomgren K (2003) Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c, and caspase-3 activation in neurons exposed to transient hypoglycemia. *J Neurochem* **85**:1431-1442.

9. Bendiske J and Bahr BA (2003) Lysosomal activation is a compensatory response against protein accumulation and associated synaptopathogenesis. *J Neuropathol Exp Neurol* **62**:451–463.

10. Ekdahl CT, Mohapel P, Weber E, Bahr B, Blomgren K, and Lindvall O (2002) Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *Eur J Neurosci* **16**:1463-1471.

11. Munirathinam S, Rogers G, and Bahr BA (2002) Positive modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors elicits protection after trimethyltin exposure in hippocampus. *Toxicol Appl Pharmacol* **185**:111-118.

12. Bahr BA and Bendiske J (2002) The neuropathogenic contributions of lysosomal dysfunction. *J Neurochem* **83**:481-489.

- ABSTRACTS

1. Bahr BA and Munirathinam S, Synaptic vulnerability following low-level soman exposures is reduced through glutamatergic signals. *FASEB J* (Suppl.), **16**, A947 (2002).

2. Bendiske J and Bahr BA, Lysosomal modulation leads to neuroprotection in a model of protein deposition and synaptic decline. *The Pharmacologist* (ASPET) (2002).

3. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, The role of caspases, calpains, and cathepsins in the degeneration of newly formed neurons in the dentate gyrus following status epilepticus. *International Stem Cell Meeting (France)* (2002).

4. Volbracht C, Chua BT, Bahr BA, and Li P, Neuronal apoptosis dependent on calpain activation. *Cell Death Meeting, Australian Neuroscience Society - Noosaville* (2002).

5. Bahr BA, The pathogenic and protective contributions of lysosomal dysfunction and modulation, respectively. *Soc. Neurosci. Abstr.*, **28**, 192.15 (2002).

6. Caba E, Giardina C, and Bahr BA, The two phases of excitotoxic NF-κB activation have different effects on pro- and anti-apoptotic gene regulation. *Soc. Neurosci. Abstr.*, **28**, 250.9 (2002).

7. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Evidence that glutamatergic and endocannabinoid signaling utilize different pathways to promote neuronal survival. Soc. *Neurosci. Abstr.*, **28**, 202.16 (2002).

8. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *Soc. Neurosci. Abstr.*, **28**, 618.6 (2002).

9. Subramaniam T, Leshchynska I, Kanju PM, Bahr BA, Dityatev A, and Suppiramaniam V, AMPA receptor-mediated currents in trans-golgi network (TGN)-derived organelles. Soc. *Neurosci. Abstr.*, **28**, 139.10 (2002).

10. Suppiramaniam VD, Subramaniam T, Kanju PM, Coleman E, Bahr BA, and Wang Z, Modulation of synaptosomal AMPA receptors by a member of the Ampakine family of drugs. *Soc. Neurosci. Abstr.*, **28**, 540.4 (2002).

11. Kanju PM, Subramaniam T, Karanja P, Brown Q, Tyler J, Bahr BA, and Suppiramaniam V, Lysosomal dysfunction leads to altered AMPA channel properties in hippocampal neurons. *Soc. Neurosci. Abstr.*, **28**, 139.11 (2002).

12. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, Calpain activation occurs early after intracerebral transplantation. *8th Internatl. Conf. Neural Transplantation Repair* (2002).

13. Bahr BA and Brown QB, Lysosomal activation in guinea pig hippocampus: Compensatory signaling against protein accumulation events. *The Pharmacologist* (ASPET) (2003).

14. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Synaptic Maintenance in Hippocampus Appears to Require Endocannabinoid-Mediated Activation of MAPK and FAK Pathways. Soc. *Neurosci. Abstr.*, **29**, 464.9 (2003).

15. Brown QB, Baude AS, Gilling K, Bahr BA, and Parsons CG, Memantine and Neramexane Protect Against Semi-chronic 3-NP Toxicity in Organotypic Hippocampal Cultures. *Soc. Neurosci. Abstr.* **29**, 153.8 (2003).

16. Chicoine LM and Bahr BA, Neuroprotection Through Polysaccharide Modulation of AMPA Receptors, *Soc. Neurosci. Abstr.*, **29**, 895.2 (2003).

17. Bahr BA, Brown QB, and Hubbard AK, Pathogenic vs. Compensatory Activation of the Lysosomal System in the Hippocampal Slice Model of Protein Accumulation and Synaptopathogenesis. *Soc. Neurosci. Abstr.*, **29**, 877.11 (2003).

18. Caba E, Elliot R, Giardina C, and Bahr BA, Opposing early and delayed gene regulation events in the excitotoxic hippocampus. *Soc. Neurosci. Abstr.*, **29**, 545.10 (2003).

19. Kosten TA, Bahr BA, Karanian DA, Yeh JC, and Kehoe P, Neonatal isolation enhances hippocampal cellular pathogenesis and impairs performance on memory tasks in adult rats. *Soc. Neurosci. Abstr.*, **29**, 833.14 (2003).

20. Vaithianthan T, Bedi D, Kanju PM, Wang Z, Bahr BA, Dityatev A, Judd RL, and Suppiramaniam VD, Evidence of AMPA–glutamate receptor dysfunction in brain of streptozotocin-diabetic rats. *Soc. Neurosci. Abstr.*, (2003).

21. Wang Z, Clark R, Vaithianathan T, Kanju P, Bahr BA, Dey P, and Suppiramaniam V, Benzamide anticonvulsants modulate the channel properties of AMPA–glutamate receptors. *Soc. Neurosci. Abstr.*, (2003).

22. Bahr BA and Caba E, The threat of sub-toxic soman exposures: Enhanced vulnerability in brain tissue. *Internatl. Conf. on Advanced Technologies for Homeland Security* (2003).

- PRESENTATIONS

1. 9th Meeting of the International Neurotoxicology Association: "Enhanced vulnerability in hippocampus following repeated exposures to low-level soman", Germany (2003).

2. International Brain Research Organization, World Congress of Neuroscience: "Dysfunction and Activation of the Lysosomal System in the Central Nervous System", Czech Republic (2003).

3. University of Connecticut Health Center/Medical School Seminar Series: "Selective Compensatory Signaling in Response to Neuropathogenic Conditions", Farmington, Connecticut (2003).

8. CONCLUSIONS

Our findings have several important implications. Although acute exposure of animals to soman causes the death of neurons, the present data indicate that synapses express the initial damage elicited by repetitive contact with the nerve agent. Proteolytic activation and synaptic decline occurred after the soman exposure, both indicators of neurotoxicity (Bahr et al., 2002). In fact, inhibition of calpain proteolytic activity has been proposed as a treatment for exposure to neurotoxins including soman (Vanderklish and Bahr, 2000; Cowan et al., 2001). Persistent synaptopathogenesis in the hippocampal region may explain the delayed behavioral abnormalities and memory loss in exposed rodents and humans. Interestingly, hippocampal tissue treated with sub-toxic soman levels becomes markedly vulnerable to excitotoxic activation of glutamate receptors. Calpain is also a mediator of the toxic action of glutamate-based excitotoxicity (Siman et al., 1989; Bahr et al., 1995b, 2002; Caba et al., 2002), suggesting an initial role for the protease in the tissue vulnerability.

Hence, these findings imply that imperceptible soman contact can leave the brain susceptible to glutamate-based injuries of which there are many. Similarly, tissue compromised by a prior excitotoxic episode is made more sensitive to soman. The enhanced vulnerability does not appear to involve the disruption of cellular repair responses as determined through the potentiation of compensatory signaling. The results reported here emphasize the need for early indicators of low-level soman exposure so that subsequent brain injury can be adequately assessed and treatment/prevention measures executed in a timely manner.

Long after exposure to an environmental toxin or military threat agent, select brain regions can remain acutely vulnerable to stroke events and age-related neurodegenerative disorders such as Parkinson's disease. Similarly, low-level chronic exposure of slice cultures to toxic agents leads to increased vulnerability to subsequent insults. Compensatory responses including the activation of gene regulation events appear to be involved in offsetting the increased vulnerability. It may indeed be the case that exposures to differently acting toxicants can be treated with the same protection strategy that exploit endogenous repair systems.

As a result of the identified biphasic cellular response to toxin exposure, enhanced vulnerability may stem from disruption of the early gene regulation events that promote survival as part of the initial phase of the response. For example, IL-6, junB, Egr-1, HES-1, SOCS-3, MAPK phosphatase (cpg21), and HO-1 are upregulated while ICE is down-regulated early after neurotoxin exposure. Thus, besides protective genes being upregulated, the decrease in the expression levels of such apoptotic genes as ICE may also designate the early phase as protective. Disruption of these types of protective changes would affect neuronal vulnerability. BDNF stands out as a protective gene whose expression is disrupted by both NMDA-mediated excitotoxicity and repeated low-level soman exposures.

Alternatively, enhanced vulnerability may be due to exacerbation of the pathogenic phase of the cellular reponse. Among the genes upregulated early that may be contributing to injury were the pathogenic genes TNF-α, MIP1α, NGFI-B, NOR-1, fra-1 and ICAM-1. It is possible to envision that the survival genes are over-powered by the upregulated pathogenic genes, resulting

in the initiation of a pathogenic phase that enhances the risk of onset of neuropathogenesis including synaptic decline and loss of function. Evidence contributing to this hypothesis comes from pathogenic genes that are upregulated early after NMDA exposure (1 h post-insult) and remain upregulated over the course of the whole period such as MIP1α and C/EBP.

An array of apoptotic genes may be affected to alter neuronal vulnerability. Some are upregulated early while a different set of pathogenic genes is upregulated 24 h post-insult. Among the unique changes occurring 24 h post-insult, the potent pathogenic genes calpain and ICE were upregulated. Conversely, such genes as BDNF, trkB, Egr-1, neuroD4, and neuronatin α, which have been shown to be protective, were down-regulated in the delayed phase. It might be these changes whose effects are compounded by the slow upregulation of more pathogenic genes such as ICE and calpain.

Calpain, in particular, has been implicated in the pathogenesis of neurotoxin exposure. During such injury, calcium influx into the cell is increased resulting in the activation of calcium-dependent proteins, some of which will promote pathogenesis, such as calpain (for review see (Vanderklish and Bahr, 2000). It has been shown previously that delayed antagonism of calpain results in neuroprotection in response to excitotoxicity (Brorson et al., 1995). Accordingly, using a specific inhibitor for calpain we have shown that signaling cascades such as MAPK and NF-κB are not altered, therefore this may be an avenue of neuroprotection to follow in preventing the detrimental effects of neurotoxins (Caba et al., 2002). Pathogenic consequences of calpain upregulation are likely compounded by the down-regulation of the proteins involved in ion homeostasis (NVP, PC3, PEP-19, and hippocalcin) and has been proposed to make pathogenic proteins more potent (Toyota et al., 2003).

According to our findings, it may be possible to design specific treatment strategies where intervention or activation of specific signaling pathways may be beneficial at different times. This means of identification of gene regulation events that potentially are most influential to survival can be used to target treatment strategies appropriately. There are appropriate therapeutic targets to be explored, and it may be possible to further filter the gene data by examining the altered genes during effective neuroprotectant treatment. Additionally, these findings may enable us to construct the sequence of events following neurotoxin exposure and enhanced vulnerability.

9. REFERENCES

Bahr BA (1995) Long-term hippocampal slices: A model system for investigating synaptic mechanisms and pathologic processes. J Neuroscience Res 42:294-305.

Bahr, B. A., Abai, B., Gall, C., Vanderklish, P. W., Hoffman, K. B., and Lynch, G. (1994). Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. *Exp. Neurol.* **129**, 81-94.

Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Singh Mutneja M, Gall C, and Hoffman KB (1995a) Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 5:425-439.

Bahr, B. A., Tiriveedhi, S., Park, G. Y., and Lynch, G. (1995b). Induction of calpain-mediated spectrin fragments by pathogenic treatment in long-term hippocampal slices. *J. Pharmacol. Exp. Ther.* **273**, 902-908.

Bahr, B. A., Hoffman, K. B., Kessler, M., Hennegriff, M., Park, G. Y., Yamamoto, R. S., Kawasaki, B. T., Vanderklish, P. W., Hall, R. A., and Lynch, G. (1996). Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (*GR53*) in brain tissue. *Neuroscience* **74**, 707-721.

Bahr, B. A., Bendiske, J., Brown, Q. B., Munirathinam, S., Caba, E., Rudin, M., Urwyler, S., Sauter, A., and Rogers, G. (2002). Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp. Neurol.* **174**, 37-47.

Brorson, J. R., Marcuccilli, C. J., and Miller, R. J. (1995) Delayed Antagonism of Calpain Reduces Excitotoxicity in Cultured Neurons. Stroke 26(7), 1259-1267.

Buchan AM, H Li, S Cho, WA Pulsinelli 1991. Blockade of AMPA receptor prevents CA1 hippocampal injury following transient forebrain ischemia. *Neurosci. Lett.* **132**: 255-258.

Caba E and Bahr BA (2003) Biphasic activation of NF-κB in the excitotoxic hippocampus. Submitted to *J Neuropathol Exp Neurol.*

Caba, E., Brown, Q. B., Kawasaki, B., and Bahr, B. A. (2002). Peptidyl α-keto amide inhibitors of calpain block excitotoxic damage without affecting signal transduction events. *J Neuroscience Res* **67**, 787-794.

Carpentier, P., Lambrinidis, M., and Blanchet, G. (1991). Early dendritic changes in hippocampal pyramidal neurons (field CA1) of rats subjected to acute soman intoxication: A light microscopic study. *Brain Res.* **541**, 293–299.

Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1, 623-634.

Cowan, F. M., Broomfield, C. A., Lenz, D. E., and Shih, T. M. (2001). Protective action of the serine protease inhibitor N-tosyl-L-lysine chloromethyl ketone (TLCK) against acute soman poisoning. *J. Appl. Toxicol.* **21**, 293-296.

de Groot, D. M., Bierman, E. P., Bruijnzeel, P. L., Carpentier, P., Kulig, B. M., Lallement, G., Melchers, B. P., Philippens, I. H., and van Huygevoort, A. H. (2001). Beneficial effects of TCP on soman intoxication in guinea pigs: Seizures, brain damage and learning behaviour. *J. Appl. Toxicol.* **21**, S57-65.

Feldman, R. G., White, R. R., and Eriator, I. I. (1993). Trimethyltin encephalopathy. *Arch. Neurol.* **50**, 1320-1324.

Filliat, P., Baubichon, D., Burckhart, M. F., Pernot-Marino, I., Foquin, A., Masqueliez, C., Perrichon, C., Carpentier, P., and Lallement, G. (1999). Memory impairment after soman intoxication in rat: Correlation with central neuropathology: Improvement with anticholinergic and antiglutamatergic therapeutics. *Neurotoxicology* **20**, 535–549.

Granger, R., Deadwlyer, S., Davis, M., Moskovitz, B., Kessler, M., Rogers, G., and Lynch, G. (1996). Facilitation of glutamate receptors reverses age-associated memory impairment in rats. Synapse 22, 332-337.

Hampson, R. E., Rogers, G., Lynch, G., and Deadwyler, S. A. (1998). Facilitative effects of the ampakine CX516 on short-term memory in rats: correlations with hippocampal neuronal activity. J. Neurosci. 18, 2748-2763.

Hayashi, T., Umemori, H., Mishina, M., and Yamamoto, T. (1999). The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. Nature 397, 72-76.

Ishida, N., Akaike, M., Tsutsumi, S., Kanai, H., Masui, A., Sadamatsu, M., Kuroda Y., Watanabe, Y., McEwen, B. S., and Kato, N. (1997). Trimethyltin syndrome as a hippocampal degeneration model: temporal changes and neurochemical features of seizure susceptibility and learning impairment. *Neuroscience* **81**, 1183-1191.

Lallement, G., Carpentier, P., Collet, A., Pernot-Marino, I., Baubichon, D., and Blanchet, G. (1991). Effects of soman-induced seizures on different extracellular amino acid levels and on glutamate uptake in rat hippocampus. *Brain Res.* **563**, 234-240.

Lallement, G., Denoyer, M., Collet, A., Pernot-Marino, I., Baubichon, D., Monmaur, P., and Blanchet, G. (1992). Changes in hippocampal acetylcholine and glutamate extracellular levels during soman-induced seizures: Influence of septal cholinoceptive cells. *Neurosci. Lett.* **139**, 104–107.

Lallement, G., Delamanche, I. S., Pernot-Marino, I., Baubichon, D., Denoyer, M., Carpentier, P., and Blanchet, G. (1993). Neuroprotective activity of glutamate receptor antagonists against soman-induced hippocampal damage: Quantification with a w3-site ligand. *Brain Res.* **618**, 227–237.

Lebrun, C., Pilliere, E., and Lestage, P. (2000). Effects of S 18986-1, a novel cognitive enhancer, on memory performances in an object recognition task in rats. Eur. J. Pharmacol. 401, 205-212.

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 organophosphorous compound. *Acta Neuropathol.* **61**, 123-129.

Limatola, C., Ciotti, M. T., Mercanti, D., Santoni, A., and Eusebi, F. (2002). Signaling pathways activated by chemokine receptor CXCR2 and AMPA-type glutamate receptors and involvement in granule cells survival. J. Neuroimmunol. 123, 9-17.

Mattson, M. P. (1990). Antigenic changes similar to those seen in neurofibrillary tangles are elicited by glutamate and Ca^{2+} influx in cultured hippocampal neurons. *Neuron* **4**, 105-117.

McKinney, R. A., Capogna, M., Durr, R., Gahwiler, B. H., and Thompson, S. M. (1999). Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat. Neurosci. 2, 44-49.

Munirathinam, S., and Bahr, B. A. (2002). Low-level soman exposure leads to synaptic damage in the hippocampus and potentiates neuronal vulnerability. *Toxicol. Sci.* **58** (Suppl.), 1551 (Abstract).

Munirathinam, S., Rogers, G., and Bahr, B. A. (2002). Positive modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus. *Toxicol. Appl. Pharmacol.* **185**, 111-118.

Pazdernik, T. L., Cross, R. S., Giesler, M., Samson, F. E., and Nelson, S. R. (1985). Changes in local cerebral glucose utilization induced by convulsants. *Neuroscience* **14**, 823-835.

Raveh, L., Weissman, B. A., Cohen, G., Alkalay, D., Rabinovitz, I., Sonego, H., and Brandeis, R. (2002). Caramiphen and scopolamine prevent soman-induced brain damage and cognitive dysfunction. *Neurotoxicology* **23**, 7–17.

Raveh, L., Brandeis, R., Gilat, E., Cohen, G., Alkalay, D., Rabinovitz, I., Sonego, H., and Weissman B. A. (2003). Anticholinergic and antiglutamatergic agents protect against soman-induced brain damage and cognitive dysfunction. *Toxicol. Sci.* **75**, 108-116.

Sheardown, M. J., Nielsen, E. O., Hansen, A. J., Jacobsen, P., and Honore, T. (1990). 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: A neuroprotectant for cerebral ischemia. *Science* **247**, 571-574.

Siman, R., Noszek J. C., and Kegerise, C. (1989). Calpain I activation is specifically related to excitatory amino acid induction of hippocampal damage. *J. Neurosci.* **9**, 1579-1590.

Slemmon JR, Feng B, and Erhardt JA (2000) Small proteins that modulate calmodulin-dependent signal transduction: effects of PEP-19, neuromodulin, and neurogranin on enzyme activation and cellular homeostasis. *Mol Neurobiol* **22**:99-113.

Sparenborg, S., Brennecke, L. H., Jaax, N. K., and Braitman, D. J. (1992). Dizocilpine (MK-801) arrests status epilepticus and prevents brain damage induced by soman. *Neuropharmacol.* **31**, 357-368.

Staubli U, Ambros-Ingerson J, Lynch G. (1992) Receptor changes and LTP: an analysis using aniracetam, a drug that reversibly modifies glutamate (AMPA) receptors. Hippocampus 2(1):49-57.

Staubli, U., Rogers, G. and Lynch, G. (1994). Facilitation of glutamate receptors enhances memory. Proc. Natl. Acad. Sci. U.S.A. 91, 777-781.

Taylor, P. (2001). Anticholinesterase agents. In The Pharmacological Basis of Therapeutics, 10th ed. (J. G. Hardman, L. E. Limbird, and A. G. Gilman, Eds.), pp. 175–191. McGraw-Hill Companies, Inc., New York.

Toyota H, Yanase N, Yoshimoto T, Moriyama M, Sudo T, and Mizuguchi J (2003) Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. *Cancer Lett* **189** :221-230.

Vanderklish, P. W., and Bahr, B. A. (2000). The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. *Internatl. J. Exp. Pathol.* **81**, 323-339.

Wade, J. V., Samson, F. E., Nelson, S. R., and Pazdernik, T. L. (1987). Changes in extracellular amino acids during soman- and kainic acid-induced seizures. *J. Neurochem. 49*, 645-650.

Wang, Y., D. L. Small, D. B. Stanimirovic, P. Morley, and J. P. Durkin. 1997. AMPA receptor-mediated regulation of the G_i-protein in cortical neurons. *Nature* **389**: 502-504.

10. APPENDICES

▫ MANUSCRIPTS (attached at the end)

1. Munirathinam S and Bahr BA (2003) Enhanced vulnerability in hippocampus following repeated exposures to low-level soman. Submitted to *Science*.

2. Caba E and Bahr BA (2003) Biphasic activation of NF-κB in the excitotoxic hippocampus. Submitted to *J Neuropathol Exp Neurol.*

3. Chicoine LM, Suppiramaniam V, Vaithianathan T, Gianutsos G, and Bahr BA (2003) Sulfate- and size-dependent polysaccharide modulation of AMPA receptor properties. *J Neurosci Res*, in press.

4. Emgard M, Hallin U, Karlsson J, Bahr BA, Brundin P, and Blomgren K (2003) Both apoptosis and necrosis occur early after intracerebral grafting of ventral mesencephalic tissue: A role for protease activation. *J Neurochem* **86**:1223-1232.

5. Ferrand-Drake M, Zhu C, Gidö G, Hansen A, Karlsson J-O, Bahr BA, Zamzami N, Kroemer G, Chan P, Wieloch T, and Blomgren K (2003) Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c, and caspase-3 activation in neurons exposed to transient hypoglycemia. *J Neurochem* **85**:1431-1442.

6. Bendiske J and Bahr BA (2003) Lysosomal activation is a compensatory response against protein accumulation and associated synaptopathogenesis. *J Neuropathol Exp Neurol* **62**:451–463.

7. Ekdahl CT, Mohapel P, Weber E, Bahr B, Blomgren K, and Lindvall O (2002) Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *Eur J Neurosci* **16**:1463-1471.

8. Munirathinam S, Rogers G, and Bahr BA (2002) Positive modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors elicits protection after trimethyltin exposure in hippocampus. *Toxicol Appl Pharmacol* **185**:111-118.

- ABSTRACTS (pages 34-54)

1. Bahr BA and Munirathinam S, Synaptic vulnerability following low-level soman exposures is reduced through glutamatergic signals. *FASEB J* (Suppl.), **16**, A947 (2002).

2. Bendiske J and Bahr BA, Lysosomal modulation leads to neuroprotection in a model of protein deposition and synaptic decline. *The Pharmacologist* (ASPET) (2002).

3. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, The role of caspases, calpains, and cathepsins in the degeneration of newly formed neurons in the dentate gyrus following status epilepticus. *International Stem Cell Meeting (France)* (2002).

4. Bahr BA, The pathogenic and protective contributions of lysosomal dysfunction and modulation, respectively. *Soc. Neurosci. Abstr.*, **28**, 192.15 (2002).

5. Caba E, Giardina C, and Bahr BA, The two phases of excitotoxic NF-κB activation have different effects on pro- and anti-apoptotic gene regulation. *Soc. Neurosci. Abstr.*, **28**, 250.9 (2002).

6. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Evidence that glutamatergic and endocannabinoid signaling utilize different pathways to promote neuronal survival. Soc. *Neurosci. Abstr.*, **28**, 202.16 (2002).

7. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *Soc. Neurosci. Abstr.*, **28**, 618.6 (2002).

8. Subramaniam T, Leshchynska I, Kanju PM, Bahr BA, Dityatev A, and Suppiramaniam V, AMPA receptor-mediated currents in trans-golgi network (TGN)-derived organelles. Soc. *Neurosci. Abstr.*, **28**, 139.10 (2002).

9. Suppiramaniam VD, Subramaniam T, Kanju PM, Coleman E, Bahr BA, and Wang Z, Modulation of synaptosomal AMPA receptors by a member of the Ampakine family of drugs. *Soc. Neurosci. Abstr.*, **28**, 540.4 (2002).

10. Kanju PM, Subramaniam T, Karanja P, Brown Q, Tyler J, Bahr BA, and Suppiramaniam V, Lysosomal dysfunction leads to altered AMPA channel properties in hippocampal neurons. *Soc. Neurosci. Abstr.*, **28**, 139.11 (2002).

11. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, Calpain activation occurs early after intracerebral transplantation. *8th Internatl. Conf. Neural Transplantation Repair* (2002).

12. Bahr BA and Brown QB, Lysosomal activation in guinea pig hippocampus: Compensatory signaling against protein accumulation events. *The Pharmacologist* (ASPET) (2003).

13. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Synaptic Maintenance in Hippocampus Appears to Require Endocannabinoid-Mediated Activation of MAPK and FAK Pathways. Soc. *Neurosci. Abstr.*, **29**, 464.9 (2003).

14. Brown QB, Baude AS, Gilling K, Bahr BA, and Parsons CG, Memantine and Neramexane Protect Against Semi-chronic 3-NP Toxicity in Organotypic Hippocampal Cultures. *Soc. Neurosci. Abstr.* **29**, 153.8 (2003).

15. Chicoine LM and Bahr BA, Neuroprotection Through Polysaccharide Modulation of AMPA Receptors, *Soc. Neurosci. Abstr.*, **29**, 895.2 (2003).

16. Bahr BA, Brown QB, and Hubbard AK, Pathogenic vs. Compensatory Activation of the Lysosomal System in the Hippocampal Slice Model of Protein Accumulation and Synaptopathogenesis. *Soc. Neurosci. Abstr.*, **29**, 877.11 (2003).

17. Caba E, Elliot R, Giardina C, and Bahr BA, Opposing early and delayed gene regulation events in the excitotoxic hippocampus. *Soc. Neurosci. Abstr.*, **29**, 545.10 (2003).

18. Kosten TA, Bahr BA, Karanian DA, Yeh JC, and Kehoe P, Neonatal isolation enhances hippocampal cellular pathogenesis and impairs performance on memory tasks in adult rats. *Soc. Neurosci. Abstr.*, **29**, 833.14 (2003).

19. Vaithianthan T, Bedi D, Kanju PM, Wang Z, Bahr BA, Dityatev A, Judd RL, and Suppiramaniam VD, Evidence of AMPA–glutamate receptor dysfunction in brain of streptozotocin-diabetic rats. *Soc. Neurosci. Abstr.*, (2003).

20. Wang Z, Clark R, Vaithianathan T, Kanju P, Bahr BA, Dey P, and Suppiramaniam V, Benzamide anticonvulsants modulate the channel properties of AMPA–glutamate receptors. *Soc. Neurosci. Abstr.*, (2003).

21. Bahr BA and Caba E, The threat of sub-toxic soman exposures: Enhanced vulnerability in brain tissue. *Internatl. Conf. on Advanced Technologies for Homeland Security* (2003).

- CURRICULUM VITAE: Ben A. Bahr (pages 55-78)

11. PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Ben A. Bahr, Ph.D., P.I.

Ebru Caba, Ph.D., postdoctoral researcher

Queenie Brown, M.S., research technician

David Karanian, B.S., graduate student/research assistant

1. Bahr BA and Munirathinam S, Synaptic vulnerability following low-level soman exposures is reduced through glutamatergic signals. *FASEB J* (Suppl.), **16**, A947 (2002).

Synaptic vulnerability following low-level soman exposures is reduced through glutamatergic signals.

Ben A. Bahr and Subramani Munirathinam

Neurosciences Program and the Center for Drug Discovery, University of Connecticut

Hippocampal slice cultures were used to study a potential treatment against the action of the military-threat agent soman (GD). The organophosphate anticholinesterase causes accumulated levels of ACh, epileptiform bursting, and increased glutamate release that promote seizure-related damage. After a week of daily GD exposures, the cultured slices exhibited dose-dependent breakdown of spectrin by the pathogenic protease calpain as well as declines in synaptic proteins. Synaptophysin staining was reduced in dendritic fields of CA1 and the dentate gyrus. Low-level GD (150 nM) for 6 days caused little synaptic or cytoskeletal effects. However when the 6-day GD treatment was combined with a 3-min excitotoxic insult (via AMPA), marked synaptic deterioration and spectrin breakdown were produced. The AMPA-insult applied alone had no effect. To test for protection, glutamatergic signaling including its link to MAPK activation was promoted by the Ampakine CX516 over the 6-day GD exposure. After the 3-min AMPA insult, CX516- treated slices exhibited complete protection against spectrin breakdown ($p<0.01$) and synaptophysin loss ($p=0.0001$) and 74% protection against altered synaptic receptors (GluR1; $p=0.01$). Thus, prolonged exposure to dilute GD enhances neuronal vulnerability and facilitation of glutamatergic signals protects against this. (U.S. Army DAMD17-99-C9090, NIH 1R43NS38404-01).

2. Bendiske J and Bahr BA, Lysosomal modulation leads to neuroprotection in a model of protein deposition and synaptic decline. *The Pharmacologist* (ASPET) (2002).

Lysosomal modulation leads to neuroprotection in a model of Alzheimer-type protein deposition and synaptic decline

Jennifer Bendiske and Ben A. Bahr
Department of Pharmaceutical Sciences and the Neurosciences Program
University of Connecticut
Storrs, Connecticut

Aberrant protein processing/deposition in Alzheimer's disease has been shown to be associated with enhanced levels of lysosomes and their enzymes. This activation of the lysosomal system may be a compensatory response against protein deposits. In hippocampal slice cultures, abnormal protein processing mediated by the lysosomal disruptor chloroquine (Cqn) led to tau deposition, buildup of amyloidogenic fragments, microtubule destabilization, transport failure, and selective decline in synaptic markers. As the pathogenic cascade progressed, there was concomitant lysosomal activation as indicated by increased levels of cathepsin isoforms, elastase and β-glucuronidase. To test whether the activation represents a compensatory response, Z-Phe-Ala-diazomethylketone (PADK) was identified as a positive modulator at low concentrations (1-10 μM), generating robust up-regulation of lysosomal enzymes (400-600% increases) over that which occurred in response to Cqn (70-100%). PADK-mediated lysosomal modulation was stable for >20 days while synaptic protein levels remained normal. When PADK and Cqn were co-infused into slice cultures, Cqn exposure no longer was associated with increased levels of 55-69-kDa tau species. To assess pre-existing conditions, Cqn was applied for 6 days after which removing the agent resulted in continued pathogenesis over subsequent days. In contrast, replacing Cqn with PADK after the 6-day treatment led to reduced deposition of PHF material and β-amyloid-containing APP fragment. Suppression of protein deposits was linked to restoration of acetylated tubulin, a marker of stable microtubules. Transport processes lost during Cqn exposure also were re-established by PADK modulation, resulting in up to 70% recovery in expression of synaptic markers. These data indicate that the lysosomal system can be pharmacologically modulated, thereby enhancing cellular repair potential during pathogenic episodes.

3. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, The role of caspases, calpains, and cathepsins in the degeneration of newly formed neurons in the dentate gyrus following status epilepticus. *International Stem Cell Meeting (France)* (2002).

We sought to determine which cell death pathways are involved in the degeneration of newly formed neurons in the rat dentate gyrus following 2 h of electrically induced status epilepticus. We were particularly interested in the role of cysteine proteases, caspases, calpains, and cathepsins, which all can participate in apoptotic cell death. Status epilepticus increased the number of bromodeoxyuridine (BrdU) -positive proliferated cells in the subgranular zone of the dentate gyrus. At the time of maximum cell proliferation 8 days after the insult, immunohistochemical analyses revealed cellular protein expression of active caspase 3-cleaved poly (ADP-ribose) polymerase (PARP) and cathepsin B, but not of the calpain-produced fodrin breakdown product 150 kDa in the proliferative subgranular zone. Only cells expressing cleaved PARP were co-labeled with BrdU. Approximately 66 % of the BrdU-positive cells expressed cleaved PARP. We gave intracerebroventricular infusions of a caspase inhibitor cocktail (zVAD-fmk, zDEVD-fmk and zLEHD-fmk) over a 2 week-period, starting at one week after the epileptic insult. Increased numbers of surviving cells double-labeled with BrdU and the neuron-specific marker NeuN were detected in the subgranular zone and granule cell layer of the caspase inhibitor-treated rats.

4. Bahr BA, The pathogenic and protective contributions of lysosomal dysfunction and modulation, respectively. *Soc. Neurosci. Abstr.*, **28**, 192.15 (2002).

The pathogenic and protective contributions of lysosomal dysfunction and modulation, respectively

BA Bahr, Dept Pharm Sci & Neurosci Prgm, Univ Connecticut, Storrs

Multiple lines of evidence implicate lysosomes in pathogenic events that produce neurodegeneration. Lysosomal storage disorders are associated with abnormal brain development. These disorders are characterized by intracellular deposits and protein aggregation, and similar events occur in Alzheimer's disease and related tauopathies. In fact, lysosomal disturbances are common in age-related diseases. Lysosomes are highly susceptible to free radical damage in the aging brain, leading to the gradual loss of their protein processing capacity. Such lysosomal dysfunction has been implicated in amyloidogenic chemistries and the formation of PHFs. In the hippocampal slice model, lysosomal disruption recapitulates important pathological features of age-related diseases including the link between protein deposition and synaptic loss. Treatment with the lysosomal disrupter chloroquine (Cqn) led to tau and amyloidogenic deposits, microtubule instability, transport failure, and synaptic decline. As in AD, this cascade is associated with up-regulated levels of lysosomal enzymes including cathepsin isoforms, elastase, and B-glucuronidase. To determine the role of lysosomal activation, Z-Phe-Ala-diazomethylketone (PADK) was identified as a positive modulator at low concentrations, generating robust up-regulation of lysosomal enzymes (400-600%) over that which occurred in response to Cqn alone (70-100%). PADK blocked Cqn-induced increases in 55-69-kDa tau species, and facilitated the clearance of protein deposits. Reduced deposition was linked to restored microtubule integrity, transport capability, and expression of synaptic markers. These data indicate that lysosomal activation can act as a compensatory response.

5. Caba E, Giardina C, and Bahr BA, The two phases of excitotoxic NF-κB activation have different effects on pro- and anti-apoptotic gene regulation. *Soc. Neurosci. Abstr.*, **28**, 250.9 (2002).

THE TWO PHASES OF EXCITOTOXIC NF-ᴷB ACTIVATION HAVE DIFFERENT EFFECTS ON PRO- AND ANTI-APOPTOTIC GENE REGULATION.

E. Caba[1], C. Giardina[2], B.A. Bahr[1]
Depts Pharm Sci/Neurosci Prgm[1] and Mol Cell Biol,[2] Univ Connecticut, Storrs.

NF-kB has been implicated in both survival and cell death. A corresponding dual response occurs during NF-kB's activation following an excitotoxic insult in hippocampal slices, consisting of an early (0.5-2 h post-insult) and delayed phase (8-24 h). The biphasic activation was specific for NF-ᴷB since no response was exhibited by the transcription factor SP-1. Using antibodies to the p50 subunit of NF-ᴷB, neuronal nuclear staining was observed in the three subfields. Through correlations with MEK activation and neuroprotectant action, we hypothesized that the early phase of the NF-kB response promotes survival signaling whereas the delayed phase corresponds with pathogenic processes. To examine this hypothesis further, we measured the anti-apoptotic and pro-apoptotic genes bcl-2 and bax, respectively, by RT-PCR. Expression levels of the bcl-2 gene exhibited a 35% increase in the early phase after the NMDA-induced excitotoxicity (20-min exposure), and the increase persisted into the delayed phase 8-24 h post-insult ($p<0.01$, post-hoc test). Message levels for bax, on the other hand, were unchanged in the early phase, but increased by 30-35% during the delayed phase ($p<0.05$). These findings provide further support that the two phases promote distinct cellular pathways: the early phase of NF-kB activation is associated with the up-regulation of anti-apoptotic gene expression, while NF-kB's delayed phase is involved in the expression of pro-apoptotic genes. Such opposing gene regulation events will be examined further using gene microarray analysis.

6. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Evidence that glutamatergic and endocannabinoid signaling utilize different pathways to promote neuronal survival. Soc. *Neurosci. Abstr.*, **28**, 202.16 (2002).

Evidence that Glutamatergic and Endocannabinoid Signaling Utilize Different Pathways to Promote Neuronal Survival.

David Karanian, Queenie B. Brown, Alex Makriyannis, and Ben A. Bahr. Center for Drug Discovery, Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, CT.

In the central nervous system, glutamatergic communication and cannabimimetic activity can promote distinct types of synaptic plasticity (Bahr et al., *Exp Neurol* 174:37-47, 2002; Gerdeman et al., *Nature Neurosci* 5:446-51, 2002). Interestingly, AMPA-type glutamate receptors and the cannabinoid system also play a role in cell survival. Here we used the hippocampal slice model to address whether the endogenous glutamatergic and cannabimimetic activities utilize the same survival signaling pathway. Positive modulation of glutamatergic responses through AMPA receptors significantly reduced cytoskeletal and synaptic damage resulting from an excitotoxic episode. The modulation caused a corresponding activation of the ERK1/2 MAPK through the upstream activator MAPK kinase (MEK). Blocking either component of the AMPA receptor-MAPK pathway has been previously shown to promote cellular damage, further indicating that this pathway facilitates compensatory signals in response to injury. In order to enhance cannabimimetic signals, we used compounds AM404 and AM374 to block cellular endocannabinoid uptake and catabolism, respectively. Increasing endocannabinoid tone in this way completely eliminated calpain-mediated proteolysis of the spectrin cytoskeleton evident 24 h after the excitotoxic insult. The cytoskeletal protection was accompanied by significant restoration of pre- and postsynaptic markers. In contrast to the results from enhanced glutamatergic signaling, the increased endocannabinoid availability did not activate MAPK measured as the phosphorylation of the catalytic core of the ERK isoforms. Combination studies also suggest that the neuroprotective effects mediated by glutamatergic and endocannabinoid responses are additive. These results provide evidence that the glutamate and cannabinoid receptor systems are involved in distinct cell survival pathways. This work was supported by U.S. Army Medical Res. grant DAMD17-99-C9090, NIH 1R43NS38404-01, NIH/NIDA DA07312 and DA09158.

7. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *Soc. Neurosci. Abstr.*, **28**, 618.6 (2002).

A large proportion of cells that proliferate in the adult dentate gyrus under normal conditions or in response to brain insults exhibit only short-term survival. Here we sought to determine which cell death pathways are involved in the degeneration of newly formed neurons in the rat dentate gyrus following 2 h of electrically induced status epilepticus. We were particularly interested in the role of 3 families of cysteine proteases, caspases, calpains, and cathepsins, which all can participate in apoptotic cell death. Status epilepticus increased the number of bromodeoxyuridine (BrdU) - positive proliferated cells in the subgranular zone of the dentate gyrus. At the time of maximum cell proliferation 8 days after the insult, immunohistochemical analyses revealed cellular protein expression of active caspase 3-cleaved poly (ADP-ribose) polymerase (PARP) and cathepsin B, but not of the calpain-produced fodrin breakdown product 150 kDa in the proliferative subgranular zone. Only cells expressing cleaved PARP were co-labeled with BrdU. Approximately 66 % of the BrdU-positive cells expressed cleaved PARP. To evaluate the role of the caspases in the newly formed cells, we gave intracerebroventricular infusions of a caspase inhibitor cocktail (zVAD-fmk, zDEVD-fmk and zLEHD-fmk) over a 2 week-period, starting at one week after the epileptic insult. Increased numbers of surviving cells double-labeled with BrdU and the neuron-specific marker NeuN were detected in the subgranular zone and granule cell layer of the caspase inhibitor-treated rats. Our data indicate that caspase-mediated cell death pathways regulate the survival of neurons generated in response to status epilepticus.

8. Subramaniam T, Leshchynska I, Kanju PM, Bahr BA, Dityatev A, and Suppiramaniam V, AMPA receptor-mediated currents in trans-golgi network (TGN)-derived organelles. Soc. *Neurosci. Abstr.*, **28**, 139.10 (2002).

AMPA RECEPTOR - MEDIATED CURRENTS IN TRANS - GOLGI NETWORK (TGN) - DERIVED ORGANELLES

T.Subramaniam [1,2*]; I.Leshchyns'ka[3]; P.M.Kanju [1,2]; B.A.Bahr[4]; A.Dityatev [3] ; V.Suppiramaniam [1,2]

1. Dept Pharmacal Sci, Auburn Univ, Auburn, AL, USA; 2. Dept. Biology, Tuskegee Univ, Tuskegee, AL, USA; 3. Center for Mol.Neurobiology, University of Hamburg, Hamburg, Germany; 4. Dept. of Pharmaceutical Sciences, Univ. of Connecticut, Storrs, CT, USA

During their maturation in endoplasmatic reticulum, cis- and trans-Golgi organelles, transmitter receptors and ion channels undergo several posttranslational modifications and changes in their binding partners. As the first step toward elucidation how the processing along biosynthetic pathway affects functional properties of AMPA receptors, we compared AMPA-evoked currents recorded in membranes of TGN-derived organelles reconstituted in lipid bilayers with our previous recordings in synaptosomal preparations (Suppiramaniam et al., Soc. Neurosci. Abstr., 502.10, 2001). The TGN-derived organelles were isolated according to the method of Fath et al. (1997, J. Cell Biol. 139, 1169-1181) and proved to be immunopositive for TGN marker gamma-adaptin and postsynaptic form of spectrin. Voltage clamp recordings showed that in the presence of substances that blocked voltage-gated Na+ and K+ currents and NMDA responses, 3μM AMPA elicited single channel currents with conductances of 40-60 pS in reconstituted TGN organelles. These AMPA-evoked currents were sensitive to 1 μM CNQX. The synaptosomal AMPA receptors and purified AMPA receptors exhibited two major conductance states of 10 pS and 50 pS. Thus, our data indicate the existence of functional AMPA receptors in TGN-derived organelles.

9. Suppiramaniam VD, Subramaniam T, Kanju PM, Coleman E, Bahr BA, and Wang Z, Modulation of synaptosomal AMPA receptors by a member of the Ampakine family of drugs. *Soc. Neurosci. Abstr.*, **28**, 540.4 (2002).

MODULATION OF SYNAPTOSOMAL AMPA RECEPTORS BY A MEMBER OF THE AMPKINE FAMILY OF DRUGS

V.D.Suppiramaniam [1,2]; T.Subramaniam [1,2]; P.M.Kanju [1,2]; E.Coleman [3*]; B.A.Bahr [4] ; W.Zang [1]

1. Department of Pharmacal Science, Auburn University, Auburn, AL, USA; 2. Dept. Biology, Tuskegee Univ, Tuskegee, AL, USA; 3. Dept. of Anatomy, Physiol & Pharmacology, Auburn Univ, Auburn, AL, USA; 4. Dept. of Pharmaceutical Sciences, Univ. of Connecticut, Storrs, CT, USA

The direct modulatory effects of an ampakine 1-(quinoxalin-6-ylcarbonyl)piperidine (CX516) on purified and reconstituted AMPA (alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors have been previously demonstrated (Suppiramaniam et al., Synapse 40: 154-158, 2001). The current study focuses on the effects of this compound on synaptosomal AMPA receptors. Synatoneurosomes were isolated from the rat hippocampus and the presence of AMPA receptor subunits were confirmed by western blot analysis. Isolated synatoneurosomes were incorporated into tip-dip bilayers and AMPA receptor channel activity was elicited by the addition of 150 nM of AMPA. Addition of 3.3 uM of CX 516 to the pseudo-extracellular fluid exhibited profound channel activity that resulted in 70% increase in the open channel probability compared to that elicited by AMPA alone. The above findings suggest that CX 516 can serve as a potent modulator of synaptic AMPA receptors.

10. Kanju PM, Subramaniam T, Karanja P, Brown Q, Tyler J, Bahr BA, and Suppiramaniam V, Lysosomal dysfunction leads to altered AMPA channel properties in hippocampal neurons. *Soc. Neurosci. Abstr.*, **28**, 139.11 (2002).

LYSOSOMAL DYSFUNCTION LEADS TO ALTERED AMPA CHANNEL PROPERTIES IN HIPPOCAMPAL NEURONS

P.M.Kanju [1,2]; T.Subramaniam [1,2]; P.Karanja [2]; Q.Brown [3]; J.Tyler [4]; B.Bahr [3]; V.Suppiramaniam [1,2*]

1. Dept. of Pharmacal Sci., Auburn Univ., Auburn, AL, USA; 2. Dept. Biol., Tuskegee Univ., Tuskegee, AL, USA; 3. Dept. of Pharmaceut. Sci., Univ. of Connecticut, Storrs, CT, USA; 4. Dept. of Neurobiol., UAB, Birmingham, AL, USA

There is evidence that experimentally induced lysosomal dysfunction exhibits features similar to those observed in aged human brain. Considerable reduction in AMPA receptor subunit GluR1 has been observed in in vitro studies following lysosomal dysfunction. However, electrical properties of AMPA receptors following lysosomal dysfunction have not been studied. This study aimed to elucidate the electrical properties of AMPA receptors in hippocampal neurons exhibiting experimentally induced lysosomal dysfunction. Hippocampal slice cultures were treated with lysosomotropic agent chloroquine to induce lysosomal disturbances. Electrophysiological recordings in whole cell configuration and single channel recordings of synaptoneurosomes revealed a significant reduction in AMPA currents in treated slices. Depending on the number of days of Chloroquine treatment, the open probabilities of AMPA channels were as follows: Control/untreated (70-75%), 3 days (40-50%), 6 days (30-45%) and 9 days (5-15%). Addition of ampakine 1-(quinoxalin-6-ylcarbonyl)piperidine (CX516) revealed a significant increase in open probabilities in 3 days (60-65%) and 6 days (55-60%) treated samples but no significant increase was recorded for the 9 days. In addition, western blot analysis of synaptoneurosomes indicated a reduction in AMPA receptor subunit GluR1 in samples treated for six days. These findings suggest that lysosomal disturbances result in alteration of AMPA channel properties that are essential for synaptic plasticity.

11. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, Calpain activation occurs early after intracerebral transplantation. *8th Internatl. Conf. Neural Transplantation Repair* (2002).

Neural transplantation is an experimental treatment for Parkinson's disease. Widespread clinical application of the grafting technique is hampered by a relatively poor survival (around 10%) of implanted embryonic dopamine neurons. Earlier animal studies have indicated that a large proportion of the grafted cells die during graft tissue preparation and within the first few days after intracerebral implantation. The present study was designed to reveal the prevalence of cell death in rat intrastriatal grafts at 90 minutes, 1 day, 3 days, 6 days and 42 days after implantation. We examined apoptotic cell death using semi-thin and paraffin sections stained with methylene blue and an antibody against activated caspase-3, respectively. We identified abundant apoptotic cell death up to 3 days after transplantation. In addition, we studied calpain activation using an antibody specific for calpain-cleaved fodrin. We report a peak in calpain activity 90 minutes after grafting. Surprisingly, we did not observe any significant difference in the number of dopaminergic neurons over time. The present results imply that grafted cells may be victims of either an early necrotic or a later apoptotic cell death, and that there is a substantial cell death as early as 90 minutes after implantation.

12. Bahr BA and Brown QB, Lysosomal activation in guinea pig hippocampus: Compensatory signaling against protein accumulation events. *The Pharmacologist* (ASPET) (2003).

Bahr BA and Brown QB, Lysosomal activation in guinea pig hippocampal slice cultures: Compensatory signaling against protein accumulation events. *The Pharmacologist* (ASPET) (2003).

Age-related lysosomal changes likely contribute to the risk factor that enhances the brain's susceptibility to neurodegenerative disorders including Alzheimer's disease. Previous reports have shown that lysosomal dysfunction facilitates protein aggregation, intracellular deposits, and microtubule destabilization. These events are part of a deleterious cascade that leads to transport failure and synaptopathogenesis. While lysosomal disruption promotes neuronal atrophy, evidence from Alzheimer brains suggests that activation of the lysosomal system constitutes a compensatory response. In guinea pig hippocampal slice cultures, the compensatory signaling in response to protein accumulation was found to be capable of neuroprotection. Lysosomal activation, as indicated by the up-regulation of catabolic enzymes, corresponded with the degree of synaptic decline, perhaps to slow the progressive neurodegeneration. Enhancing the lysosomal activation response with a selective modulator restored synaptic integrity by clearing protein deposits and re-establishing microtubule-based transport mechanisms. The need for protein clearance is critical for a variety of diseases, and the lysosomal compensatory response indicates that the brain is not a passive recipient of age-related pathology. Moreover, positive modulation of the lysosomal response represents a therapeutic avenue against protein accumulation diseases.

13. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Synaptic Maintenance in Hippocampus Appears to Require Endocannabinoid-Mediated Activation of MAPK and FAK Pathways. Soc. *Neurosci. Abstr.*, **29**, 464.9 (2003).

Synaptic Maintenance in Hippocampus Appears to Require Cannabinoid-Mediated Activation of MAPK and FAK Pathways

D.A.Karanian*; Q.B.Brown; A.Makriyannis; B.A.Bahr
Center for Drug Discovery, Department of Pharmaceutical Sciences & Neuroscience Program, Univ of Connecticut, Storrs, CT, USA

Cannabinoid signaling through the G-protein-coupled CB1 receptor has been implicated in neuroprotection against excitotoxicity, oxidative stress, hypoxia, ischemia, and trauma. Pathways regulated by the coupled G proteins include those involving ERK1/ERK2 mitogen-activated protein kinase (MAPK) and focal adhesion kinase (FAK). Here we determined the role of CB1 activity and related signal transduction events in the maintenance of synaptic contacts using hippocampal slice cultures. Disrupting endocannabinoid signaling with a CB1-selective antagonist (10 μM AM281) caused gradual decline of the synaptic markers GluR1, synapsin IIa and synaptophysin over 5 days. In addition, blocking the MAPK pathway with the MEK inhibitor U0126 has been shown to promote synaptic decay (Bahr et al. 2002, *Exp Neurol* 174:37) as did blocking integrin-related FAK signaling with GRGDSP. The integrin antagonist caused pre- and postsynaptic decline after 4 days, and enhanced neuronal vulnerability to an excitotoxic insult in the hippocampal slices. To test if cannabinoid signaling is indeed linked to the activation of MAPK and FAK, the CB1 receptors were activated with 10 μM R-methanandamide (AM356). Within 30 min, AM356 induced MAPK activation measured by selective antibody staining of phosphorylated Thr-183 and Tyr-185 ERK residues through the upstream activator MEK. The 3-4-fold increase in pERK was blocked by both AM281 and U0126. CB1 stimulation also caused FAK activation, and the 3-5-fold increase in pFAK was blocked by AM281 and GRGDSP. Interestingly, similar levels of MAPK and FAK activation were produced when endocannabinoid signaling was enhanced with inhibitors of endocannabinoid transport and hydrolysis (AM404 and AM374, respectively). These data indicate that the cannabinoid system is involved in neuronal maintenance through linkages to MAPK and FAK compensatory signaling. *Supported by: US Army DAMD17-99-C9090, NIH 1R3NS38404-1, NIH/NIDA DA07312 AND DA09158.*

14. Brown QB, Baude AS, Gilling K, Bahr BA, and Parsons CG, Memantine and Neramexane Protect Against Semi-chronic 3-NP Toxicity in Organotypic Hippocampal Cultures. *Soc. Neurosci. Abstr.* **29**, 153.8 (2003).

MEMANTINE AND NERAMEXANE PROTECT AGAINST SEMI-CHRONIC 3-NP TOXICITY IN ORGANOTYPIC HIPPOCAMPAL CULTURES.

Q.B. Brown, A.S. Baude, K. Gilling, G. Gianutsos, B.A. Bahr, C.G. Parsons

Dept. of Pharmaceutical Sci., Univ. of Connecticut
Precl. R&D, Merz Pharmaceuticals GmbH, Frankfurt, Germany

Memantine recently received registration for the treatment of Alzheimer's disease (AD) in Europe. Clinical data indicate the symptomatic utility of this moderate affinity uncompetitive NMDA receptor antagonist in AD. Unfortunately, proof of the predicted neuroprotective effects of such agents is difficult to obtain in clinical trials. While memantine has been shown to be neuroprotective in several *in vivo* tests modelling prolonged disturbances in glutamatergic transmission, no *in vitro* models have adequately addressed this same question. Such models would be useful to help elucidate further the mechanism of action and functional consequences of such neuroprotection. Here we wished to assess the neuroprotective potencies of memantine and neramexane (MRZ 2/579) against mitochondrial toxin-induced, semi-chronic excitotoxicity in organotypic hippocampal slice cultures over 4-20 days (both at 1, 3 and 10 μM against 35-60 μM 3-nitropropionate (3-NP)). For biochemical quantification, calpain-mediated spectrin breakdown product, postsynaptic marker GluR1 immunoreactivity, and LDH release were measured. The neuroprotective effects of neramexane on 3-NP-induced cytotoxicity (LDH release) were seen in three independent assays with a mean IC_{50} of 1.72 ± 0.02 μM, 2.24 ± 0.03 μM and 2.73 ± 0.19 μM, on days 7, 9 and 12 of exposure, respectively. Under similar conditions, memantine was neuroprotective with respective IC_{50}s of 1.85 ± 0.02 μM, 1.91 ± 0.12 μM and 2.53 ± 0.18 μM. In addition, 3-NP-induced spectrin breakdown and synaptic decline were both significantly reduced by memantine at 1 μM ($p<0.0001$). For the neramexane and memantine effects at day 12-20 of exposure, phase contrast as well as PI fluorescence images and cresyl violet staining confirmed neuroprotection including the maintenance of neuronal morphology and cell density in major hippocampal subfields. These data indicate that both memantine and neramexane are likely to slow the progression of human diseases hypothesised to involve chronic excitotoxic processes such as AD, vascular dementia, Parkinson's and Huntington's disease and ALS.

15. Chicoine LM and Bahr BA, Neuroprotection Through Polysaccharide Modulation of AMPA Receptors, *Soc. Neurosci. Abstr.*, **29**, 895.2 (2003).

Neuroprotection Through Polysaccharide Modulation of AMPA Receptors

L. Chicoine and B.A. Bahr
Dept. Pharm. Sci./Neurosci. Prgm, Univ. Connecticut, Storrs

AMPA-type glutamate receptors are functionally coupled to a variety of signal transduction events, likely explaining the receptors' involvement in synaptic maintenance and neuronal repair in the hippocampus (McKinney et al. 1999, Nature Neurosci 2:44; Bahr et al. 2002, Exp Neurol 174:37). In fact, allosteric regulation of the receptors with Ampakines promotes both pro-survival cell signaling and associated neuroprotection (Bahr et al. 2002). Here, we tested whether positive modulation of AMPA receptors with a large, 500-kDa dextran sulfate (DS-L) is also protective. Using primary hippocampal cultures, an excitotoxin was administered in the absence or presence of DS-L, a smaller 5-kDa species (DS-S), or non-sulfated dextran of 500 kDa. Only DS-L prevented neuronal damage as determined by a membrane permeability assay and phase contrast morphological examination. Thus, the protection was sulfate- and size-dependent as is the case for DS-L modulation of AMPA-mediated Na^+/Ca^{2+} currents and ligand binding. In addition, there was a close correspondence between the DS-L dose requirement for neuroprotection (EC_{50}=12 nM) and AMPA receptor modulation (EC_{50}=5 nM). Concentrations of dextran (0.001-10 μM), DS-S (0.001-10 μM), glucosamine D-2,3 disulfate (100 nM), and sodium sulfate (1 mM) had no protective effects. To test for related survival signaling linked to polysaccharide modulation, 100 nM DS-L was applied to hippocampal slice cultures for 60 min. This resulted in PD98059- and U0126-sensitive MAPK activation measuring pERK2. DS-L activation of the MEK-MAPK pathway was blocked by AMPA receptor antagonists but not with an NMDA receptor antagonist. DS-L was also neuroprotective in the slice model. Interestingly, DS-L modulation was initiated post-insult, reducing both the calpain-mediated spectrin breakdown and synaptic decline evident 24 h after a 20-min NMDA exposure. Together, the results raise the idea that synaptic polysaccharides elicit compensatory/repair responses through AMPA receptors.

16. Bahr BA, Brown QB, and Hubbard AK, Pathogenic vs. Compensatory Activation of the Lysosomal System in the Hippocampal Slice Model of Protein Accumulation and Synaptopathogenesis. *Soc. Neurosci. Abstr.*, **29**, 877.11 (2003).

Pathogenic vs. Compensatory Activation of the Lysosomal System in the Hippocampal Slice Model of Protein Accumulation and Synaptopathogenesis

Bahr BA, Brown QB, and Hubbard AK. Dept. Pharm. Sci./Neurosci. Prgm, Univ. Connecticut, Storrs

In hippocampal slice cultures, chloroquine-mediated lysosomal disturbances contribute to intracellular deposits and microtubule destabilization as part of the induced progressive neurodegeneration (Bendiske et al. 2002, *J Neuropathol Exp Neurol* 61:640). These events implicate lysosomal dysfunction as an age-related risk factor that promotes susceptibility to protein accumulation, transport failure, and synaptic decline. Evidence from the model system suggests that synaptopathogenesis is not irreversible and that concomitant lysosomal activation is a compensatory response (Bendiske & Bahr 2003, *J Neuropathol Exp Neurol* 62:451). Here, it was found that lysosomal activation is a reversible response that affects a number of enzymes, especially the cathepsin family. Enhancing the lysosomal response with a selective modulator (10 uM PADK) caused clearance of protein deposits and re-established microtubule-based transport mechanisms. This led to restored synaptic integrity measuring synaptophysin, GluR1 carboxyterminal domain, and NR1 aminoterminal domain (N1) in slice cultures from rat and guinea pig. Cathepsin activity may have a protective role as indicated by the correlation between cathepsin levels and the degree of synaptic recovery ($r=0.95$). We are also testing for correlated changes in caspase-3 activation, tau deposition, and synaptic functionality. Next, we determined whether PADK modulation is distinct from the lysosomal activation produced by chloroquine and Aβ1-42. Indeed, the chloroquine-induced upregulation of cathepsin D was enhanced by PADK but not by Aβ1-42. The PADK effect was enhanced by Aβ1-42 but not by a control peptide. Thus, chloroquine and Aβ1-42 produce additive effects with PADK but not with each other, raising the idea that PADK causes a distinct, non-pathogenic activation of the lysosomal system. Such lysosomal modulation may be a therapeutic approach for clearing early deposits in protein accumulation diseases.

Support Contributed By: U.S. Army DAMD17-99-C9090, Boehringer Ingelheim fellowship.

17. Caba E, Elliot R, Giardina C, and Bahr BA, Opposing early and delayed gene regulation events in the excitotoxic hippocampus. *Soc. Neurosci. Abstr.*, **29**, 545.10 (2003).

OPPOSING EARLY AND DELAYED GENE REGULATION EVENTS IN THE EXCITOTOXIC HIPPOCAMPUS.

E. Caba[1*]; R.C. Elliott[2]; C. Giardina[3]; B.A. Bahr[1]

[1]Pharm Sci/Neurosci Prgm, Univ Connecticut, Storrs, CT, USA [2]Neurol, Beth Israel-Deaconess, Boston, MA, USA [3]Mol Cell Biol, Univ Connecticut, Storrs, CT, USA

Disparate signaling responses result from a variety of neuro-pathogenic insults. For instance, NF-κB activation post-insult has been implicated in promoting pro- and anti-apoptotic pathways. In examining the distinct signaling events, we discovered that excitotoxic NF-κB response, resulting from biphasic IκBβ degradation, was comprised of early activation of p50/p50 and p65/p50 complexes as well as a delayed phase involving primarily p50/p50. Such NF-κB responses indeed resulted in opposing gene regulation events. Here, we sought to determine whether distinct gene regulation processes are activated in hippocampal slices by a 20 min excitotoxic NMDA exposure. Tissue harvested 1 h post-insult were screened with the Affymetrix Rat Neurobiology U34 Array (n=2 per cond.) revealing a distinct pattern of survival changes from controls. These include the upregulation of transcription factors (Egr-1 and junB) as well as the down-regulation of such genes as ICE. At the same time, several pathogenic gene regulation events were evident, possibly to initiate the injury process. Slices harvested 24 h post-insult showed a much different pattern. While only a few survival events were evident, a small group of pathogenic changes emerged clearly unique from the initial injury responses. The delayed pathogenic changes include a marked down-regulation of Egr-1, BDNF and trkB and the upregulation of ICE and calpain. Therefore, while Egr-1 and ICE may represent early compensatory changes, they also may be determining factors in the delayed injury process. Together, these data indicate that beneficial gene regulation events are activated early to potentially offset initial pathogenesis. However, the main pathogenic phase of excitotoxicity may involve a small group of genes that are altered 10-20 h post-insult. *Support Contributed By: U.S. Army DAMD17-99-C9090, Boehringer Ingelheim fellowship.*

18. Kosten TA, Bahr BA, Karanian DA, Yeh JC, and Kehoe P, Neonatal isolation enhances hippocampal cellular pathogenesis and impairs performance on memory tasks in adult rats. *Soc. Neurosci. Abstr.*, **29**, 833.14 (2003).

Neonatal isolation enhances hippocampal cellular pathogenesis and impairs performance on memory tasks in adult rats.

TA Kosten, BA Bahr, DA Karanian, JC Yeh, P Kehoe*. Yale University School of Medicine, University of Connecticut, Storrs, University of California, Irvine.

Stress impairs performance on memory tasks in adult rats, an effect that reflects, in part, deficits in hippocampal processes. Early life stress, such as maternal separation, alters hippocampal function and, in some cases, impairs memory in adult rats. The aims of this study were to test if the early life stress of neonatal isolation (individual isolation for 1-hr/day on PN 2-9) impairs performance on memory tasks and affects hippocampal cellular pathogenesis in the adult. Litters were assigned to isolate or non-handled conditions and to one of three experiments. In Experiment 1, rats were assessed for spatial memory in an 8-arm radial arm maze (4 arms baited) procedure. Working and reference memory errors decreased over trials, but did not differ by group. However, isolate rats made more errors during initial habituation sessions. In Experiment 2, separate groups of rats were tested for object recognition memory by assessing preference for a novel vs. a pre-exposed (familiar) object at 4-hr or 24-hr post-exposure. Isolate rats showed impairment at the 24-hr test but not at the 4-hr test. In Experiment 3, hippocampus samples were obtained and levels of calpain-mediated spectrin breakdown, a marker of neurodegeneration, and the synaptic marker GluR1 were analyzed by Western blot. Results show significant increased spectrin damage in the hippocampus of isolate rats (+73%, $P<0.01$) and a trend towards synaptic decline compared to non-handled rats. These data show that neonatal isolation has enduring effects resulting in some memory impairments and that these may reflect compromised hippocampal cellular integrity.

19. Vaithianthan T, Bedi D, Kanju PM, Wang Z, Bahr BA, Dityatev A, Judd RL, and Suppiramaniam VD, Evidence of AMPA–glutamate receptor dysfunction in brain of streptozotocin-diabetic rats. *Soc. Neurosci. Abstr.*, (2003).

EVIDENCE OF AMPA - GLUTAMATE RECEPTOR DYSFUNCTION IN BRAIN OF STREPTOZOTOCIN-DIABETIC RATS

T. Vaithianthan[1*]; D. Bedi[2]; P.M. Kanju[1]; Z. Wang[1]; B.A. Bahr[3]; A. Dityatev[4]; R.L. Judd[2]; V.D. Suppiramaniam[1]

1. Dept Pharmacal Sci, Auburn Univ, Auburn, AL, USA 2. Collage of Veterinary Medicine, Auburn Univ, Auburn, AL, USA 3. Dept Pharmaceutical Sciences, Univ. of Connecticut, Storrs, CT, USA 4. Center for Molecular Neurobiology, University of Hamburg, Hamburg, Germany

Cognitive deficits are recognized as a complication in patients with insulin dependent diabetes. Impairments of certain forms of memory have been detected in streptozotocin (STZ)-diabetic rats, an animal model for diabetes mellitus. Learning and memory acquisition depends, in part, on the appropriate expression and function of glutamate receptor subtypes in the brain. To test if the memory deficits are associated with modified function of α- amino-3-hydroxy-methyl-isoxazole (AMPA) subtype of glutamate receptors, we have studied the effects of STZ induced diabetes on the electrophysiological properties of these receptors located at hippocampal postsynaptic sites. A bilayer reconstitution technique along with patch clamp electronics were employed to determine the single channel properties of post synaptic AMPA receptors in hippocampal synaptoneurosomes obtained from control, STZ-diabetes, and insulin treated rats. The STZ-diabetes animals showed a 32% reduction in the single channel conductance of AMPA receptors, as compared to the control animals. The insulin treated group exhibited almost similar conductance as that of control. The probability of channel openings (P0) was reduced from 41% in control to 11% in diabetes animals whereas the P0 for insulin group was 50%. The mean open time of the receptor channel was decreased by 52% in STZ-diabetes animals, compared to the control. The open time of insulin group was unaffected. The electrophysiological alterations in AMPA channel conductance, opening frequency, and mean open time in diabetic animals suggest a possible involvement of AMPA receptor dysfunction in cognitive impairment of STZ-diabetic animals.

20. Wang Z, Clark R, Vaithianathan T, Kanju P, Bahr BA, Dey P, and Suppiramaniam V, Benzamide anticonvulsants modulate the channel properties of AMPA–glutamate receptors. *Soc. Neurosci. Abstr.*, (2003).

BENZAMIDE ANTICONVULSANTS MODULATE THE CHANNEL PROPERTIES OF AMPA-GLUTAMATE RECEPTORS

Z. Wang[1]; R. Clark[1]; T. Vaithianathan[1]; P. Kanju[1]; B. Bahr[2]; P. Dey[1]; V. Suppiramaniam[1*]
1. Pharmacal Science, Auburn University, Auburn, AL, USA 2. Pharmaceutical Sciences, Univ. of Connecticut, Storrs, CT, USA

Dysfunction of glutamatergic neurotransmission has been strongly implicated in the propagation and spread of epileptiform activity. While the anticonvulsant effect of glutamate receptor antagonists have been known for over two decades, poor receptor selectivity and unacceptable toxicity profiles has limited their therapeutic exploitation. A new class of 4-aminobenzamide compounds synthesized in our laboratory exhibits anticonvulsant activity in vivo against electroshock and metrozole induced seizures. While the mechanism of anticonvulsant activity of these compounds is unknown, structure-activity relationship indicates that these compounds may interact with AMPA receptor. The current study tested the hypothesis that the anticonvulsant activity of these benzamide molecules is due to modulation of α-amino-3-hydroxy-4-methyl-isoxazole propionic acid (AMPA) glutamate receptors. Purified AMPA receptors and synaptoneurosomes isolated from rodent hippocampus were incorporated into lipid bilayers to investigate the effects of benzamide compounds AMPA receptor channel properties. Synaptoneurosomal AMPA receptors were activated by 290 nM of AMPA resulting in single channel fluctuations. Infusion of a benzamide (30 μM) anticonvulsant reduced the single channel conductance by 14-22% and the open channel probability by 44- 52%. Purified receptors showed 19-23% % reduction in conductance and 36-48 % decrease in open probability of AMPA activated channels. The data indicate that 4-aminobenzamide anticonvulsants can directly bind and modulate purified and synaptic AMPA receptors.

21. Bahr BA and Caba E, The threat of sub-toxic soman exposures: Enhanced vulnerability in brain tissue. *Internatl. Conf. on Advanced Technologies for Homeland Security* (2003).

Ben A. Bahr and Ebru Caba

Dept. Pharmaceutical Sciences, the Neurosciences Program, and the Center for Drug Discovery, University of Connecticut, Storrs

Hippocampal slice cultures were used to study the action of the military-threat agent soman (GD). Soman, a chemical weapon of mass destruction, produces convulsions and cell loss in the brain, especially in the hippocampus. The organophosphate anticholinesterase causes accumulated levels of ACh, epileptiform bursting, and increased glutamate release that promote seizure-related damage. The excitatory neurotransmitter glutamate has been linked to soman-induced neuropathology. We found that after repeated exposures to sub-toxic levels of soman, hippocampal tissue exhibits enhanced vulnerability to brief episodes of glutamate receptor over-stimulation. After a week of daily GD exposures, the cultured slices exhibited dose-dependent breakdown of spectrin by the pathogenic protease calpain as well as declines in synaptic proteins. Synaptophysin staining was reduced in dendritic fields of CA1 and the dentate gyrus. Low-level GD (150 nM) for 6 days caused little synaptic or cytoskeletal effects. However when the 6-day GD treatment was combined with a 3-min excitotoxic insult (via AMPA), marked synaptic deterioration and spectrin breakdown were produced. The AMPA-insult applied alone had no effect. The enhanced vulnerability does not appear to involve the disruption of cellular repair responses as determined through the potentiation of compensatory signaling with a neuroprotectant. In fact, the neuroprotectant that acts against excitotoxicity through the MAP kinase pathway eliminates soman-induced neuronal vulnerability. Thus, seemingly innocuous soman exposures leave the brain vulnerable to excitotoxic insults implicated in traumatic brain injury and stroke. Preliminary gene array analyses are helping to identify gene regulation events that may underlie the enhanced vulnerability following low-level neurotoxin exposure. (U.S. Army DAMD17-99-C9090, NIH 1R43NS38404-01).

Ben A. Bahr, Ph.D.

Associate Professor of Pharmacology and Neurotoxicology

Department of Pharmaceutical Sciences and the Neurosciences Program
Center for Drug Discovery
University of Connecticut, Storrs, Connecticut 06269-2092
Bahr@uconn.edu tel 860-486-6043 fax 860-486-6858

DEGREES

1984; BA (Molecular Biology) Dept. of Biological Sciences, University of California, Santa Barbara
1984; BA (Biochemistry) Dept. of Biological Sciences, University of California, Santa Barbara
1989; PhD (Chemistry) Dept. of Chemistry, University of California, Santa Barbara

POSITIONS

2001 -	Associate Professor (tenured), Dept. of Pharm. Sciences, University of Connecticut
1998-01	Assistant Professor, Dept. of Pharmaceutical Sciences, University of Connecticut
1995-97	Nontenure-track faculty (Associate), Ctr Neurobiol Learning/Memory, Univ. of Calif.
1991-94	Nontenure-track faculty (Assistant), Ctr Neurobiol Learning/Memory, Univ. of Calif.
1990-94	Lecturer, Dept. Psychobiol. and Ctr Neurobiol Learning/Memory, Univ of Calif, Irvine
1989-91	NRSA Postdoctoral Fellow, Ctr Neurobiol Learning/Memory, Univ. of Calif., Irvine
1985-89	Predoctoral Fellow, Dept. of Chemistry and the Neuroscience Institute, Univ. of Calif.

Consultantships:

2001 -	Consultant/Collaborator, Bristol-Myers Squibb, Wallingford, Connecticut
2001 -	Consultant/Collaborator, Merz Pharmaceuticals, Inc., Frankfurt, Germany
1999 -	Consultant, Cognetix, Inc., Ivorytown, Connecticut
1996 -	Consultant/Collaborator, Cortex Pharmaceuticals, Inc., Irvine, California

DISTINCTIONS

The Professor B.R. Baker Memorial Award in Chemistry, 1987
The American Chemical Society Pacific Division Invited Keynote Address, 1991
Young Investigator Award from the University of California, Irvine, 1995
Faculty Career Development Award from the University of California, Irvine, 1996
Colloquium Organizer and Chair for the American Society for Neurochemistry, 2000
Young Investigator Award from the International Society for Neurochemistry, 2001

RESEARCH INTERESTS

- Links Between Receptor Signaling Events, Synaptic Regulation, and Cellular Repair
- Neuroprotection in Models of Age-Related Diseases, Stroke, and Neurotoxin Exposure
- Pathogenic Cascades Involving Aberrant Protein Processing and Synaptic Failure

PATENTS

1999 Brain Aging Assay (U.S. Patent Application WO1998US0001140)

2000 Positive Lysosomal Modulation and Use for Treating Neurodegeneration (U.S. 20020094958)

PROFESSIONAL AFFILIATIONS

Society for Neuroscience, Member since 1985
American Society for Cell Biology, Member since 1990
American Society for Pharmacology & Experimental Therapeutics, Member since 1995
Society of Toxicology, Member since 1998
American Society for Neurochemistry, Member since 1998
Proteome Society, Member since 2001

PROFESSIONAL ACTIVITIES

1989-93	Organization Committee Member, Human Frontiers Science Program, Univ. of Calif.
1990-91	Member of the Health Sciences Advisory Committee, Univ. of Calif., Irvine
1990-92	Chair of the Hebb Seminars, Ctr. Neurobio. Learning/Memory, Univ. of Calif., Irvine
1990-92	Research Advisor: The Gerard Award Program, Dept. of Psychobiol., Univ. of Calif.
1990-92	Member of the Seminar and Workshop Program Committee, Univ. of Calif., Irvine
1991	NSF/NATO/FEBS International Travel Award, symposium presenter
1992-93	Research Advisor: The Dean's Res. Award Program, College of Med., Univ. of Calif.
1992-95	Organization Committee Member, Irvine Institute for Brain Aging & Dementia
1992-93	Research Advisor: NSF Undergrad. Research Program in Psychobiol., Univ. of Calif.
1992-94	Advisor for President's Undergrad. Res. Fellowship, Sch. of Biol. Sci., Univ. of Calif.
1993	Research Proposal Reviewer, NSF Program of Neuroscience
1993-	Peer Review Consultant, National Institutes of Health / ADAMHA
1993-94	Research Advisor: Instructional Development Fellowship Program, Univ. of Calif.
1995-97	Reviewer for *J Neuroscience, J Neurobiology of Learning and Memory, Hippocampus*
1996-97	Chair, Institutional Animal Care and Use Committee, Cortex Pharmaceuticals

PROFESSIONAL ACTIVITIES (continued)

1998-01	Member of the Pharmacology Faculty Search Committees, University of Connecticut
1998-01	Program Member for Tuskegee-Auburn Universities Neuroscience Conference Series
1998-	Reviewer for *J Neuroscience, J Comparative Neurology, J Neurochemistry, Synapse, Mol Brain Research, Neuroscience*
1999-00	Symposium Program, American Society for Neurochemistry Meeting
1999-01	Secretary of the School of Pharmacy Faculty, University of Connecticut
2000	Organizer for the University of Connecticut School of Pharmacy Hewitt Symposium
2000	Colloquium organizer/chair for American Society for Neurochemistry annual meeting
2000-	Review board member for the *Journal of Molecular Biology and Biotechnology*
2000-	Officer of Animal Care and Safety, School of Pharmacy, University of Connecticut
2001-	Member of the Neurosciences Program Steering Committee, University of Connecticut
2001	Reviewer for the Alzheimer's Association's 2001 Grants Program
2001-	Consultant for the Virginia Commonwealth Neurotrauma Initiative Grant
2001	Newspaper interview: Associated Press (health segment on memory overload).
2001-02	Mentor for UConn Neurosciences Graduate Student Fellow
2001-	Mentor of Boehringer-Ingelheim Graduate Student Fellow
2001	Honorary membership to the Australian Neuroscience Society, presenter
2001-	Consultant, Univ. of Connecticut-Hartford Hospital Huntington's Disease Program
2001-	Pharm/Tox Representative for the Dept. Pharmaceutical Sci. Steering Committee
2001-03	University Undergraduate Research Advisory Committee member, Univ. of Conn.
2001	UConn Advance newspaper interview: New treatment strategy for Alzheimer's disease.
2002	Reviewer of MedChem dissertation prospectus, Center for Drug Discovery, UConn
2002	Reviewer for Research Foundation Internal Awards Program, Univ. of Connecticut
2002	UConn Tradition magazine interview: How to study neurological disorders.
2002	Reviewer for professor DSc promotion, University of London
2002	Television interview: Channel 30 News (self-repair systems in the brain).
2002-	Professional Student Research Advisory Committee member, Univ. of Conn.
2002-	University Strategic Plan Task Force Committee member, University of Connecticut
2002	Reviewer of Physiology-Neurobiology PhD dissertation prospectus, UConn
2002	Reviewer of Molecular and Cell Biology PhD dissertation prospectus, UConn
2003	American Federation Aging Research: National Scientific Advisory Council member
2003	Boehringer-Ingelheim Pharmaceuticals – UConn Relations Committee member

TEACHING

University of California - Irvine, Undergraduate Student Education

Neural Systems - Mechanisms of Brain Function	1990-94
Biology of Behavioral Disorders	1991

University of Connecticut, Pharm.D. Program

Neuropharmacology	1998 -
Biostatistics / Literature Evaluation Skills	1999 -
Neurotoxicology Workshop	1999 -
General principles/organ systems (membrane transport)	2001 -

University of Connecticut, Graduate Student Education

Advanced Pharmacology	1998 -
Special Topics in Pharmacology/Neuroscience	1998 -
Synaptic Plasticity (Dept. of Psychology)	1999
Memory Disorders Discussion Lab	1999 -
Advanced Toxicology (pesticides/excitotoxicity)	2001
Function and Dysfunction of Brian Synapses	2001 -

MENTORING: University of California - Irvine

Undergraduate Researchers:

Peter Vanderklish		1989-92
Mo Tin		1989-91
Lu Ha		1989-91
Henry Ta		1990-92
Barbara Bakus	(Dean's Res. Award Program Fellow)	1990-92
Dino Capaldi		1990-93
Sheri Viteri		1990-93
Nam Lam		1991-93
Babak Abai	(NSF Undergrad. Res. Program Fellow)	1991-94
Steve Shahrestani		1991-93
D. Tyler McQuade	(President's Undergrad. Res. Fellowship)	1991-94
Annette Godshall		1991-94
Everard Esteban	(Gerard Award Fellow)	1991-94
Ramon Rosario		1992-94
Joycelyne Vicente		1993-96
Jason Sharp		1993-95
Keith Hoffman	(Instructional Development Fellowship)	1993-95
Suma Tiriveedhi		1993-96
George Park		1994-96
Manpreet Singh (Mutneja)		1994-96
Brian Kawasaki		1994-97
Tu Pham		1995-96

Co-Advisor for Graduate Students:

Randy Hall	1991-95
Peter Vanderklish	1992-93
Deena Smith	1995-96
Keith Hoffman	1995-98
Eric Bednarski	1995-96

Co-Mentorship of Postdoctoral Fellow:

Dr. Peng Xiao	1991-92

MENTORING: University of Connecticut

Major Advisor:

Kevin Quackenbush	(Pharmacology)	1998
Queenie Brown	(Pharmacology)	1999-00
Jennifer Bendiske	(Ph.D., Pharm/Toxicol) (Neurosci. Fellow)	1997-01
Ebru Caba	(Ph.D., Pharm/Toxicol) (Neurosci. & B.I. Fellow)	1998-03
Linda Chicoine	(Ph.D., Pharm/Toxicol)	1999-03
Sum Lam	(Pharm.D. Honors Thesis)	1999-01
David Karanian	(Ph.D., Pharmacology)	2000-
Sokhom Pin	(Ph.D., Pharmacology)	2001-
Dave Butler	(Ph.D., Pharmacology)	2003-

Member, Thesis Examining Committee:

Mehmet Inan	(Ph.D., Molecular & Cell Biology)	1999-00
Brian Carlson	(Ph.D., Psychology)	1999-01
Sean Christie	(Ph.D., Physiology and Neurobiology)	2002-
Erik Charych	(Ph.D., Physiology and Neurobiology)	2002-
Robert Place	(Ph.D., Molecular & Cell Biology)	2002-
Mark Slein	(M.S., Pharmaceutical Sciences)	2003
Zhongshu Yang	(Ph.D., Physiology and Neurobiology)	2003-

Postdoctoral Mentorship:

Dr. Subramani Munirathinam	1999-02
Dr. Ebru Caba	2003-

Research Scientists:

Queenie Brown	2000-03
Melissa Mendez	2000-02
Regina Vontell	2000-02
Seyda Caskurlu	2003-

Undergraduate Researchers:

Ellen Chung	(Pharmacy)	1999-02
Osama Abdelghany	(Pharmacy)	1999-02
Ben Enos	(Pharmacy)	2001-02
Courtney Doyle	(Pharmacy)	2002-03
Jeannette Yeh	(Pharmacy)	2002-
Kehinde Ajayi	(Dept. of Chemistry) (Undergrad Research Fellow)	2002-
Xiaohang Zhang	(Pharmacy)	2003-
Julie Goetter	(Pharmacy)	2003-
Erin Brennan	(Dept. of Animal Science)	2003-
Emily Luther	(Skidmore College, NY)	2003
Danielle Boschetto	(Pharmacy) (grad student assistant)	2003-

Visiting Scientists:

Dr. Alison Beckmann	Yale University	1998
Dr. Yasuji Matsuoka	New York University	2000
Dr. Andrea Baude	Merz Pharmaceuticals	2001

INVITED SEMINAR PRESENTATIONS

1989, University of Washington Department of Physiology and Biophysics Seminar, "Copurification of the Vesamicol Receptor and the SV1 Antigen of *Torpedo* Synaptic Vesicles: Is the Acetylcholine Transporter a Proteoglycan?", Seattle, Washington.

1989, University of California Molecular Biology and Biochemistry Section Seminar, "Neurotransmitter Storage Properties in Cholinergic Neurons", Santa Barbara, California.

1990, Academy of Sciences Institute of Neurobiology and Brain Research Seminar, "Transmembrane Linkages Between the Extracellular Matrix and the Cytoskeleton Affecting Long-Term Synaptic Modulation", Magdeburg, East Germany.

1990, Univ. of California Neuroscience Research Seminar, "Identification of Synaptosomal Adhesion Components and their Role in Neuronal Plasticity", Santa Barbara, California.

1990, Univ. of California Medical Center Seminar, "Disassembling and Reassembling Synapses: Points of Contact Between Plasticity and Pathology", City of Orange, Calif.

1991, The American Chemical Society Conference: Keynote Address for the ACS-CACT Joint Chemistry Update Meeting, "In Search for the Molecules of Memory", Anaheim, CA.

1992, Beckman Institute Neuronal-Pattern-Analysis Seminar, "Adhesion Molecules, AMPA Receptors, and Long-Term Potentiation", University of Illinois, Urbana-Champaign, Illinois.

1993, Winter Conference on Neural Plasticity, Fifth Annual Meeting: Symposium on Long-Term Potentiation - Mechanisms of Maintenance, "Involvement of Adhesion Molecules in the Functional Reorganization of Synaptic Contacts", Saint Lucia, West Indies.

1994, Univ. of California Learning and Memory Seminar, "Alternative Approach Toward the Understanding of Brain Aging: A Model that Can Express Cellular Events Prominent in the Aged Human Brain and Alzheimer's Disease in Days Instead of Decades", Irvine, California.

1995, The American Association of Retired Persons (AARP): Keynote Address for Nevada Regional Meeting, "New Strategies, Research Tools, and Memory Drugs Directed at Alzheimer's Disease and Other Related Pathologies", Reno, Nevada.

1996, Spring Hippocampus Research Conference (V), "Distinct Adhesive Chemistries and the Functional Modulation of Synapses: A Possible Connection Between Synaptic Architecture and the Stabilization of Receptor Regulation Systems", Grand Cayman, British West Indies.

1997, University of Oxford Calpain Symposium: New Therapeutics, "Calpain Activation in Long-Term Hippocampal Slices: A Model of Neurotoxicity", Oxford, United Kingdom.

1997, Auburn University Receptor Physiology Seminar: "Regulation of Glutamatergic Activity by Structural Signaling Elements, Extrasynaptic Matrix Components, and Novel Allosteric Modulators", Auburn, Alabama.

1998, University of Connecticut Department of Physiology and Neurobiology Seminar: "Cytoskeletal Reorganization Events Underlying Synaptic Plasticity and Their Potential Link to Neuroprotection", Mansfield-Storrs, Connecticut.

1999, Connecticut Center on Aging Seminar: "An Alternative for Studying Age-Related Neuropathology and Potential Treatment with New Memory Enhancing Drugs", Storrs, CT.

1999, University of Connecticut Department of Psychology: "Development of an Aging Model to Study Processes that Lead to Synaptic Pathology", Mansfield-Storrs, CT.

2000, Pfizer Inc., Central Research Division Invited Speaker: "Alzheimer-Type Pathogenesis and Potential Neuroprotection Avenues: Unique Ideas from a Unique System", Groton, CT.

2000, American Society for Neurochemistry Colloquium: "Multifaceted Properties of AMPA Receptors Include Links to Neuroprotective Signaling Pathways", Chicago, Illinois.

2000, Hewitt Symposium on Neurotherapeutic Research: "Advances in an *In Vitro* Model of Age-Related Neuropathogenesis", Storrs, Connecticut.

2000, Nathan Kline Institute/NYU Medical School Seminar: "Abnormal Protein Processing is Linked to Microtubule Destabilization and Synaptic Decline", Orangeburg, New York.

2001, Workshop on DoD Sponsored Parkinson's Related Research: "A Novel Protective Signaling Pathway Identified in a Model of Neurotoxin Exposure", Potomac, Maryland.

2001, Joint Asian Pacific Society for Neurochemistry/Australian Neuroscience Society, Alzheimers Pathology Symposium: "A Link Between Microtubule and Synaptic Failure in an *In Vitro* Model of Alzheimer-Type Abnormal Protein Processing," Brisbane, Australia.

2001, Joint International Society for Neurochemistry/American Society for Neurochemistry Colloquium: "Therapeutic Strategies Identified with an *In Vitro* Model of Stroke and Alzheimer's Disease", Rio de Janeiro, Brazil.

2001, International Society for Neurochemistry Colloquium: "Dynamic Structural Chemistries Underlying Synaptic Plasticity", Buenos Aires, Argentina.

2001, Pfizer Inc., CNS Program Invited Speaker: "Lypex-Induced Reversal of Alzheimer-Type Pathogenesis", Groton, Connecticut.

2002, International Conference on Alzheimer's Disease: "Activation of the Lysosomal System Reverses Neuropathogenesis in the Slice Model of Protein Deposition", Stockholm, Sweden.

2003, International Brain Research Organization: "Dysfunction and Activation of the Lysosomal System: Implications For and Against Alzheimer's Disease", Czech Republic.

2003, University of Connecticut Health Center Seminar Series: "Selective Compensatory Signaling in Response to Neuropathogenic Conditions", Farmington, Connecticut.

PUBLICATIONS

1. Bahr BA and Parsons SM (1986) Demonstration of a receptor for the acetylcholine storage blocker *l-trans*-2-(4-phenyl-[3,4-^{3}H]piperidino)cyclohexanol. *Proc Natl Acad Sci USA* **83**:2267-2270.

2. Bahr BA and Parsons SM (1986) Acetylcholine transport and drug inhibition kinetics in *Torpedo* synaptic vesicles. *J Neurochem* **46**:1214-1218.

3. Parsons SM, Anderson DC, Bahr BA, and Rogers GA (1986) A new pharmacological tool to study acetylcholine storage in nerve terminals. In Hanin I (ed): *Dynamics of Cholinergic Function*. New York, NY: Plenum Press, pp 1169-1176.

4. Anderson DC, Bahr BA, and Parsons SM (1986) Stoichiometries of acetylcholine uptake, release, and drug inhibition in *Torpedo* synaptic vesicles. *J Neurochem* **46**:1207-1213.

5. Parsons SM, Bahr BA, Gracz LM, Kornreich WD, and Rogers GA (1987) Uptake system for acetylcholine in isolated *Torpedo* synaptic vesicles and its pharmacology. In Dowdall MJ, Hawthorne JN (eds): *Cellular and Molecular Basis of Cholinergic Function*. Chichester, U.K.: E. Horwood Ltd., pp 303-315.

6. Parsons SM, Bahr BA, Gracz LM, Kaufman R, Kornreich WD, Nilsson LM, and Rogers GA (1987) Acetylcholine transport: Fundamental properties and effects of pharmacologic agents. *Ann NY Acad Sci* **493**:220-233.

7. Rogers GA, Nilsson LM, Bahr BA, Kornreich WD, and Parsons SM (1987) The most interesting members of the AH5183 family of drugs. In Dowdall MJ, Hawthorne JN (eds): *Cellular and Molecular Basis of Cholinergic Function*. Chichester, U.K.: E. Horwood Ltd., pp 333-337.

8. Parsons SM, Rogers GA, Anderson DC, Bahr BA, Gracz LM, and Kornreich WK (1987) Inhibition of acetylcholine transport by AH5183. In Tucek S (ed): *Synaptic Transmitters and Receptors*. Prague, Czechoslovakia: J. Wiley and Sons, pp 51-54.

9. Parsons SM, Noremberg K, Rogers GA, Gracz LM, Kornreich WD, Bahr BA, and Kaufman R (1988) Complexity and regulation in the acetylcholine storage system of synaptic vesicles. In Zimmermann H (ed): *NATO ASI Series: Cellular and Molecular Basis of Neuronal Signalling (Synaptic Transmission)*. Berlin, FRG: Springer-Verlag, vol. H21, pp 325-335.

10. Bahr BA (1989) *The Acetylcholine Storage System and Drug Inhibition in Cholinergic Synaptic Vesicles*. Ann Arbor, MI: University Microfilms, Inc., 221 pp.

11. Rogers GA, Parsons SM, Anderson DC, Nilsson LM, Bahr BA, Kornreich WD, Kaufman R, Jacobs RS, and Kirtman B (1989) Synthesis, *in vitro* acetylcholine-storage-blocking activities, and biological properties of derivatives and analogues of *trans*-2-(4-phenylpiperidino)cyclohexanol (vesamicol). *J Med Chem* **32**:1217-1230.

12. Bahr BA and Parsons SM (1989) The synaptic vesicle vesamicol (AH5183) receptor contains a low affinity acetylcholine binding site. In Snoek G (ed): *NATO ASI Series: Receptors, Membrane Transport, and Signal Transduction*. Heidelberg, FRG: Springer-Verlag, vol. H29, pp 233-241.

13. Bahr BA, Sheppard A, and Lynch G (1991) Fibronectin binding by brain synaptosomal membranes may not involve conventional integrins. *NeuroReport* **2**:13-16.

14. Sheppard A, Wu J, Bahr BA, and Lynch G (1991) Compartmentation and glycoprotein substrates of calpain in the developing rat brain. *Synapse* (NY) **9**:231-234.

15. Lynch G, Bahr BA, and Vanderklish PW (1991) Induction and stabilization of long-term potentiation. In Ascher P, Choi D, Christen Y (eds): *Glutamate, Cell Death and Memory*. Heidelberg, FRG: Springer-Verlag, pp 45-60.

16. Bahr BA, Sheppard A, Vanderklish PW, Bakus BL, Capaldi D, and Lynch G (1991) Antibodies to the $\alpha_v\beta_3$ integrin label a protein concentrated in brain synaptosomal membranes. *NeuroReport* **2**:321-324.

17. Bahr BA, Vanderklish PW, Ha LT, Tin M, Kessler M, and Lynch G (1991) Spectrin breakdown products increase with age in telencephalon of mouse brain. *Neurosci Lett* **131**:237-240.

18. Xiao P, Bahr BA, Staubli U, Vanderklish PW, and Lynch G (1991) Evidence that matrix recognition contributes to stabilization but not induction of LTP. *NeuroReport* **2**:461-464.

19. Bahr BA and Lynch G (1992) Purification of an Arg-Gly-Asp selective matrix receptor from brain synaptic plasma membranes. *Biochem J* **281**:137-142.

20. Bahr BA and Parsons SM (1992) Purification of the vesamicol receptor. *Biochemistry* **31**:5763-5769.

21. Bahr BA, Clarkson ED, Rogers GA, Noremberg K, and Parsons SM (1992) A kinetic and allosteric model for the acetylcholine transporter-vesamicol receptor in synaptic vesicles. *Biochemistry* **31**:5752-5762.

22. Bahr BA, Noremberg K, Rogers GA, Hicks BW, and Parsons SM (1992) Linkage of the acetylcholine transporter-vesamicol receptor to proteoglycan in synaptic vesicles. *Biochemistry* **31**:5778-5784.

23. Bahr BA, Godshall AC, Hall RA, and Lynch G (1992) Mouse telencephalon exhibits an age-related decrease in glutamate (AMPA) receptors but no change in nerve terminal markers. *Brain Res* **589**:320-326.

24. Vanderklish P, Neve RL, Bahr BA, Arai A, Hennegriff M, and Lynch G (1992) Suppression of a glutamate receptor subunit impairs long-term potentiation. *Synapse* (NY) **12**:333-337.

25. Bahr BA, Vodyanoy V, Hall RA, Suppiramaniam V, Kessler M, Sumikawa K, and Lynch G (1992) Functional reconstitution of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors from rat brain. *J Neurochem* **59**:1979-1982.

26. Clarkson ED, Bahr BA, and Parsons SM (1993) Classical noncholinergic neurotransmitters and the vesicular transport system for acetylcholine. *J Neurochem* **61**:22-28.

27. Parsons SM, Bahr BA, Rogers GA, Clarkson ED, Noremberg K, and Hicks BW (1993) Acetylcholine transporter-vesamicol receptor pharmacology and structure. *Prog Brain Res* **98**:175-181.

28. Vodyanoy V, Bahr BA, Suppiramaniam V, Hall RA, Baudry M, and Lynch G (1993) Single channel recordings of reconstituted AMPA receptors reveal low and high conductance states. *Neurosci Lett* **150**:80-84.

29. Neve RL, Ivins KJ, Neve KA, Bahr BA, Vanderklish PW, Arai A, and Lynch G (1993) The use of antisense intervention to decipher the role of the neuronal growth-associated protein GAP-43. *Neuroprotocols* **2**:39-49.

30. Bahr BA, Godshall AC, Murray BA, and Lynch G (1993) Age-related changes in neural cell adhesion molecule (NCAM) isoforms in the mouse telencephalon. *Brain Res* **628**:286-292.

31. Hall RA and Bahr BA (1994) AMPA receptor development in rat telencephalon: [^{3}H]AMPA binding and Western blot studies. *J Neurochem* **63**:1658-1665.

32. del Cerro S, Arai A, Kessler M, Bahr BA, Vanderklish P, Rivera S, and Lynch G (1994) Stimulation of NMDA receptors activates calpain in cultured hippocampal slices. *Neurosci Lett* **167**:149-152.

33. Bahr BA, Lam N, and Lynch G (1994) Changes in the concentrations of tau and other structural proteins in the brains of aged mice. *Neurosci Lett* **175**:49-52.

34. Bahr BA, Neve RL, Sharp J, Geller AI, and Lynch G (1994) Rapid and stable gene expression in hippocampal slice cultures from a defective HSV-1 vector. *Mol Brain Res* **26**:277-285.

35. Bahr BA, Abai B, Gall C, Vanderklish PW, Hoffman KB, and Lynch G (1994) Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. *Exp Neurol* **129**:81-94.

36. Bahr BA, Tiriveedhi S, Park GY, and Lynch G (1995) Induction of calpain-mediated spectrin fragments by pathogenic treatments in long-term hippocampal slices. *J Pharmacol Exp Ther* **273**:902-908.

37. Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Singh Mutneja M, Gall C, and Hoffman KB (1995) Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. *Hippocampus* **5**:425-439.

38. Bednarski E, Vanderklish PW, Gall CM, Saido TC, Bahr BA, and Lynch G (1995) Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices. *Brain Res* **694**:147-157.

39. Bahr BA (1995) Long-term hippocampal slices: A model system for investigating synaptic mechanisms and pathologic processes. *J Neuroscience Res* **42**:294-305.

40. Bahr BA, Hoffman KB, Kessler M, Hennegriff M, Park GY, Yamamoto R S, Kawasaki BT, Vanderklish PW, Hall RA, and Lynch G (1996) Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (*GR53*) in brain tissue. *Neuroscience* **74**:707-721.

41. Hall RA, Vodyanoy V, Quan A, Sinnarajah S, Suppiramaniam V, Kessler M, and Bahr BA (1996) Effects of heparin on the properties of solubilized and reconstituted rat brain AMPA receptors. *Neurosci Lett* **217**:179-183.

42. Bahr BA, Staubli U, Xiao P, Chun D, Zhan-Xin J, Esteban ET, and Lynch G (1997) Arg-Gly-Asp-Ser-selective adhesion and the stabilization of long-term potentiation: Pharmacological studies and the characterization of the candidate matrix receptor synaptegrin-1. *J Neuroscience* **17**:1320-1329.

43. Capaldi D, Rosario R, Esteban ET, and Bahr BA (1997) A 27-kDa matrix receptor from rat brain synaptosomes: Selective recognition of the Arg-Gly-Asp-Ser domain and unique resistance to calcium-dependent proteolysis. *Neuroscience Res* **28**:275-279.

44. Kawasaki BT, Hoffman KB, Yamamoto RS, and Bahr BA (1997) Variants of the receptor/channel clustering molecule gephyrin in brain: Distinct distribution patterns, developmental profiles, and proteolytic cleavage by calpain. *J Neuroscience Res* **49**:381-388.

45. Edelstein CL, Ling H, Gengaro PE, Nemenoff RA, Bahr BA, and Schrier RW (1997) Effect of glycine on prelethal and postlethal increases in calpain activity in rat renal proximal tubules. *Kidney Internatl* **52**:1271-1278.

46. Bahr BA, Hoffman KB, Yang AJ, Hess US, Glabe CG, and Lynch G (1998) Amyloid β protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. *J Comp Neurol* **397**:139-147.

47. Bahr BA and Vicente JS (1998) Age-related phosphorylation and fragmentation events influence the distribution profiles of distinct tau isoforms in mouse brain. *J Neuropathol Exp Neurol* **57**:111-121.

48. Hoffman KB, Larson J, Bahr BA, and Lynch G (1998) Activation of NMDA receptors stimulates extracellular proteolysis of cell adhesion molecules in hippocampus. *Brain Res* **811**:152-155.

49. Blomgren K, Hallin U, Andersson A-L, Puka-Sundvall M, Bahr BA, McRae A, Saido TC, Kawashima S, and Hagberg H (1999) Calpastatin is up-regulated in response to hypoxia, and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. *J Biol Chem* **274**:14046-14052.

50. Sabatini DM, Barrow RK, Blackshaw S, Burnett PE, Lai MM, Field ME, Bahr BA, Kirsch J, Betz H, and Snyder SH (1999) Interaction of RAFT1 with gephyrin required for rapamycin-sensitive signaling. *Science* **284**:1161-1164.

51. Sinnarajah S, Suppiramaniam V, Kumar KP, Hall RA, Bahr BA, and Vodyanoy V (1999) Heparin modulates the single channel kinetics of reconstituted AMPA receptors from rat brain. *Synapse* (NY) **31**:203-209.

52. Bahr BA, Yilma S, and Suppiramaniam V (1999) Structural chemistries underlying synaptic signaling and regulation. *J Mol Biol Biotech* **1**:35-48 (http://www.jmbab.com/a5bahr.htm).

53. Bahr BA (2000) Integrin-type signaling has a distinct influence on NMDA-induced cytoskeletal disassembly. *J Neuroscience Res* **59**:827-832.

54. Rohn TT, Ivins KJ, Bahr BA, Cotman CW, and Cribbs DH (2000) A monoclonal antibody to amyloid precursor protein induces neuronal apoptosis. *J Neurochem* **74**:2331-2342.

55. Vanderklish PW and Bahr BA (2000) The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. *Internatl J Exp Pathol* **81**:323-339.

56. Suppiramaniam V, Bahr BA, Sinnarajah S, Owens K, Rogers G, Yilma S, and Vodyanoy V (2001) Member of the Ampakine class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. *Synapse* (NY) **40**:154-158.

57. Hoffman KB, Murray BA, Lynch G, Munirathinam S, and Bahr BA (2001) Delayed and isoform-specific effect of NMDA exposure on neural cell adhesion molecules in hippocampus. *Neuroscience Res* **39**:167-173.

58. Rohn TT, Head E, Su JH, Anderson AJ, Bahr BA, Cotman CW, and Cribbs DH (2001) Correlation between caspase activation and neurofibrillary tangle formation in Alzheimer's disease. *Amer J Pathol* **158**:189-198.

59. Wang X, Karlsson J-O, Zhu C, Bahr BA, Hagberg H, and Blomgren K (2001) Caspase-3 activation after neonatal rat cerebral hypoxia-ischemia. *Biol Neonate* **79**:172-179.

60. Bahr BA (2001) Adhesion chemistries and synaptic regulation. *Neurochemistry News* **1**:78-81.

61. Blomgren K, Zhu C, Wang X, Karlsson J-O, Andersson A-L, Bahr BA, Mallard C, and Hagberg H (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia - A mechanism of "pathological apoptosis"? *J Biol Chem* **276**:10191-10198.

62. Caba E, Brown QB, Kawasaki B, and Bahr BA (2002) Peptidyl α-keto amide inhibitors of calpain block excitotoxic damage without affecting signal transduction events. *J Neuroscience Res* **67**:787-794.

63. Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A, and Rogers G (2002) Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp Neurol* **174**:37-47.

64. Vicente JS, Munirathinam S, and Bahr BA (2002) Calpain-mediated spectrin breakdown identifies vulnerable brain regions at middle age. *J Mol Biol Biotech* **3**:1-9 (http://www.jmbab.com/vol3p1pdf.pdf).

65. Ekdahl CT, Mohapel P, Weber E, Bahr B, Blomgren K, and Lindvall O (2002) Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *Eur J Neurosci* **16**:1463-1471.

66. Munirathinam S, Rogers G, and Bahr BA (2002) Positive modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors elicits neuroprotection after trimethyltin exposure in hippocampus. *Toxicol Appl Pharmacol* **185**:111-118.

67. Bendiske J, Caba E, Brown QB, and Bahr BA (2002) Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. *J Neuropathol Exp Neurol* **61**:640-650.

68. Bahr BA and Bendiske J (2002) The neuropathogenic contributions of lysosomal dysfunction. *J Neurochem* **83**:481-489.

69. Bendiske J and Bahr BA (2003) Lysosomal activation is a compensatory response against protein accumulation and associated synaptopathogenesis – An approach for slowing Alzheimer's disease? *J Neuropathol Exp Neurol* **62**:451–463.

70. Bahr BA (2003) Dysfunction and activation of the lysosomal system: Implications for and against Alzheimer's disease. In Columbus F (ed): *Focus on Alzheimer's Disease*. Hauppauge, New York: Nova Science, pp. 117-148.

71. Ferrand-Drake M, Zhu C, Gidö G, Hansen A, Karlsson J-O, Bahr BA, Zamzami N, Kroemer G, Chan P, Wieloch T, and Blomgren K (2003) Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c, and caspase-3 activation in neurons exposed to transient hypoglycemia. *J Neurochem* **85**:1431-1442.

72. Chicoine LM, Suppiramaniam V, Vaithianathan T, Gianutsos G, and Bahr BA (2003) Sulfate- and size-dependent polysaccharide modulation of AMPA receptor properties. *J Neurosci Res*, in press.

73. Emgard M, Hallin U, Karlsson J, Bahr BA, Brundin P, and Blomgren K (2003) Both apoptosis and necrosis occur early after intracerebral grafting of ventral mesencephalic tissue: A role for protease activation. *J Neurochem* **86**:1223-1232.

74. Ekdahl CT, Zhu C, Bonde S, Bahr BA, Blomgren K, and Lindvall O (2003) Death mechanisms in status epilepticus-generated neurons and effects of additional seizures on their survival. *Neurobiol Disease*, in press.

75. Caba E and Bahr BA (2003) Biphasic activation of NF-κB in the excitotoxic hippocampus. Submitted.

76. Chicoine LM and Bahr BA (2003) Polysaccharide modulation of AMPA receptors results in neuroprotection against excitotoxicity. Submitted.

77. Munirathinam S and Bahr BA (2003) Enhanced vulnerability in hippocampus following repeated exposures to low-level soman. Submitted.

78. Vaithianathan T, Matthias K, Bahr BA, Schachner M, Suppiramaniam V, Dityatev A, and Steinhäuser C (2003) Neural cell adhesion molecule-associated polysialic acid potentiates AMPA receptor currents. Submitted.

79. Bahr BA, Bendiske J, Caba E, Chicoine L, Karanian D, and Suppiramaniam V (2003) Role of adhesion molecules in the adult brain. Involvement in plasticity and neuronal maintenance. Invited review for *J Neuroscience Res*, to be submitted.

80. Brundin P, Karlsson J-O, Hagberg H, Bahr BA, and Blomgren K (2003) Effects of caspase-3 inhibition in an animal model of Parkinson's disease. In preparation.

81. Caba E, Giardina C, and Bahr BA (2003) Biphasic NF-κB activation in response to NMDA exposure denotes two opposing signaling pathways. In preparation.

82. Suppiramaniam, Subramaniam, Hammond, Bahr, Schachner,and Dityatev (2003) Polysialic acid modulates single channel properties of synaptosomal AMPA receptors. In preparation.

83. Baude A, Brown QB, Bahr BA, and Parsons C (2003) Protective effects against semi-chronic 3-NP exposure in hippocampal slices. In preparation.

84. Bendiske J, Michaelis ML, Georg G, and Bahr BA (2003) Disruption of microtuble integrity accounts for protein deposition-induced synaptic decline. In preparation.

ABSTRACTS

1. Bahr BA, Anderson D, Parsons SM, Kinetic mechanism of a new anticholinergic that inhibits acetylcholine transport by synaptic vesicles, *Pacific Slope Biochem Conf*, IX-49 (1984).

2. Parsons SM, Anderson DC, Bahr BA, Quion R, Kirtman B, Nilsson LM, Rogers GA, Optimal structure of AH5183 for anticholinergic function, *Pacific Slope Biochem Conf*, IX-50 (1984).

3. Parsons, S.M., Anderson, D.C., Bahr, B.A., Nilsson, L.M., and Rogers, G.A., A Structure-Activity Study of AH5183, a New Anticholinergic Which Blocks Synaptic Vesicles, *Soc. Neurosci. Abstr.*, **10**, 275 (1984).

4. Parsons, S.M., Anderson, D.C., Bahr, B.A., and Rogers, G.A., A New Type of Intraterminal Receptor at the Cholinergic Synapse, *FASEB Abstr. Fed. Proc.*, **43**, 289 (1984).

5. Parsons, S.M., Bahr, B.A., and Anderson, D.C., Mechanistic Studies of Acetylcholine Storage and Drug Inhibition, *Soc. Neurosci. Abstr.*, **11**, 347 (1985).

6. Parsons SM, Anderson DC, and Bahr BA, Acetylcholine Storage, Toxin and Drug Action in *Torpedo* Synaptic Vesicles, *Tenth Meeting of the Internatl. Soc. Neurochem.*, **38**, 389 (1985).

7. Bahr BA and Parsons SM, Acetylcholine storage inhibition and binding behavior of the anticholinergic AH5183 in synaptic vesicles, *Pacific Slope Biochem Conf*, II-14 (1985).

8. Parsons, S.M., Gracz, L.M., Bahr, B.A., and Kornreich, W.D., The AH5183 Receptor in VP_1 and VP_2 Cholinergic Synaptic Vesicles, *Soc. Neurosci. Abstr.*, **12**, 239 (1986).

9. Bahr, B.A. and Parsons, S.M., The Vesamicol (AH5183) Receptor in VP_1 Cholinergic Synaptic Vesicles - Partial Purification, *Soc. Neurosci. Abstr.*, **13**, 670 (1987).

10. Bahr BA and Parsons SM, An acetylcholine binding site copurifies with the vesamicol (AH5183) receptor from cholinergic synaptic vesicles, *Soc. Neurosci. Abstr.*, **14**, 682 (1988).

11. Bahr, B.A. and Parsons, S.M., The Purified Vesamicol (AH5183) Receptor from Cholinergic Synaptic Vesicles May Contain an Allosteric Acetylcholine Binding Site, *NATO/FEBS Internatl. Meeting:Receptors, Membrane Transport, and Signal Transduction*, P-4 (1988).

12. Parsons SM, Yamagata S, Rogers G, and Bahr BA, Newly Identified Proteins of Cholinergic Synaptic Vesicle, *12th Meeting of the Internatl. Soc. Neurochem.*, **42**, 441 (1989).

13. Bahr, B.A. and Parsons, S.M., Characterization of the Vesamicol (AH5183) Receptor in VP_1 Cholinergic Synaptic Vesicles, *Soc. Neurosci. Abstr.*, **15**, 814 (1989).

14. Parsons SM, Rogers G, Bahr BA, Yamagata S, Kornreich W, Regulation of vesicular storage of acetylcholine, *Internatl. Soc. Neurochem. Presynaptic Function Symposium*, 203 (1989).

15. Bahr BA, Acetylcholine storage system and drug inhibition in synaptic vesicles, *Dissertation Abstr. Internatl.*, Sec. B, University Microfilms Internatl., **vol. 147**, 872-873 (1989).

16. Bahr BA, Staubli U, Lynch G, Transmembrane linkages between the extracellular matrix and the cytoskeleton affecting long-term synaptic modulation, *Eighth Internatl. Meeting on Cellular Mechanisms of Memory Formation and Long-Term Potentiation*, **8**, 12 (1990).

17. Bahr BA, Bakus B, Lynch G, Identification of synaptic antigens from rat brain that are related to Arg-Gly-Asp binding adhesion proteins, *UCI/USC Joint Neurosci. Symposium*, **1** (1990).

18. Lynch, G., Wu, J., Bahr, B.A. and Sheppard, A.M., Aspects of Calpain Biochemistry and Neuronal Membrane Plasticity, *Soc. Neurosci. Abstr.*, **16**, 1012 (1990).

19. Bahr, B.A., Sheppard, A., Vanderklish, P.W., Bakus, B., Capaldi, D.K., Kessler, M., Ha, L.T., Tin, M.T., and Lynch, G., Fibronectin Binding to Brain Synaptosomal Membrane May Involve Novel Matrix Receptors, *Soc. Neurosci. Abstr.*, **16**, 1011 (1990).

20. Bahr BA, Xiao P, Staubli U, Vanderklish P, Bakus B, Capaldi D, Sheppard A, Lynch G, Transmembrane linkages between the extracellular matrix and the cytoskeleton affecting LTP, *Fourth Internatl. Conference on the Neurobiology of Learning and Memory*, **4**, 49 (1990).

21. Lynch G, Bahr BA, and Vanderklish PW, Disassembling and Reassembling Synapses: Some Points of Contact Between Plasticity and Pathology, *Fondation IPSEN pour la Recherche Therapeutique Paris Meeting: Glutamate, Cell Death and Memory*, **1**, 121 (1990).

22. Bahr, B.A. and Lynch, G., Brain Synaptosomal Membranes Contain Novel Matrix Receptors with Conserved Integrin Epitopes, *Mol. Biol. Cell* **111** (Suppl.), 401a (1990).

23. Bahr BA, Staubli U, Ambros-Ingerson J, Larson J, Kessler M, Xiao P, Godshall AC, Hall RA, Lynch G (1991) Receptor changes and LTP: Mechanisms of expression and stabilization and implications for age-related disorders of brain function. *Du Pont Biotech Update* **6**:63.

24. Capaldi, D.K., Bahr, B.A., and Lynch, G., Identification of a Synaptosomal Proteoglycan that has an Arg-Gly-Asp Binding Site for Extracellular Matrix Components, *UCI/USC Joint Neurosci. Symposium*, **2** (1991).

25. Bahr, B.A., and Lynch, G., Purification of the Matrix Recognition Molecule *F55* from Synaptic Membranes, *UCI/USC Joint Neurosci. Symposium*, **2** (1991).

26. Clarkson, E.D., Bahr, B.A., Rogers, G.A., and Parsons, S.M., Kinetic and Allosteric Model for the Acetylcholine Transporter-Vesamicol Receptor: A Very Low Specificity Transporter, *Soc. Neurosci. Abstr.*, **17**, 1550 (1991).

27. Vanderklish P, Kessler M, Hall R, Bahr BA, Sumikawa K, Lynch G, The AMPA/Quisqualate Receptor is a Substrate for Calpain, *Soc. Neurosci. Abstr.*, **17**, 1536 (1991).

28. Ha, L.T., Bahr, B.A., Vanderklish, P.W., Tin, M.T., Murray, B., and Lynch, G., Spectrin Breakdown Product and Changes in N-CAM Increase with Age in Specific Brain Areas, *Soc. Neurosci. Abstr.*, **17**, 53 (1991).

29. Bakus, B.L., Bahr, B.A., Esteban, E.T., Godshall, A.C., Black, J., and Lynch, G., Groups of Structural Components Co-localized in Brain Subcellular Fractions are Differentially Affected by a Calcium Activated Protease, *Soc. Neurosci. Abstr.*, **17**, 1153 (1991).

30. Bahr BA, Xiao P, Lynch G, Evidence that the matrix recognition molecule *F55* is present in synapses and contributes to the stabilization of LTP, *Soc. Neurosci. Abstr.*, **17**, 387 (1991).

31. Capaldi, D.K., Bahr, B.A., Lim, H., Noremberg, K., and Lynch, G., A Proteoglycan-like Heparin Binding Protein of High Molecular Weight Found Concentrated in Synaptosomal Membranes, *Soc. Neurosci. Abstr.*, **17**, 1154 (1991).

32. Hall, R., Bahr, B.A., Kessler, M., Sumikawa, K., and Lynch, G., Partial Purification and Specific Immunoreactivity of the AMPA-type Glutamate Receptor from Rat Brain, *Soc. Neurosci. Abstr.*, **17**, 74 (1991).

33. Xiao P, Bahr BA, Staubli U, Ambros-Ingerson J, Hall RA, Kessler M, Larson J, Capaldi D, Esteban ET, Lynch G, Selective changes of AMPA receptors in the mechanisms of LTP expression and stabilization, *5th Conf on Neurobiol of Learning and Memory*, **5**, 14 (1992).

34. Vodyanoy V, Bahr BA, Suppiramaniam V, Hall R, Hennegriff M, Hoffman K, Baudry M, Lynch G, Ligand-Gated Ion Channel Activity from Rat Brain AMPA Receptors Reconstituted in Lipid Bilayers, *5th Conf on Neurobiol of Learning and Memory*, **5**, 34 (1992).

35. Clarkson, E.D., Bahr, B.A., and Parsons, S.M., Classical Noncholinergic Neurotransmitters and the Vesicular Transport System for Acetylcholine, *Soc. Neurosci. Abstr.*, **18**, 94 (1992).

36. McQuade D, Bahr BA, Kessler M, Esteban E, Bakus B, Hall R, Vanderklish P, Shaw G, Sumikawa K, Lynch G, Antibodies Against the GluR-A AMPA Receptor Subunit Label 98 and 57 kDa Proteins Concentrated in PSDs, *Soc. Neurosci. Abstr.*, **18**, 88 (1992).

37. Hennegriff, M., Bahr, B.A., Hall, R., Guthrie, K.M., Gall, C.M., Yamamoto, R.S., and Lynch, G., Antibodies to the GluR-A, GluR-B/C, GluR-D, and NMDA-R1 Glutamate Receptor Subunits: Western Blot Studies, *Soc. Neurosci. Abstr.*, **18**, 88 (1992).

38. Lam N, Bahr BA, Godshall A, Granger R, Lynch G, Different age-related decreases of ankyrin and spectrin occur in mouse telencephalon, *Soc. Neurosci. Abstr.*, **18**, 1487 (1992).

39. Vodyanoy, V., Bahr, B.A., Suppiramaniam, V., Hall, R.A., Kessler, M., Baudry, M., and Lynch, G., Rat Brain Glutamate (AMPA) Receptors Reconstituted in Lipid Bilayers Exhibit Low and High Conductance States, *Soc. Neurosci. Abstr.*, **18**, 652 (1992).

40. Bahr, B.A., Abai, B., Shahrestani, S., Viteri, S., and Lynch, G., Evidence that Brain Synaptic Membranes Contain a Receptor for the Alzheimer Amyloid βA4 Peptide, *Soc. Neurosci. Abstr.*, **18**, 1438 (1992).

41. Bahr BA and Lynch G, Involvement of Adhesion Molecules in the Functional Reorganization of Synaptic Contacts, *Fifth Winter Conference on Neural Plasticity*, **5**, 20 (1993).

42. Sharp, J., Bahr, B.A., Neve, R.L., Geller, A.I., and Lynch, G., The Use of Organotypic Hippocampal Cultures for the Study of HSV-mediated Gene Transfer, *The Third UCI/USC Joint Neurosci. Symposium*, **3** (1993).

43. del Cerro, S., Arai, A., Kessler, M., Bahr, B., Vanderklish, P., Rivera, S., and Lynch, G., Activation of Calpain by NMDA Receptor Stimulation in Organotypic Hippocampal Slice Culture, *Soc. Neurosci. Abstr.*, **19**, 1349 (1993).

44. Hall RA, Bahr BA, and Lynch G, Developmental Shift Toward Low-Affinity AMPA Binding Occurs During Synaptogenesis in Rat Telencephalon, *Soc. Neurosci. Abstr.*, **19**, 721 (1993).

45. Hoffman KD, Bahr BA, Rivera S, Vanderklish P, Hall RA, Kessler M, Arai A, Gall C, and Lynch G, Stable Maintenance of Glutamate Receptors and Other Synaptic Components in Organotypic Hippocampal Slice Cultures, *Soc. Neurosci. Abstr.*, **19**, 1357 (1993).

46. Bahr BA, Sharp J, Neve R, Geller A, Lynch G, A herpes simplex virus for gene transfer into neurons in organotypic hippocampal slice cultures, *Soc. Neurosci. Abstr.*, **19**, 1690 (1993).

47. Hennegriff, M., Hall, R.A., Bahr, B.A., Neve, R.L., and Lynch, G., Effects of Cyclothiazide on [^{3}H]AMPA Binding to AMPA Receptor Subunits Stably Expressed in HEK 293 Cells, *Soc. Neurosci. Abstr.*, **20**, 484 (1994).

48. Bahr, B.A., Abai, B., Gall, C., Vanderklish, P.W, Hoffman, K.B., Singh, M., and Lynch, G., Induction of β-Amyloid Containing Polypeptides and Concomitant Changes in Synaptic Properties in Rat Hippocampus, *Soc. Neurosci. Abstr.*, **20**, 48 (1994).

49. Bahr BA, Park GY, Singh M, and Lynch G, Induction of Aging and Excitotoxicity Processes in Long-Term Hippocampal Slice Cultures, *Mol. Biol. Cell* **5** (Suppl.), 468a (1994).

50. Bahr, B.A., Xiao, P., Esteban, E.T., and Lynch, G., Adhesion Molecules and Hippocampal Plasticity: Integrin-Type Matrix Recognition Contributes to the Stabilization of LTP, Possibly Involving the RGDS-Selective Matrix Receptor *F55*, *Soc. Neurosci. Abstr.*, **21**, 1323 (1995).

51. Hoffman, K.B., Murray, B.A., and Bahr, B.A., Adhesion Molecules and Hippocampal Plasticity: Evidence that a Selective, Delayed Change in Neural Cell Adhesion Molecule-180 ($NCAM_{180}$) is Associated with LTP Mechanisms, *Soc. Neurosci. Abstr.*, **21**, 1323 (1995).

52. Bednarski, E., Vanderklish, P., Gall, C.M., Saido, T., Bahr, B.A., Shaw, G., and Lynch, G., Antisense Oligonucleotides to Calpain I mRNA Protect Cultured Hippocampal Slices from NMDA-Induced Pathophysiology, *Soc. Neurosci. Abstr.*, **21**, 608 (1995).

53. Suppiramaniam V, Sinnarajah S, Vodyanoy V, Hall R, Rogers G, Bahr BA, Modulation of Channel Open Time in Reconstituted AMPA Receptors, *Soc. Neurosci. Abstr.*, **21**, 81 (1995).

54. Suppiramaniam, V., Sinnarajah, S., Vodyanoy, V., Rogers, G., Lynch, G., and Bahr, B.A., The Benzoylpiperidine Compound BDP-5 Prolongs Single Channel Open Times of AMPA Receptors Reconstituted in Bilayers, *FASEB J (Suppl.) Exp. Biol. 95*, **9**, A373 (1995).

55. Sinnarajah, S., Suppiramaniam, V., Vodyanoy, V., Hall, R.A., and Bahr, B.A., Channel Modulating Effects of Heparin in AMPA Receptors Reconstituted in Bilayers, *FASEB J (Suppl.) Exp. Biol. 95*, **9**, A373 (1995).

56. Bahr BA, Hoffman K, Vanderklish P, Kawasaki B, Yang A, Hess H, Gall C, Glabe C, Lynch G, The Alzheimer's Aβ Peptide Induces CA1-Specific Aβ Immunostaining and Synaptic Decay in Hippocampal Slice Cultures, *Soc. Neurosci. Abstr.*, **22**, 1209 (1996).

57. Kawasaki BT and Bahr BA, Selective Calpain Inhibitors Reduce Residual Spectrin Proteolysis and the Delayed Loss of Pre- and Postsynaptic Markers When Applied After Severe NMDA Exposure in Cultured Hippocampal Slices, *Soc. Neurosci. Abstr.*, **22**, 1907 (1996).

58. Bahr BA and Kawasaki BT, Hippocampal Slice Cultures as an *In Vitro* Model of Toxicity: Sensitive Assessment of Pathogenic Agents and Development of Therapeutics for Neurodegenerative Disorders, *The Pharmacologist* (ASPET), **39**, 48 (1997).

59. Bahr, BA, Vanderklish, P, Bednarski, E, Lynch, G, Calpain Activation in Long-Term Hippocampal Slices: A Model of Neurotoxicity, *First International Symposium on Calpains: Their Role in Pathology and New Therapeutic Opportunities* (1997).

60. Bahr BA, Kawasaki B, Quach C, Rogers G, Attenuation of excitotoxic damage by nanomolar levels of Ampakines in long-term hippocampal slices, *Soc. Neurosci. Abstr.*, **23**, 2306 (1997).

61. Suppiramaniam V, Kawasaki B, Causey Z, Yilma Y, Sinnaraja S, Vodyanoy V, Bahr BA, AMPA receptors exhibit corresponding changes in binding and channel properties induced by nanomolar levels of sulfated polysaccharides, *Soc. Neurosci. Abstr.*, **23**, 1203 (1997).

62. Edelstein CL, Ling H, Gengaro PE, Wangsiripaisan A, Bahr BA, Nemenoff RA, and Schrier RW, Calpain mediated changes in spectrin and NaK-ATPase in rat renal proximal tubules (PT), *J. Amer. Soc. Nephrol.* **8**, 2729 (1997).

63. Bahr BA, Kawasaki BT, and Hoffman KB, Variants of the receptor/channel clustering molecule gephyrin in brain: Distinct distribution patterns are influenced by calpain-mediated cleavage events. *Sixth Conference on the Neurobiology of Learning & Memory*, **6**, 17 (1997).

64. Bahr BA, Glabe C, Lynch G, Hippocampal CA1 neurons are selectively targeted by Aβ1–42 resulting in accumulation of carboxyterminal APP fragments and loss of synaptophysin. *IBC 7th Annual Conf. on Alzheimer's Disease: Breakthroughs from Leaders in the Field* (1998).

65. Suppiramaniam V, Prem Kumar S, Yilma S, Sinnarajah S, Vodyanoy V, Manivannan K, Bahr B, Polysaccharides Enhance the Cooperative Gating Behavior of AMPA Receptor Channels Reconstituted in Lipid Bilayers, *Soc. Neurosci. Abstr.*, **24**, 98 (1998).

66. Bahr BA, Calpain-Mediated Cytoskeletal Disassembly Following Brief NMDA Exposure is Attenuated by an Integrin Antagonist in Hippocampus, *Soc. Neurosci. Abstr.*, **24**, 866 (1998).

67. Blomgren K, Hallin U, Puka-Sundvall M, Bahr BA, Hagberg H, Selective interactions between caspase-3, calpain, and stress protein calpastatin in neonatal rat hypoxia-ischemia, *XIXth Internatl. Conf. on Cerebral Blood Flow and Metabolism, Brain '99;* Denmark (1999).

68. Blomgren K, Hallin U, Puka-Sundvall M, Bahr BA, and Hagberg H, Caspase-Calpain Interaction and Calpastatin as a Stress Protein in Neonatal Rat Hypoxia-Ischimia, *XIXth International Symposium on Cerebral Blood Flow and Metabolism, Brain '99* (1999).

69. Bendiske J, Rogers G, Rudin M, Urwyler S, Sauter A, and Bahr BA, Ampakine Modulators of AMPA-Type Glutamate Receptors Promote Neuronal Recovery after Excitotoxic Injury in Brain Tissue, *Mol. Biol. Cell* **10** (Suppl.), 230 (1999).

70. Suppiramaniam V, Yilma S, Bowens A, Manivannan K, Bahr BA, and Dityatev A, Colominic Acid (polysialic acid) Alters the Channel Properties of AMPA Receptors Reconsitituted in Lipid Bilayers, *Soc. Neurosci. Abstr.*, **25**, 1489 (1999).

71. Blomgren K, Wang X, Hagelberg C, Karlsson J-O, Bahr BA, and Hagberg H, Caspase-3-calpain interaction in neonatal rat hypoxia-ischemia, *Soc. Neurosci. Abstr.*, **25**, 1585 (1999).

72. Bahr BA, Bendiske J, Rudin M, Urwyler S, Sauter A, and Rogers G, Positive Modulation of AMPA Receptors Promotes Cellular Repair Following Excitotoxic Injury to Brain Tissue *In Vitro* and *In Vivo*, *Toxicol. Sci.* **54** (Suppl.), 179 (2000).

73. Bendiske J, Caba E, and Bahr BA, Tau modifications and concomitant transport disturbances following lysosomal dysfunction, *J. Neurochem.* **74** (Suppl.), S30C (2000).

74. Brown QB and Bahr BA, Phosphorylation of the ERK1/ERK2 MAP kinase can be influenced by AMPA-type glutamate receptors and their positive modulation in hippocampus, *J. Neurochem.* **74** (Suppl.), S20D (2000).

75. Bahr BA, Bendiske J, Brown QB, Rogers G, Rudin M, Urwyler S, and Sauter A, Multifaceted properties of AMPA receptors include links to neuroprotective signaling pathways, *J. Neurochem.* **74** (Suppl.), S50C (2000).

76. Caba E, Brown QB, and Bahr BA, Evaluation of mitogen-activated protein kinase and NF-κB in an *in vitro* model of neuroprotection, *J. Neurochem.* **74** (Suppl.), S20C (2000).

77. Bendiske J, Caba E, Lam S, Gianutsos G, Bahr BA, Tau deposition is linked to microtubule destabilization and concomitant synaptic decline, *Soc. Neurosci. Abstr.*, **26**, 1023 (2000).

78. Munirathinam S and Bahr BA, Positive modulation of hippocampal AMPA receptors promotes recovery several hours after trimethyltin-induced excitotoxicity, *Soc. Neurosci. Abstr.*, **26**, 503 (2000).

79. Bahr BA, Bendiske J, Brown QB, Caba E, Rogers G, Rudin M, Urwyler S, and Sauter A, Links to metabotropic signaling allow AMPA receptors to counteract the neurodegeneration resulting from their own ionotropic over-activity, *Soc. Neurosci. Abstr.*, **26**, 503 (2000).

80. Suppiramaniam V, Manivannan K, Subramaniam T, Dityatev A, and Bahr BA, Interaction between AMPA receptor channels: A mechanism for modifying synaptic strength, *Soc. Neurosci. Abstr.*, **26**, 902 (2000).

81. Blomgren K, Wang X-Y, Zhu C-L, Karlsson J-O, Bahr BA, Hagberg H, Activation of caspase-2, -3, -8, -9 in neonatal rat hypoxia-ischemia, *Soc. Neurosci. Abstr.*, **26**, 245 (2000).

82. Bahr BA, Bendiske J, Caba E, A link between microtubule and synaptic failure in a model of AD-type abnormal protein processing, *Proc. Australian Neurosci. Soc.*, **12**, 50 (2001).

83. Bahr BA and Munirathinam S, A Novel Protective Signaling Pathway Identified in a Model of Neurotoxin Exposure, *Dept. of Defense Workshop of the Neurotoxin Exposure Treatment Research Program (NETRP)* (2001).

84. Caba E, Giardina C, and Bahr BA, Evidence of a Biphasic NF-κB Response to an Excitotoxin in Hippocampus, *Toxicol. Sci.* **55** (Suppl.), 1732 (2001).

85. Bahr BA and Munirathinam S, Multiple Avenues of Protection Identified in a Model of Neurotoxin Exposure, *Toxicol. Sci.* **55** (Suppl.), 899 (2001).

86. Chicoine L, Gianutsos G, and Bahr BA, Sulfated glycosaminoglycan enhances the channel properties of calcium-permeable AMPA receptors, *New England Pharmacol Meeting* (2001).

87. Caba E and Bahr BA, Biphasic response of NF-κB to stroke-type pathogenesis in organotypic hippocampal slice cultures, *GRASP Pharmacy Research Conference* (2001).

88. Bahr BA, Structural Chemistries and Synaptic Plasticity, *J Neurochem* **78** (Suppl 1), 117 (2001).

89. Brown QB, Bendiske J, Caba E, Munirathinam S, and Bahr BA, AMPA-type glutamate receptors are linked to endogenous protection mechanisms resulting in reduced excitotoxic risk, *J Neurochem* **78** (Suppl) (2001).

90. Bendiske J and Bahr BA, Characterization and slowing of the protein deposition • transport failure • synaptic decay cascade in hippocampal slice cultures, *Second Neurobiology of Aging Conference: Identifying Accelerators and Brakes of Brain Aging* (2001).

91. Caba E, Giardina C, Rogers G, Bahr BA, Ampakine effects indicate that the biphasic NF-κB response to excitotoxicity denotes two opposing signaling pathways, *Soc. Neurosci. Abstr.*, **27**, 867.2 (2001).

92. Suppiramaniam V, Subramaniam T, Kanju PM, Kleene R, Moss A, Premkumar L, Brown Q, Bahr BA, and Dityatev A, Single channel recordings from mossy fiber synaptosomes: Evidence for the presence of high-affinity synaptic AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 502.10 (2001).

93. Subramaniam T, Kleene R, Manivannan K, Dityatev A, Bahr BA, and Suppiramaniam V, Polysialic acid modulates single channel properties of synaptosomal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 610.5 (2001).

94. Chicoine L, Suppiramaniam V, Gianutsos G, Kawasaki BT, and Bahr BA, Sulfated polysaccharides modulate calcium permeability and binding properties of hippocampal AMPA receptors, *Soc. Neurosci. Abstr.*, **27**, 480.6 (2001).

95. Bahr BA, Bendiske J, Michaelis ML, Georg G, Caba E, and Brown Q, Compensatory responses in the brain yield new treatment strategies for neurodegenerative disorders, *Soc. Neurosci. Abstr.*, **27**, 581.2 (2001).

96. Bendiske J and Bahr BA, Positive lysosomal modulation reverses the PHF-tau accumulation • microtubule destabilization • transport failure • synaptic deterioration cascade, *Soc. Neurosci. Abstr.*, **27**, 425.15 (2001).

97. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, The majority of cell death occurs early after intracerebral grafting of ventral mesencephalic tissue: A role for calpain activation. *Soc. Neurosci. Abstr.*, **27**, 197.4 (2001).

98. Bahr BA, Caba E, Giardina C, Brown QB, and Bendiske J, Biphasic NF-κB activation in brain tissue denotes protective and pathogenic signaling pathways, *Mol. Biol. Cell* **12** (Suppl.), 2233 (2001).

99. Munirathinam S and Bahr BA, Low-level soman exposure leads to synaptic damage in the hippocampus and potentiates neuronal vulnerability. *Toxicol. Sci.* **58** (Suppl.), 1551 (2002).

100. Bahr BA and Bendiske J, Activation of the lysosomal system reverses the neuropathogenic cascade induced in a model of abnormal protein processing/deposition. *8th Internatl.Conf. on Alzheimer's Disease and Related Disorders* (Alzheimer's Association), 1016 (2002).

101. Bahr BA and Munirathinam S, Synaptic vulnerability following low-level soman exposures is reduced through glutamatergic signals. *FASEB J* (Suppl.), **16**, A947 (2002).

102. Bendiske J and Bahr BA, Lysosomal modulation leads to neuroprotection in a model of Alzheimer-type protein deposition and synaptic decline. *The Pharmacologist* (ASPET) (2002).

103. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, The role of caspases, calpains, and cathepsins in the degeneration of newly formed neurons in the dentate gyrus following status epilepticus. *International Stem Cell Meeting (France)* (2002).

104. Volbracht C, Chua BT, Bahr BA, and Li P, Neuronal apoptosis dependent on calpain activation. *Cell Death Meeting, Australian Neuroscience Society - Noosaville* (2002).

105. Bahr BA, The pathogenic and protective contributions of lysosomal dysfunction and modulation, respectively. *Soc. Neurosci. Abstr.*, **28**, 192.15 (2002).

106. Caba E, Giardina C, and Bahr BA, The two phases of excitotoxic NF-κB activation have different effects on pro- and anti-apoptotic gene regulation. *Soc. Neurosci. Abstr.*, **28**, 250.9 (2002).

107. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Evidence that glutamatergic and endocannabinoid signaling utilize different pathways to promote neuronal survival. Soc. *Neurosci. Abstr.*, **28**, 202.16 (2002).

108. Ekdahl CT, Mohapel P, Weber E, Bahr BA, Blomgren K, and Lindvall O, Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus. *Soc. Neurosci. Abstr.*, **28**, 618.6 (2002).

109. Subramaniam T, Leshchynska I, Kanju PM, Bahr BA, Dityatev A, and Suppiramaniam V, AMPA receptor-mediated currents in trans-golgi network (TGN)-derived organelles. Soc. *Neurosci. Abstr.*, **28**, 139.10 (2002).

110. Suppiramaniam VD, Subramaniam T, Kanju PM, Coleman E, Bahr BA, and Wang Z, Modulation of synaptosomal AMPA receptors by a member of the Ampakine family of drugs. *Soc. Neurosci. Abstr.*, **28**, 540.4 (2002).

111. Kanju PM, Subramaniam T, Karanja P, Brown Q, Tyler J, Bahr BA, and Suppiramaniam V, Lysosomal dysfunction leads to altered AMPA channel properties in hippocampal neurons. *Soc. Neurosci. Abstr.*, **28**, 139.11 (2002).

112. Emgard M, Karlsson J, Bahr BA, Brundin P, and Blomgren K, Calpain activation occurs early after intracerebral transplantation. *8th Internatl. Conf. Neural Transplantation Repair* (2002).

113. Bahr BA and Brown QB, Lysosomal activation in guinea pig hippocampal slice cultures: Compensatory signaling against protein accumulation events. *The Pharmacologist* (ASPET) (2003).

114. Bahr BA, Munirathinam S, and Brown QB, Enhanced vulnerability in hippocampus following repeated exposures to low-level soman. *9th Meeting Internatl.Neurotoxicology Assoc.* (2003).

115. Bahr BA, Dysfunction and activation of the lysosomal system: Implications for and against Alzheimer's disease. *Internatl. Brain Research Organization Abstr.* (2003).

116. Karanian DA, Brown QB, Makriyannis A, and Bahr BA, Synaptic Maintenance in Hippocampus Appears to Require Endocannabinoid-Mediated Activation of MAPK and FAK Pathways. Soc. *Neurosci. Abstr.*, **29**, 464.9 (2003).

117. Brown QB, Baude AS, Gilling K, Bahr BA, and Parsons CG, Memantine and Neramexane Protect Against Semi-chronic 3-NP Toxicity in Organotypic Hippocampal Cultures. *Soc. Neurosci. Abstr.* **29**, 153.8 (2003).

118. Chicoine LM and Bahr BA, Neuroprotection Through Polysaccharide Modulation of AMPA Receptors, *Soc. Neurosci. Abstr.*, **29**, 895.2 (2003).

119. Bahr BA, Brown QB, and Hubbard AK, Pathogenic vs. Compensatory Activation of the Lysosomal System in the Hippocampal Slice Model of Protein Accumulation and Synaptopathogenesis. *Soc. Neurosci. Abstr.*, **29**, 877.11 (2003).

120. Caba E, Elliot R, Giardina C, and Bahr BA, Opposing early and delayed gene regulation events in the excitotoxic hippocampus. *Soc. Neurosci. Abstr.*, **29**, 545.10 (2003).

121. Kosten TA, Bahr BA, Karanian DA, Yeh JC, and Kehoe P, Neonatal isolation enhances hippocampal cellular pathogenesis and impairs performance on memory tasks in adult rats. *Soc. Neurosci. Abstr.*, **29**, 833.14 (2003).

122. Vaithianthan T, Bedi D, Kanju PM, Wang Z, Bahr BA, Dityatev A, Judd RL, and Suppiramaniam VD, Evidence of AMPA–glutamate receptor dysfunction in brain of streptozotocin-diabetic rats. *Soc. Neurosci. Abstr.*, **29**, 000 (2003).

123. Bedi D, Kanju PM, Bahr BA, Dityatev A, Judd RL, and Suppiramaniam VD, AMPA–glutamate receptor modulation by benzoamine agents. *Soc. Neurosci. Abstr.*, **29**, 000 (2003).

124. Bahr BA and Caba E, The threat of sub-toxic soman exposures: Enhanced vulnerability in brain tissue. *Internatl. Conf. on Advanced Technologies for Homeland Security* (2003).

August 15, 2003

report for *Science*

Manuscript ref. #35280

Enhanced Vulnerability in Hippocampus Following Repeated Exposures to Low-Level Soman

Subramani Munirathinam and Ben A. Bahr

Department of Pharmaceutical Sciences, Center for Drug Discovery, University of Connecticut, Storrs, Connecticut, USA

Subramani Munirathinam
Dept. of Pharmaceutical Sciences
University of Connecticut
Storrs, CT 06269-2092
Tel 860-697-2655, Fax 860-486-4998
e-mail: muni@neuron.uchc.edu

Ben A. Bahr (corresponding author)
Dept. of Pharmaceutical Sciences
University of Connecticut
Storrs, CT 06269-2092
Tel 860-486-6043, Fax 860-486-6858
e-mail: Bahr@uconn.edu

Abstract

Soman, a chemical weapon of mass destruction, produces convulsions and cell loss in the brain, especially in the hippocampus. Soman initiates cholinergic mechanisms responsible for the development of seizures. However, another excitatory neurotransmitter, glutamate, also has been linked to soman-induced neuropathology. We found that after repeated exposures to sub-toxic levels of soman, hippocampal tissue exhibits enhanced vulnerability to brief episodes of glutamate receptor over-stimulation. Thus, seemingly innocuous soman exposures leave the brain vulnerable to excitotoxic insults implicated in traumatic brain injury and stroke. In addition, a neuroprotectant that acts against excitotoxicity through the MAP kinase pathway eliminates soman-induced neuronal vulnerability.

Acute exposure to soman, a military-threat agent that is three times more potent than sarin, causes seizure activity and long-term damage in the central nervous system (*1-2*). Soman is a serious concern to soldiers as well as civilians during the threat of a chemical attack and the unpredictable movement of the airborne toxin. While an irreversible inhibitor of acetylcholinesterase, the nerve agent also promotes the release of excitatory amino acids such as glutamate that participate in the neurodegeneration (*3-5*). Evidence suggests that septo-hippocampal areas of the glutamatergic system are recruited early after soman exposure and the resultant accumulation of extracellular acetylcholine. Excess glutamatergic stimulation in turn causes distinct damage to brain tissue, and blocking specific glutamate receptors reduces neuropathogenic responses including soman toxicity (*5-9*).

Excitotoxic levels of glutamate may be involved in the dendritic and synaptic damage following acute soman exposure that leads to neuronal dysfunction and memory impairment (*9-12*). Over-stimulation of glutamate receptors indeed causes marked deterioration in the hippocampus (*13, 14*), a brain region involved in information processing and one that exhibits early degeneration following ischemia and related brain

trauma. The synaptic integrity required for stable neuronal connections is particularly vulnerable to damage. Hence, hippocampal circuitries that are important for memory encoding express a high susceptibility to both nerve agents and excitotoxicity.

Events of excitotoxicity in hippocampal neurons enhance the vulnerability to other types of neuropathogenesis (*15, 16*). The organotypic hippocampal slice culture is a sensitive model system that exhibits toxic responses as expected from *in vivo* studies (*13, 16-18*). Using this model, we report that the hippocampus becomes susceptible to injury after sub-toxic soman exposure. Selective deterioration of synapses is linked to the enhanced vulnerability, associated with proteolytic activation and resultant cytoskeletal damage.

Soman (nerve agent code GD) at 20 μM was administered to cultured hippocampal slices with single daily applications to produce transient exposure of the chemical agent before its rapid hydrolysis. After the first day, soman produced little to no effect on the synaptic vesicle component synaptophysin or the AMPA receptor subunit GluR1 (Fig. 1A, left lanes). Also absent was the amino-terminal spectrin breakdown product (BDP_N), a proteolytic marker that identifies toxic conditions and selective neurodegeneration. Repeated applications of soman over 7 days, on the other hand, resulted in reduced levels of the pre- and postsynaptic proteins (Fig. 1A, right lanes; left and middle graphs in Fig. 1B). The dose-dependent decline in synaptophysin was evident throughout the hippocampal subfields, especially in the dendritic zones of the stratum radiatum (Fig. 2A, top panels) and the molecular layer of the dentate gyrus (lower panels).

Besides reduced concentrations of synaptic markers, repeated exposures to 20 μM soman also activated the protease calpain as indicated by spectrin fragmentation (Fig. 1A, right lanes). The cleavage product BDP_N was measured with antibodies directed against calpain's recognition site in the spectrin sequence (*17*). Calpain-mediated spectrin cleavage results from glutamatergic excitotoxicity and is thought to be a

precursor to neuronal death (*13*, *14*). However, spectrin proteolysis caused by the transient soman exposures was not associated with extensive cell loss. Histological examination of soman-treated slice cultures revealed intact, densely packed neurons with no evident pyknotic changes in the major pyramidal fields of CA1 (Fig. 2B, top panels) and CA3 (Fig. 2B, lower panels) and in the hilus (Fig. 2C, upper panels). In the lower panels of Figure 2C, indications of pyknotic nuclei (arrows) were found among the granular neurons of the dentate gyrus, but their sparse distribution is an unlikely explanation of the marked synaptic decline in the molecular layer. Thus, soman toxicity produces synaptic decay before cell death.

Sub-toxic conditions were evident with low-level soman exposures. Slices treated with 0.15 μM soman exhibited no appreciable synaptic decline after 7 days of exposure (Fig. 1B). In addition, while slices exposed repeatedly to 20 μM soman generated pronounced levels of calpain-mediated proteolysis, daily applications of 0.15 μM soman caused only a small increase above background BDP_N levels (Fig. 1B, rightmost graph).

Surprisingly, hippocampal tissue that was treated with the sub-toxic soman concentration exhibited enhanced vulnerability to an excitotoxic insult. Excitotoxicity was induced by over-stimulating AMPA-type glutamate receptors for 3 min with 100 μM AMPA, a selective agonist known to cause neurodegeneration with longer treatment periods in the slice model (*13*, *17*). The excitotoxic insult alone did not cause significant synaptic decline or calpain-mediated spectrin breakdown (Fig. 3A, rightmost lanes), nor did repeated exposures to the low-level soman (leftmost lanes). In contrast, the brief excitotoxic insult caused obvious pathogenic changes in slice cultures that were first subjected to a week of daily exposures to sub-toxic soman (Fig. 3B; see middle lanes of Fig. 3A). Similar findings of enhanced vulnerability resulted from a single application of soman. A 1-h treatment with trimethyltin (TMT), a neurotoxin that causes selective excitotoxic damage in the brain (*18-20*), was found to be more effective at causing synaptic and cytoskeletal deterioration when followed by a single exposure to low-level soman and assessed 24 h later (Fig. 3C). Therefore, we conclude that soman exposures

either exacerbate common mechanisms of excitotoxicity or disrupt cellular repair signals that compensate for the excitotoxic consequences.

To test whether low-level soman exposures disrupt repair signaling, we attempted to offset the enhanced vulnerability by positive modulating endogenous survival signals. This was done with 1-(quinoxalin-6-ylcarbonyl)piperidine, a neuroprotectant that promotes basal glutamatergic responses and their connection to repair systems including the MAP kinase pathway (*13, 21-24*). The compound was introduced into the daily applications of sub-toxic soman, and a week later the hippocampal slices were assessed for excitotoxic vulnerability. As shown in Figure 4A, the repeated soman exposures allowed a normally undamaging AMPA treatment to produce marked synaptic decline and cytoskeletal breakdown (lane 2, compared to control slices in lane 1). The addition of 1-(quinoxalin-6-ylcarbonyl)piperidine completely prevented the AMPA-induced pathogenic responses (Fig. 4A, lane 3, and Fig. 4B). Pre- and postsynaptic markers remained at levels similar to those in control slices, and calpain activation was not evident. These data indicate that soman-induced neuronal vulnerability does not involve disruption of endogenous repair responses.

Our findings have several important implications. Although acute exposure of animals to soman causes the death of neurons, the present data indicate that synapses express the initial damage elicited by repetitive contact with the nerve agent. Proteolytic activation and synaptic decline occurred after the soman exposure, both indicators of neurotoxicity (*13*). Inhibition of proteolytic processes has been proposed as a treatment for exposure to neurotoxins including soman (*25*). Persistent synaptopathogenesis in the hippocampal region may explain the delayed behavioral abnormalities and memory loss in exposed rodents and humans. Interestingly, hippocampal tissue treated with sub-toxic soman levels becomes markedly vulnerable to excitotoxic activation of glutamate receptors. Hence, these findings imply that imperceptible soman contact can leave the brain susceptible to glutamate-based injuries of which there are many. Similarly, tissue compromised by a prior excitotoxic episode is made more sensitive to soman. The

enhanced vulnerability does not appear to involve the disruption of cellular repair responses as determined through the potentiation of compensatory signaling.

The results reported here emphasize the need for early indicators of low-level soman exposure so that subsequent brain injury can be adequately assessed and treatment/prevention measures executed in a timely manner.

REFERENCES

1. G. Lemercier, P. Carpentier, H. Sentenac-Roumanou, P. Morelis, *Acta Neuropathol.* **61**, 123 (1983).
2. T. L. Pazdernik, R. S. Cross, M. Giesler, F. E. Samson, S. R. Nelson, *Neuroscience* **14**, 823 (1985).
3. J. V. Wade, F. E. Samson, S. R. Nelson, T. L. Pazdernik, *J. Neurochem.* **49**, 645 (1987).
4. G. Lallement *et al.*, *Brain Res.* **563**, 234 (1991).
5. G. Lallement *et al.*, *Brain Res.* **618**, 227 (1993).
6. S. Sparenborg, L. H. Brennecke, N. K. Jaax, D. J. Braitman, *Neuropharmacol.* **31**, 357 (1992).
7. D. W. Choi, *Neuron* **1**, 623 (1988).
8. M. J. Sheardown, E. O. Nielsen, A. J. Hansen, P. Jacobsen, T. Honore, *Science* **247**, 571 (1990).
9. D. M. de Groot *et al.*, *J. Appl. Toxicol.* **21**, S57 (2001).
10. P. Carpentier, M. Lambrinidis, G. Blanchet, *Brain Res.* **541**, 293 (1991).
11. P. Filliat *et al.*, *Neurotoxicol.* **20**, 535 (1999).
12. L. Raveh *et al.*, *Neurotoxicol.* **23**, 7 (2002).
13. B. A. Bahr *et al.*, *Exp. Neurol.* **174**, (2002).
14. R. Siman, J. C. Noszek, C. Kegerise, *J. Neurosci.* **9**, 1579 (1989).
15. M. P. Mattson, *Neuron* **4**, 105 (1990).
16. B. A. Bahr *et al.*, *Exp. Neurol.* **129**, 81 (1994).
17. B. A. Bahr *et al.*, *J. Pharmacol. Exp. Ther.* **273**, 902 (1995).

18. S. Munirathinam, G. Rogers, B. A. Bahr, *Toxicol. Appl. Pharmacol.* **185**, 111 (2002).

19. N. Ishida *et al.*, *Neuroscience* **81**, 1183 (1997).

20. R. G. Feldman, R. R. White, I. I. Eriator, *Arch. Neurol.* **50**, 1320 (1993).

21. Y. Wang, D. L. Small, D. B. Stanimirovic, P. Morley, J. P. Durkin, *Nature* **389**, 502 (1997).

22. R. A. McKinney, M. Capogna, R. Durr, B. H. Gahwiler, S. M. Thompson, *Nature Neurosci.* **2**, 44 (1999).

23. T. Hayashi, H. Umemori, M. Mishina, T. Yamamoto, *Nature* **397**, 72 (1999).

24. C. Limatola, M. T. Ciotti, D. Mercanti, A. Santoni, F. Eusebi, *J. Neuroimmunol.* **123**, 9 (2002).

25. F. M. Cowan, C. A. Broomfield, D. E. Lenz, T. M. Shih, *J. Appl. Toxicol.* **21**, 293 (2001).

26. This work was supported by U.S. Army Medical Research grant DAMD17-99-C9090 and NIH grant 1R43NS38404-01.

FIGURE LEGENDS

Fig. 1. Synaptic decline and cytoskeletal breakdown after repeated soman exposures. (A) Hippocampal slice cultures were prepared from postnatal day 12 Sprague-Dawley rats and maintained in culture for 3 weeks (16), after which a single daily treatment of 20 μM soman was applied for the noted number of days. Equal protein aliquots from slice homogenates were used to determine amounts of synaptophysin, AMPA receptor subunit GluR1, and calpain-mediated spectrin breakdown product BDP_N by immunoblotting (13). Blots were reprobed with an antibody to β-actin to indicate the relative amount of protein loaded in each lane. (B) Integrated optical density levels of the immunoreactive bands were determined for slices subjected to repeated exposure to 0, 0.15, or 20 μM soman over 7 days (mean ± SEM; n = 5-9 groups of 6-8 slices each). Post-hoc tests compared to non-treated control: *$p<0.01$, **$p<0.001$.

Fig. 2. Synaptic decline before neuronal loss in soman-treated hippocampus. (A) Hippocampal slice cultures were repeatedly exposed to 20 μM soman (nerve agent code GD) for 7 days, after which they were fixed, sectioned, and stained for synaptophysin (16); non-treated control slices were analyzed in parallel (NT). The immunoreactivity in the hippocampal subfield CA1 (upper panels) and dentate gyrus (lower panels) is shown. (B, C) Similarly treated slice cultures were stained with cresyl violet. Photomicrographs show pyramidal neurons in CA1 and CA3 (B; upper and lower panels, respectively), as well as the dentate gyrus zone and granular neurons from the inner blade (C; upper and lower panels, respectively). Pyknotic nuclei are noted with arrows. h, hilus; IBl, inner blade; ml, molecular layer; OBl, outer blade; sg, stratum granulosum; sp, stratum pyramidale; sr, stratum radiatum. Scale bar: upper panels of A-C, 35, 35, and 150 μm, respectively; lower panels of A-C, 50, 110, and 30 μm, respectively.

Fig. 3. Enhanced vulnerability in hippocampal slices exposed to low-level soman. (A) Slice cultures were treated *i*) with or without 0.15 μM soman (GD) for a week of daily exposures (left lanes), *ii*) with or without a 3-min excitotoxic insult using 100 μM AMPA 24 h before harvesting slices (right lanes), and *iii*) with or without the repeated soman exposures in conjunction with the AMPA insult on day 6 (middle lanes). Slices were harvested on the same day and assessed for synaptophysin (syn) and the spectrin breakdown product BDP_N by immunoblotting (13). (B) Integrated optical density levels were determined for synaptophysin and BDP_N in the different treatment groups (mean ± SEM; n = 7-15 groups of 6-8 slices each). Unpaired, two-tailed t-test: *$p<0.01$. (C) Slices were treated once with or without 0.15 μM soman (left lanes), with or without 100 μM TMT for 1 h (right lanes), and with or without the TMT exposure followed by the single soman application (middle lanes). Immunoblotting was conducted 24 h post-insult. Blots were reprobed with an antibody to β-actin to indicate the relative amount of protein loaded in each lane.

Fig. 4. Active cellular repair signaling against soman-induced neuronal vulnerability. (A) Hippocampal slice cultures were repeatedly exposed to 0.15 μM soman in the absence (lane 1) or presence of the neuroprotectant 1-(quinoxalin-6-ylcarbonyl)piperidine (100 μM; lane 3). Six days later, portions of each slice group were exposed for 3 min to a normally undamaging AMPA insult (see Figure 3). After an additional 24 h, the slices were harvested and samples assessed for synaptophysin (syn), GluR1, and the spectrin breakdown product BDP_N by immunoblotting. In slices subjected to the AMPA insult (lanes 2 and 3), only those without the protectant exhibited synaptic decline and spectrin proteolysis. (B) Integrated optical density levels were determined for synaptophysin and BDP_N in the different treatment groups (mean ± SEM; n = 7-17 groups of 6-8 slices each). Post-hoc tests of the protectant effect: *$p<0.01$, **$p<0.001$.

Figure 1

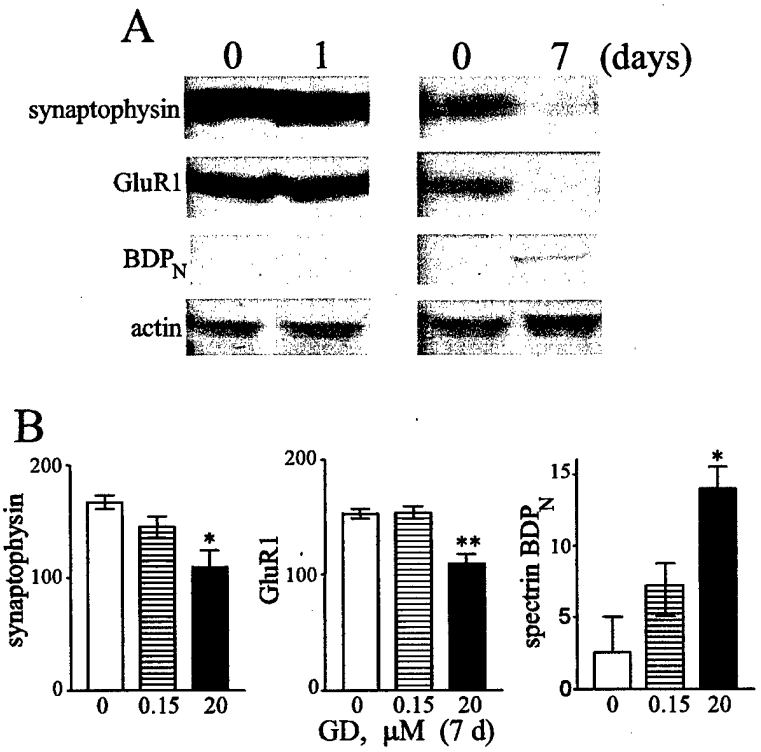

A
0
1
0
7
(days)
synaptophysin
GluR1
BDP$_N$
actin
B
synaptophysin
GluR1
spectrin BDP$_N$
200
100
0
15
10
5
0
0.15
20
*
**
GD, μM (7 d)

Figure 2

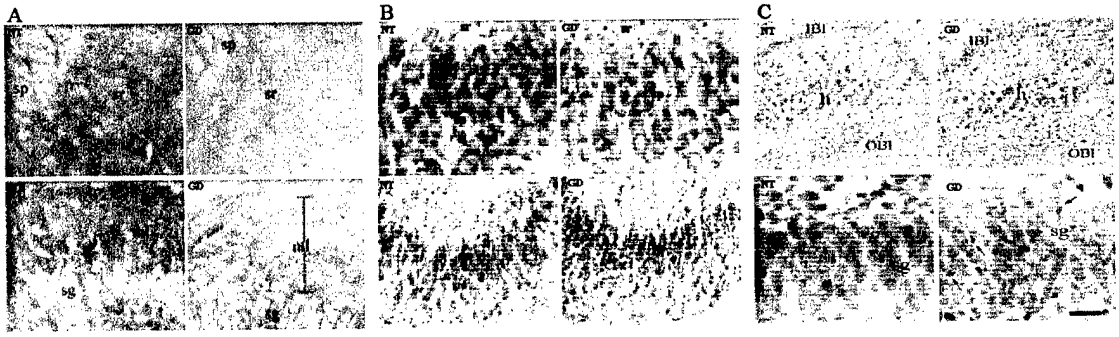


Figure 3

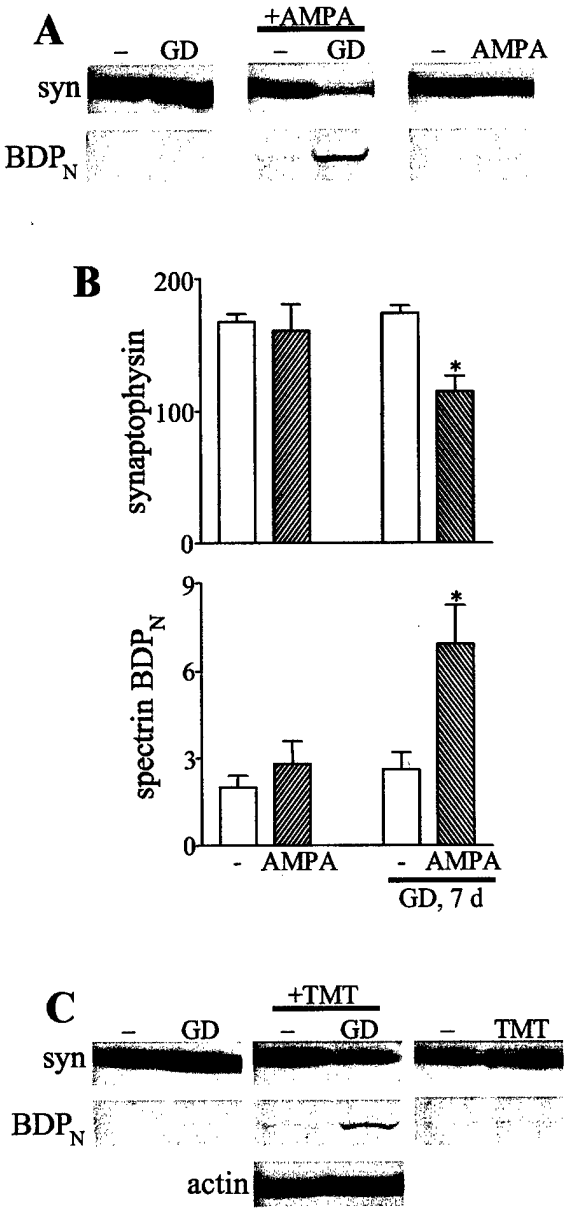
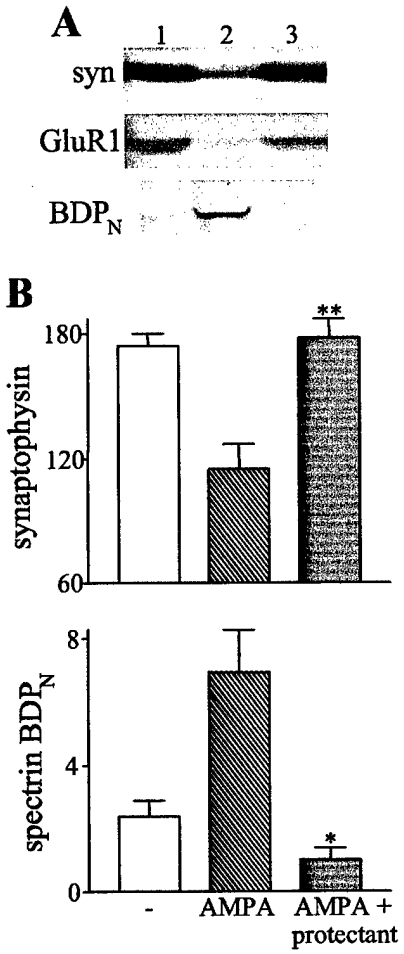

A
+AMPA
– GD
– GD
– AMPA
syn
BDP$_N$
B
synaptophysin
200
100
0
spectrin BDP$_N$
9
6
3
0
*
*
- AMPA
- AMPA
GD, 7 d
C
+TMT
– GD
– GD
– TMT
syn
BDP$_N$
actin

Figure 4


A
1
2
3
syn
GluR1
BDP$_N$
B
synaptophysin
180
120
60
**
spectrin BDP$_N$
8
4
0
*
-
AMPA
AMPA +
protectant

Submitted August 21, 2003

for Journal of Neuropathology and Experimental Neurology

Biphasic NF-κB Activation in the Excitotoxic Hippocampus

Ebru Caba, Ph.D. and Ben A. Bahr, Ph.D.

Department of Pharmaceutical Sciences and the Neurosciences Program

University of Connecticut, Storrs, Connecticut

Corresponding author: Ben A. Bahr, Dept. of Pharmaceutical Sciences

University of Connecticut, Storrs, CT 06269-2092

Tel: (860) 486-6043; fax: (860) 486-6858

e-mail: bahr@uconn.edu

Grant Information: U.S. Army Medical Research grant DAMD17-99-C9090, Boehringer Ingelheim Doctoral Fellowship.

Ben A. Bahr

Abstract

Excitotoxic stimuli that over-activate NMDA receptors result in the activation of various cellular responses. The inducible transcription factor NF-κB is known to be involved in excitotoxic responses by neurons. Here, we show that NF-κB activation occurs in a biphasic manner in hippocampal slices following a 20-min NMDA exposure. The biphasic activation profile of NF-κB consisted of an early (0.5-2 h post-insult) and delayed (10-24 h) phase, and both phases were blocked by the NMDA receptor antagonist AP5. Endogenous inhibitors of NF-κB, IκBs, were examined for their involvement in the biphasic activation. IκBβ exhibited marked degradation in response to the excitotoxity, while changes in the levels of IκBα and p105 isoforms were not detected. The initial decline in IκBβ occurred in as little as 30 min post-NMDA exposure, coinciding with the early NF-κB activation. A second, more gradual phase of IκBβ degradation was also evident, possibly giving rise to the delayed activation of the transcription factor. Interestingly, the two phases of NF-κB activity were associated with distinct gene regulation events. Upregulation of bcl-2 message occurred early after the excitotoxic insult and remained upregulated for an extended period. In contrast, bax message initially remained unchanged after the insult, but then exhibited an increase 24 h later, corresponding with the second phase of the NF-κB response. These results indicate that at least two phases of NF-κB activation are generated in the excitotoxic hippocampus, and that the phases may be involved in opposing cellular responses.

Keywords: bcl-2, bax, Excitotoxicity, Hippocampal Slice Cultures, IκB, NF-κB,

Introduction

Excessive or prolonged activation of NMDA- and AMPA-type glutamate receptors in the brain induces sustained levels of intracellular calcium, causing neuronal impairment and death, particularly in the hippocampus. This phenomenon, referred to as glutamate excitotoxicity, is characterized by damage of cellular components and disruption, or at times activation, of signal transduction events including those involving nuclear factor-κB (NF-κB) (1-5). NF-κB is a ubiquitous, inducible transcription factor that is activated by a wide range of stimuli (6-8). The complexity of this transcription factor is not only a result of the variety of signals that regulate it, but the vast number of genes that are regulated by NF-κB.

NF-κB is being extensively studied because it is a pleiotropic factor that, depending on the stimulus (9, 10), cell type (11, 12), and stage of development (13), can initiate the transcription of many genes. The diversity of NF-κB activation is illustrated by its involvement in promoting apoptosis as well as cell survival. Numerous studies have assigned such opposing roles for NF-κB in the same cell and tissue types (14-18), thus suggesting that the pathway mediated by NF-κB is determined not by the tissue type but by the stimulus. In the brain, however, a controversy exists in the fact that NF-κB activation leads to opposing pathways after the same type of stimulus. For example, several studies implicate NF-κB in apoptosis in response to excitotoxicity and global ischemia (19-23), while others point to the anti-apoptotic role played by NF-κB during the same type of excitotoxic episodes (24-28). The present study approached the dual role issue by testing for distinct events of NF-κB activation that may be involved in pro- and anti-apoptotic pathways in the excitotoxic hippocampus.

The hippocampus is particularly vulnerable to stroke-type excitotoxicity, hence we sought to explore the role of NF-κB in the established cultured slice model of excitotoxicity (4, 29-31). To study the transcription factor, we evaluated the activation profile of NF-κB in slice cultures that were

subjected to a 20-min NMDA exposure. The brief insult resulted in a biphasic activation profile for NF-κB. Interestingly, we show that the biphasic NF-κB response in the excitotoxic hippocampus coincides with early upregulation of an anti-apoptotic gene and delayed upregulation of a pro-apoptotic gene.

Methods and Materials

Organotypic hippocampal slice cultures. Sprague-Dawley rat litters (Charles River Laboratories, Wilmington, MA) were housed in accordance with guidelines from the National Institutes of Health and Institutional Animal Care and Use Committee (IUCAC). Animals 11-12 days postnatal were sacrificed by isoflurane anesthesia and decapitation. The methodology used has been approved by the Animal Care and Use Committee. Transverse slices of hippocampus (400 μm) were then quickly prepared, maintained on Millicell-CM inserts (Millipore Corporation, Bedford, MA), and periodically supplied with fresh media composed of 50% basal medium Eagle, 25% Earle's balanced salts, 25% horse serum, and defined supplements (30, 32). Slices were maintained at 37°C with a 5% CO_2 enriched atmosphere and fed every other day until use. Slices were allowed to mature for 10-15 days in culture before being used in experiments.

Induction of excitotoxicity. Brief excitotoxicity was achieved by incubating the cultured slices with media containing 200 μM NMDA (Tocris, Ellisville, MO) for 20 min. After removal of the excitoxin, the cultures were subsequently washed twice with media containing 40 μM each of CNQX and MK801 (Tocris). The slices then placed in fresh media for the recovery period of 0.5 to 24 h. In separate groups of cultures, the slices were pre-treated with AP5 (Tocris) for 5 min, and AP5 was continually present throughout the treatment paradigm.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extraction were carried out as previously described (4). Briefly, harvested slices were homogenized in ice-cold nuclear extract buffer (20 mM HEPES pH 7.9, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.7% IGEPAL CA630, 10% glycerol, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 0.1 mM phenylmethanesulfonyl fluoride, and 0.5 mM dithiothreitol). Homogenates were centrifuged at 6,500 rpm, then resuspended and re-centrifuged. The pellets were suspended in 35 μl ice-cold high salt buffer and centrifuged so that supernatant could be collected and protein concentrations determined. Equal amounts of protein were subjected to EMSA as outlined previously (4). Nuclear proteins (7.5 μg) were incubated with 2.5 μg

poly-dI•dC (Sigma-Aldrich Corporation, St. Louis, MO) and 0.75 μg BSA in 15 μl total volume for 20 min, then incubated at room temperature for 15 min with ^{32}p-labeled NF-κB consensus oligonucleotide (5'AGT TGA GGG GAC TTT CCC AGG C3'; Promega, Madison, WI). DNA-protein complexes were then resolved by 4% PAGE and visualized by Kodak X-OMAT film autoradiography. Supershift analyses entailed incubating samples with antibodies to p50, p52, and p65 (Santa Cruz Biotechnology, Santa Cruz, CA) for 20 min on ice before electrophoretic separation. NF-κB translocation was determined by integrated optical density using BIOQUANT software (R & M Biometrics, Nashville, TN). Mean integrated densities for antigens in slice samples treated with or without NMDA were compared using unpaired, two-tailed *t* tests or ANOVA.

SDS-PAGE and Western blot analysis. Control and NMDA treated slice cultures were gently removed with a soft brush and homogenized in groups of 6-8 using ice-cold homogenization buffer. Protein content was determined with a BSA standard and equal aliquots of the slice samples were denatured in sampling buffer for 5 min at 100 °C, then separated by 4-16% SDS-PAGE and blotted onto nitrocellulose. Immunodetection was accomplished by incubation overnight at 4°C with polycolonal anti-IκBα, anti-IκBβ, and anti-p105 antibodies (Santa Cruz Biotechnology). Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates, and band intensity was determined using the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system. Color development of immunoreactive bands was terminated before maximal intensity was reached in order to avoid saturation and to ensure a linear relationship with increasing amount of sample protein. Bands were scanned at high resolution and assessed for integrated density with BIOQUANT software (R & M Biometrics). Mean integrated densities for antigens in slice samples treated with or without NMDA were compared using unpaired, two-tailed *t* tests or ANOVA.

Reverse transcriptase polymerase chain reaction (RT-PCR). RNA was isolated from control and excitotoxin exposed hippocampal slices using RNeasy Mini kit according to manufacturer's recommendations (Qiagen, Valencia, CA). cDNA was generated using 2.5 μM random hexamers, 1 X PCR reaction buffer, 2.5 mM $MgCl_2$, 1 mM of each dNTP, 1U/μl MuLV reverse transcriptase

(Applied Biosystems, Branchburg, NJ) in total reaction volume of 20 μl. Using 200 ng cDNA as the template, PCR was carried out in a total volume of 25 μl containing 1 X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM forward and reverse primers (Sigma Genosys, The Woodlands, TX), and 1.25 U/μl Taq DNA polymerase (Eppendorf Scientific, Westbury, NY). Primer sequences were as follows: bcl-2 5' strand CAC CCC TGG CAT CTT CTC CTT, bcl-2 3' strand AGC GTC TTC AGA GAC AGC CAG; bax 5' strand CCA CCA GCT CTG AAC AGA TCA TGA, bax 3' strand TCA GCC CAT CTT CTT CCA GAT GGT. β-actin primers were purchased from Promega (Madison, WI). PCR products were resolved on 2% agarose gels and their images captured and analyzed using the Kodak EDAS120 system (Rochester, NY).

Results

To monitor excitotoxic activation of NF-κB, cultured hippocampal slices were infused with NMDA for 20 min followed by rapid quenching of glutamatergic receptors with selective antagonists. Increased NF-κB binding of the consensus sequence was apparent in the nuclei isolated from NMDA-treated slices (Fig. 1A). The activation was specific for NF-κB since another transcription factor (SP-1) was not influenced by the excitotoxic insult. The activated NF-κB appears to consist primarily of the p50 subunit as determined by supershift analyses (Fig. 1B). Anti-p50 antibodies shifted the NF-κB-DNA complex (arrowhead) to a higher molecular weight (arrow), while anti-p65 antibodies did not. Anti-p52 antibodies also had no effect (not shown). These experiments indicate that the excitotoxic response leads to the nuclear translocation of primarily p50/p50 homodimers.

To determine the activity profile for NF-κB, nuclei were isolated at various times post-NMDA insult. In Figure 2, biphasic activation of NF-κB is evident following the induced excitotoxicity. The EMSA shows an initial increase in activity 1 h post-insult followed by a decline to near basal levels at 5 h, and then a gradual second phase of activation between 10-24 h. Image analyses of the NF-κB activity in non-treated slice groups and slices harvested 0.5-1 h, 5 h, 10 h, and 24 h post-insult found a profile with an early peak at around 1 h and a delayed phase of activity at 24 h (ANOVA: $p<0.0001$; n=4-13 groups of 20 slices each). In a separate experiment, the NMDA receptor antagonist AP5 was present before, during, and after the excitotoxic insult. Previous studies reported that NMDA-induced NF-κB activation is blocked by AP5 (1). Similarly, Figure 3 shows that AP5 disrupts both the early (panel A) and the delayed (panel B) phases of NF-κB activity in the excitotoxic hippocampus.

Next, we tested whether IκB degradation events can account for biphasic NF-κB activation. Specific antibodies were used to monitor IκB family members, namely IκBα, IκBβ and p105, in control and NMDA-treated slices. Somewhat surprisingly, a significant decline in IκBβ (Fig. 4B) coincided with the early activation of NF-κB (Fig. 4C), while no such decline in immunoreactivity

was evident for IκBα (Fig. 4A). Interestingly, IκBβ levels appeared to be restored to or above control levels 1 h post-insult, followed by a second more gradual decline (analysis of variance: $p=0.016$). This decrease was not significant until 10 h post-insult and became more apparent after 24 h (see Fig. 5A and 5B), perhaps giving rise to the NF-κB activation in the delayed phase (Fig. 5C). No change was observed in the IκBα level 10-24 h post-insult (99±3% of control, $n=12$; see representative blot in Fig. 5A). The p105 isoform also exhibited stable levels in the slices harvested 1-24 h post-insult (data not shown).

Lastly, we tested for evidence that the early and delayed excitotoxic responses involving NF-κB lead to distinct gene regulation events. Since NF-κB has been implicated in compensatory repair signaling as well as programmed cell death (see ref. 33 for review), representatives of both anti-apoptotic (bcl-2) and pro-apoptotic (bax) pathways were examined. We chose bcl-2 and bax because these genes are known to be regulated by NF-κB (34). Specific primers were used for RT-PCR analyses of gene expression levels. Early after the excitotoxic NMDA exposure, bcl-2 message was increased and it remained at the upregulated level (Fig. 6, left panel; ANOVA $p=0.002$). Bax expression, on the other hand, did not exhibit early regulation, but did express a delayed increase in message level at 24 h post-insult (Fig. 6, right panel). This leads to the idea that the NF-κB activation profile may result in anti-apoptotic as well as pro-apoptotic chemistries in the excitotoxic hippocampus.

Discussion

The data presented indicate that NMDA-mediated excitotoxicity in hippocampal slice cultures causes activation of NF-κB with distinct phases. The study provides evidence that NF-κB responds to an excitotoxic insult in a biphasic manner to promote opposing pathways. The transcription factor indeed has been shown to have a dual role in promoting both life and death in neurons (5, 35).

NF-κB exhibited an activation profile with two distinct peaks that were both mediated by the excitotoxic over-stimulation of the NMDA receptors. Biphasic activation of the transcription factor has been shown in other model systems (36-39). For instance, Lille et al., (39) reported a comparable activation profile in response to ischemia/reperfusion in rat muscle. Similar to our findings, they described an initial peak between 30 min and 3 h post-perfusion, and a second wave of activation between 6 and 16 h. However, unlike the present study, the level of NF-κB activation diminished by 24 h post-injury. In astrocytes and neuroblastoma cells, biphasic NF-κB activity follows a shorter time course than our results, and falls within the first phase described here (38). Notwithstanding, it is of interest that differential IκB degradation was implicated in the astrocytes and neuroblastoma experiments as well as in other studies illustrating biphasic activation of NF-κB. Thompson et al. (36) have suggested the involvement of two different IκBs that give rise to two phases of NF-κB, a transient phase mediated through IκBα and an IκBβ-mediated persistent phase. Han and Brasier (37) provide evidence that while IκBβ controlled the delayed phase of NF-κB activation, it also contributes to the initial phase of activity. The latter corresponds with the present findings that implicate IκBβ in both phases of the excitotoxic NF-κB response.

Here, biphasic IκBβ degradation was found in the excitotoxic hippocampus, consisting of rapid degradation as early as 30 min post-insult as well as a second much slower degradation. The NF-κB response to the insult does not appear to involve IκBα or p105 since they did not exhibit post-insult declines in immunoreactivity levels. Although unlikely, it may be the case that the

immunoassays used are the present study is not sensitive enough to detect IκBα or p105 degradation in neurons. With that said, the biphasic IκBβ degradation appears to give rise to both phases of NF-κB activation. Other IκB molecules cannot be excluded from playing a role, although they are unlikely candidates since IκBγ expression is only found in mouse B cells (40), IκBδ associates with RelB/p52 complexes (40), and IκBε selectively inhibits RelA and cRel (41).

Perhaps the two degradation events of IκBβ occur through two distinct mechanisms. Indeed, it has been proposed that IκB proteins are degraded by a number of ways, and degradation of IκBα and IκBβ has been suggested to go through different pathways involving the calcium-dependent protease calpain and the ubiquitine/proteosome degradation system (37). Although studies have implicated calpain in the degradation of the inhibitory proteins (42-44), our previous work provides evidence that calpain is not involved in the early response of NF-κB (4). Hence, the ubiquitine/proteosome pathway is a strong candidate for playing a role in the early NF-κB activation. Calpain, on the other hand, may be involved in the delayed phase, a finding that is consistent with Han and Brasier (37) who have shown that calpain is involved in the second phase of the biphasic activation observed in their treatment paradigm. It can't be ruled out that additional pathways are involved. For instance, the lysosomal system has been suggested to degrade both IκBα and IκBβ (45). More interesting with respect to the current study, previous reports have identified an unphosphorylated, non-degradable or slowly degraded form of IκBβ that is involved in the persistent activation of NF-κB (46, 47). This novel form of IκBβ may explain the apparent rapid re-synthesis of IκBβ and its gradual degradation in the delayed phase of NF-κB activation.

Inducible transcription factors such as NF-κB are important in communicating changes in the cellular environment into specific signaling responses. The dual role of NF-κB in cell survival as well as cell death makes this transcription factor unique. NF-κB's signaling is involved in both pro- and anti-apoptotic pathways (e.g., see 18). Interestingly, the present study found that the message level of the gene bcl-2 increased in the excitotoxic hippocampus during the same time period of early NF-κB

activation. This is in agreement with several other studies that point to a role for NF-κB in anti-apoptotic mechanisms (24-28). Moreover, bcl-2 expression was found similarly regulated in excitotoxicity-related models of hypoxia and reperfusion (26, 48). Hippocampal slices exposed to excitotoxic levels of NMDA did not exhibit increased message of the pro-apoptotic gene, bax, until the delayed phase of NF-κB activation. This also is in agreement with many reports linking NF-κB signaling to apoptosis (19-23) and showing that bax is up-regulated in response to glutamate-induced neurotoxicity (19, 49-54). While bcl-2 and bax represent opposing pathways of protection vs. death, respectively, both genes appear to be regulated by the phases of NF-κB activation. Such dual regulation could explain NF-κB's role in both protection and pathogenesis. Together, the findings suggest that biphasic NF-κB activation leads to the regulation of opposing cellular responses.

References

1. Guerrini L, Blasi F, Denis-Donini S. Synaptic activation of NF-κB by glutamate in cerebellar granule neurons in vitro. Proc Natl Acad Sci USA 1995; 92: 9077-9081.

2. Kaltschmidt C, Kaltschmidt B, Baeuerle PA. Stimulation of ionotropic glutamate receptors activates transcription factor NF-κB in primary neurons. Proc Natl Acad Sci USA 1995; 92: 9618-9622.

3. Vanderklish PW, Bahr BA. The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. Int J Exp Pathol 2000; 81: 323-339.

4. Caba E, Brown QB, Kawasaki B, Bahr BA. Peptidyl α-keto amide inhibitor of calpain blocks excitotoxic damage without affecting signal transduction events. J Neurosci Res 2002; 67: 787-794.

5. Mattson MP, Camandola S. NF-κB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 2001; 107: 247-254.

6. Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 2000; 10: 381-391.

7. Grilli M, Memo M. Possible role of NF-κB and p53 in the glutamate-induced pro-apoptotic neuronal pathway. Cell Death Differ 1999; 6: 22-27.

8. Mattson MP, Culmsee C, Yu Z, Camandola S. Roles of nuclear factor κB in neuronal survival

and plasticity. J Neurochem 2000; 74: 443-456.

9. Sonenshein GE. Rel/NF-κB transcription factors and the control of apoptosis. Semin Cancer Biol 1997; 8: 113-119.

10. Mattson MP, Culmsee C, Yu ZF. Apoptotic and antiapoptotic mechanisms in stroke. Cell Tissue Res 2000; 301: 173-187.

11. Baeuerle PA. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim Biophys Acta 1991; 1072: 63-80.

12. Baeuerle PA, Baltimore D. NF-κB: ten years after. Cell 1996; 87: 13-20.

13. Guerrini L, Molteni A, Wirth T, Kistler B, Blasi F. Glutamate-dependent activation of NF-κB during mouse cerebellum development. J Neurosci 1997; 17: 6057-6063.

14. Manna SK, Aggarwal BB. Lipopolysaccharide Inhibits TNF-Induced Apoptosis: Role of Nuclear Factor-κB activation and reactive oxygen intermediates. J Immunol 1999; 162: 1510-1518.

15. Usami I, Kubota M, Bessho R, Kataoka A, Koishi S, Watanabe K, Sawada M, Lin YW, Akiyama Y, Furusho K. Role of protein tyrosine phosphorylation in etoposide-induced apoptosis and NF-κB activation. Biochem Pharmacol 1998; 55: 185-191.

16. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW, Brenner DA. NFκB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998;

101: 802-811.

17. Kuhnel F, Zender L, Paul Y, Tietze MK, Trautwein C, Manns M, Kubicka S. NF-κB mediates apoptosis through transcriptional activation of Fas (CD95) in adenoviral hepatitis. J Biol Chem 2000; 275: 6421-6427.

18. Lin B, Williams-Skipp C, Tao Y, Schleicher MS, Cano LL, Duke RC, Scheinman RI. NF-κB functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type Cell Death Differ 1999; 6: 570-582.

19. Grilli M, Pizzi M, Memo M , Spano P. Neuroprotection by aspirin and sodium salicylate through blockade of NF-κB activation. Science 1996; 274: 1383-1385.

20. Clemens JA, Stephenson DT, Dixon EP, Smalstig EB, Mincy RE, Rash KS, Little SP. Global cerebral ischemia activates nuclear factor-κB prior to evidence of DNA fragmentation. Brain Res Mol Brain Res 1997; 48: 187-196.

21. Qin ZH, Wang Y, Nakai M, Chase TN. Nuclear Factor-κB contributes to excitotoxin-induced apoptosis in rat striatum. Mol Pharmacol 1998; 53: 33-42.

22. Nakai M, Qin ZH, Chen JF, Wang Y, Chase TN. Kainic acid-induced apoptosis in rat striatum is associated with nuclear factor-κB activation. J Neurochem 2000; 74: 647-658.

23. Bachis A, Colangelo AM, Vicini S, Doe PP, De Bernardi MA, Brooker G, Mocchetti I. Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity. J Neurosci 2001; 21: 3104-3112.

24. Goodman Y, Mattson MP. Ceramide protects hippocampal neurons against excitotoxic and oxidative insults, and amyloid β-peptide toxicity. J Neurochem 1996; 66: 869-872.

25. Mattson MP. Neuroprotective signal transduction: relevance to stroke. Neurosci Biobehav Rev 1997; 21: 193-206.

26. Tamatani M, Mitsuda N, Matsuzaki H, Okado H, Miyake S, Vitek MP, Yamaguchi A, Tohyama M. A pathway of neuronal apoptosis induced by hypoxia/reoxygenation: roles of nuclear factor-κB and Bcl-2. J Neurochem 2000; 75: 683-693.

27. Lipsky RH, Xu K, Zhu D, Kelly C, Terhakopian A, Novelli A, Marini AM. Nuclear factor κB is a critical determinant in N-methyl-D-aspartate receptor-mediated neuroprotection. J Neurochem 2001; 78: 254-264.

28. Rosenberger J, Petrovics G, Buzas B. Oxidative stress induces proorphanin FQ and proenkephalin gene expression in astrocytes through p38- and ERK-MAP kinases and NF-κB. J Neurochem 2001; 79: 35-44.

29. Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Mutneja MS, Gall C, Hoffman KB. Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 1995; 5: 425-439.

30. Bahr BA. Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathologic processes. J Neurosci Res 1995; 42: 294-305.

31. Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A,

Rogers G. Survival signaling and selective neuroprotection through glutamatergic transmission. Exp Neurol 2002; 174: 37-47.

32. Bahr BA, Arai B, Gall CM, Vanderklish PW, Hoffman KB, Lynch G. Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Experimental Neurology 1994a; 129: 81-94.

33. Perkins ND. The Rel/NF-κB family: friend and foe. Trends Biochem Sci 2000; 25: 434-440.

34. Viatour P, Bentires-Alj M, Chariot A, Deregowski V, de Leval L, Merville MP, Bours V. NF-κB2/p100 induces Bcl-2 expression. Leukemia 2003; 17:1349-1356.

35. Lezoualc'h F, Sagara Y, Holsboer F, Behl C. High constitutive NF-κB activity mediates resistance to oxidative stress in neuronal cells. J Neurosci 1998; 18: 3224-3232.

36. Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P, Ghosh S. IκB-β regulates the persistent response in a biphasic activation of NF-κB. Cell 1995; 80: 573-582.

37. Han Y, Brasier AR. Mechanism for biphasic rel A. NF-κB1 nuclear translocation in tumor necrosis factor α-stimulated hepatocytes. J Biol Chem 1997; 272: 9825-9832.

38. Kemler I, Fontana A. Role of IκBα and IκBβ in the biphasic nuclear translocation of NF-κB in TNFα-stimulated astrocytes and in neuroblastoma cells. Glia 1999; 26: 212-220.

39. Lille ST, Lefler SR, Mowlavi A, Suchy H, Boyle EMJ, Farr AL, Su CY, Frank N, Mulligan DC. Inhibition of the initial wave of NF-κB activity in rat muscle reduces ischemia/reperfusion

injury. Muscle Nerve 2001; 24: 534-541.

40. Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996; 14: 649-683.

41. Whiteside ST, Epinat JC, Rice NR, Israel A. IκBε, a novel member of the IκB family, controls RelA and cRel NF-κB activity. EMBO J 1997; 16: 1413-1426.

42. Liu ZQ, Kunimatsu M, Yang JP, Ozaki Y, Sasaki M, Okamoto T. Proteolytic processing of nuclear factor κB by calpain in vitro. FEBS Lett 1996; 385: 109-113.

43. Pianetti S, Arsura M, Romieu-Mourez R, Coffey RJ, Sonenshein GE. Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκB-α that can be inhibited by the tumor suppressor. PTEN. Oncogene 2001; 20: 1287-1299.

44. Shen J, Channavajhala P, Seldin DC, Sonenshein GE. Phosphorylation by the protein kinase CK2 promotes calpain-mediated degradation of IκBα. J Immunol 2001; 167: 4919-4925.

45. Cuervo AM, Hu W, Lim B, Dice JF. IκB is a substrate for a selective pathway of lysosomal proteolysis. Mol Biol Cell 1998; 9: 1995-2010.

46. Suyang H, Phillips R, Douglas I, Ghosh S. Role of unphosphorylated, newly synthesized IκBβ in persistent activation of NF-κB. Mol Cell Biol. 1996; 16: 5444-5449.

47. Weil R, Laurent-Winter C, Israel A. Regulation of IκBβ degradation. Similarities to and differences from IκBα. J Biol Chem 1997; 272: 9942-9949.

48. Poulaki V, Mitsiades CS, Joussen AM, Lappas A, Kirchhof B, Mitsiades N. Constitutive nuclear factor-κB activity is crucial for human retinoblastoma cell viability. Am J Pathol 2002; 161: 2229-2240.

49. McInnis J, Wang C, Anastasio N, Hultman M, Ye Y, Salvemini D, Johnson KM. The role of superoxide and nuclear factor-κB signaling in N-methyl- D-aspartate-induced necrosis and apoptosis. J Pharmacol Exp Ther 2002; 301: 478-487.

50. Furukawa K, Mattson MP. The transcription factor NF-κB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainate-induced currents induced by tumor necrosis factor-α in hippocampal neurons. J Neurochem 1998; 70: 1876-1886.

51. Ko HW, Park KY, Kim H, Han PL, Kim YU, Gwag BJ, Choi EJ. Ca2+-mediated activation of c-Jun N-terminal kinase and nuclear factor κB by NMDA in cortical cell cultures. J Neurochem 1998; 71: 1390-1395.

52. Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS. Bax involvement in p53-mediated neuronal cell death. J Neurosci 1998; 18: 1363-1373.

53. Qin ZH, Chen RW, Wang Y, Nakai M, Chuang DM, Chase TN. Nuclear factor κB nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. J Neurosci 1999; 19: 4023-4033.

54. Djebaili M, Rondouin G, Baille V, Bockaert J. p53 and Bax implication in NMDA induced-apoptosis in mouse hippocampus. Neuroreport 2000; 11: 2973-2976.

Legends

Figure 1. Specific NF-κB activation in the excitotoxic hippocampus. NMDA (200 μM) was infused into hippocampal slice cultures for 20 min, followed by rapid washout and quenching of glutamatergic receptors (see Materials and Methods). Nuclei were isolated 1h post-insult and NF-κB activation was assessed by probe binding. The EMSA shows that NF-κB translocation is increased following the insult, while there was no increase in SP-1 binding in similar samples (A). Additional nuclear extracts from NMDA-treated slices were incubated with different antibodies for supershift analyses (B). The NF-κB–DNA complex (arrowhead) from samples harvested 24 h post-insult exhibited a supershift with anti-p50 antibody (arrow) but not with anti-p65 antibodies.

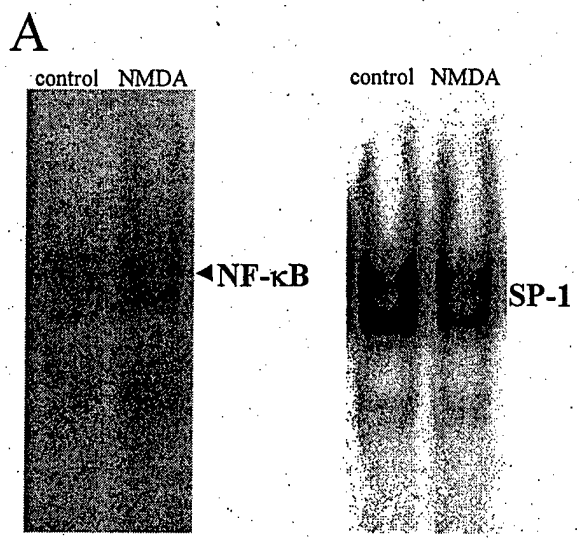
Figure 2. NF-κB activation is biphasic following excitotoxicity in hippocampal slices. The hippocampal slices were exposed to 200 μM NMDA for 20 min, and following quenching with glutamate receptor antagonists, were harvested 1, 5, 10, and 24 h later. EMSA of nuclei preparations shows NF-κB activation in response to the excitotoxic paradigm (arrowhead). Image analyses across 4-13 groups of slices confirmed two peaks; one at 1 h and the other at 24 h post-insult. nt, no treatment.

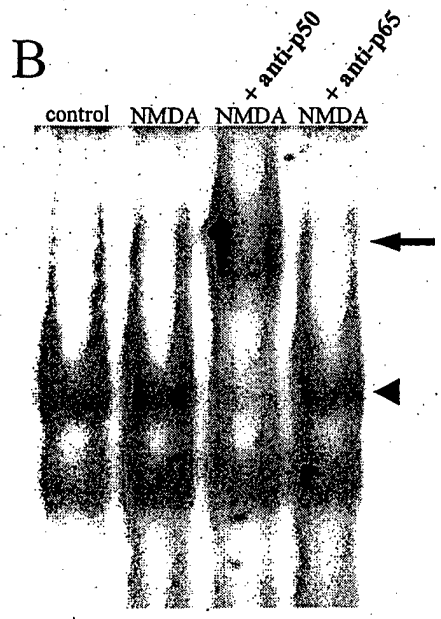
Figure 3. NF-κB activation is mediated through NMDA-type glutamate receptors. Hippocampal slices were treated with NMDA as in Figure 2, in a separate group of slices, AP5 was applied to hippocampal slices before, during, and after the NMDA insult (see Materials and Methods). Nuclear extractions were carried out on slices harvested 0.5-1 h (A) and 24 h (B) post-insult and used for EMSA. AP5 diminished NMDA receptor-mediated NF-κB activation to basal levels in both the early and the delayed phase of the response.

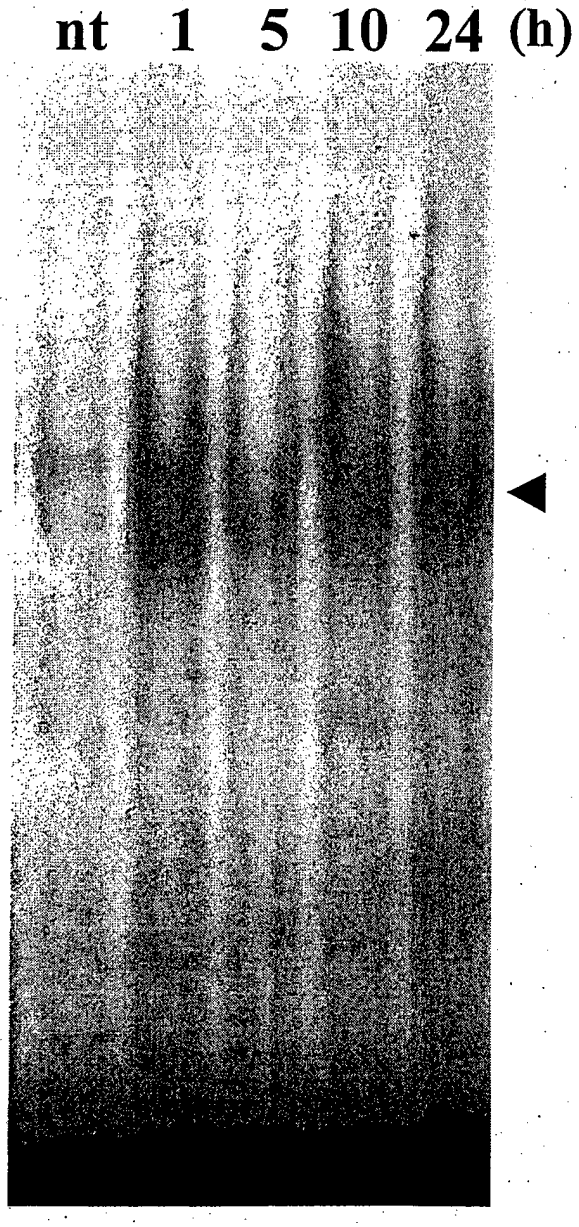
Figure 4. Gradual IκBβ degradation corresponds with early NF-κB translocation in excitotoxic hippocampus. Cultured slices were subjected to the 20-min NMDA insult, and the harvested tissue was analyzed by immunoblot for IκB isoforms (A-B; see representative blots; Lane 1: nt; Lane 2: 0.5 h post-insult) and by EMSA for nuclear NF-κB binding activity in corresponding samples (C). Quantifications of immunoreactivity were determined by image analysis (mean ± SEM; n=3-5). NF-κB activation was measured as the integrated optical density of the NF-κB-DNA complex (see representative EMSA in Fig. 1A; n=3-12). Unpaired, two-tailed t-test compared NMDA-treated slices to control. * $p<0.05$; *** $p<0.0001$.

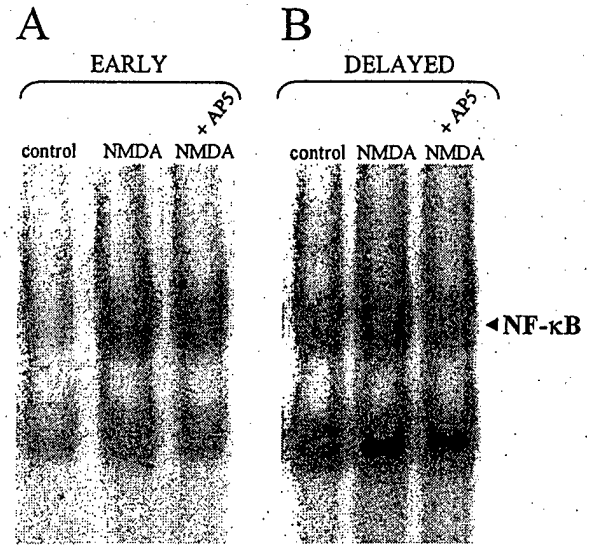
Figure 5. IκBβ degradation corresponds with the delayed activation of NF-κB. Following the NMDA insult, cultured slices were harvested 1, 5, 10, and 24 h later (lanes 1-4 respectively) and analyzed by immunoblot for IκBα and IκBβ. IκBβ levels were determined by image analyses (B) and similar samples were analyzed for NF-κB activity at the indicated post-insult times (C). The gradual decrease in IκBβ (ANOVA: $p=0.016$) corresponds with the increased translocation of NF-κB (ANOVA: $p<0.001$). Tukey's post-hoc test compared to 1 h point. *$p<0.05$.

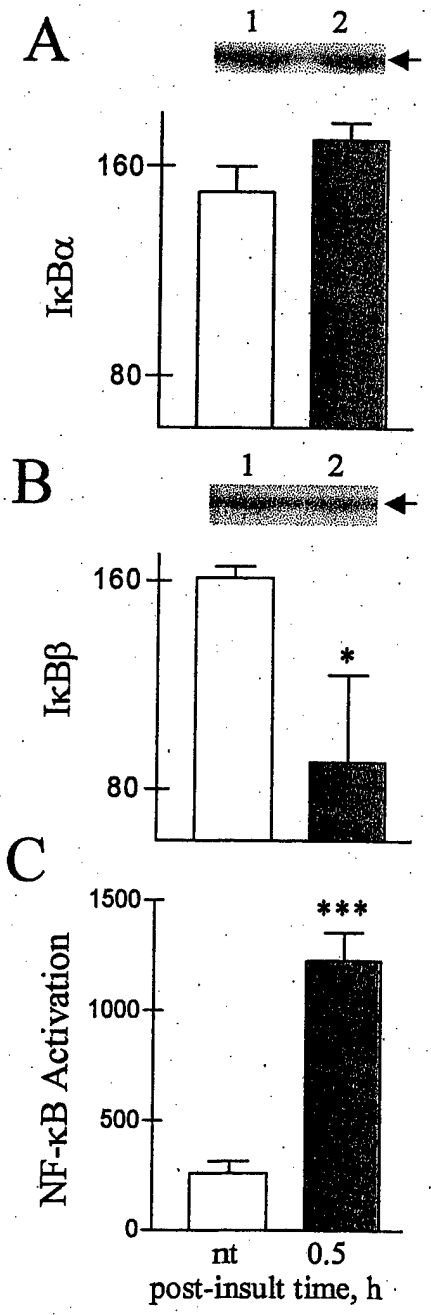
Figure 6. Differential gene regulation is evident in the two phases of NF-κB activation. Non-treated (nt) control and NMDA insulted cultured slices were harvested in groups of 35-40 either in the early or delayed phase of NF-κB. RT-PCR products of bcl-2, bax, and β-actin, amplified from the same RNA preparations, were quantified by image analysis, normalized to respective β-actin message, and plotted as percent change from non-treated control values. The early bcl-2 message upregulation corresponds with the rapid initial NF-κB translocation. The late message increase of bax coincides with the gradual delayed phase activation. Analysis of variance was applied to bcl-2 ($p=0.002$) and bax ($p=0.03$) data. Tukey's post-hoc test compared non-treated control. *$p<0.05$ **$p<0.01$.

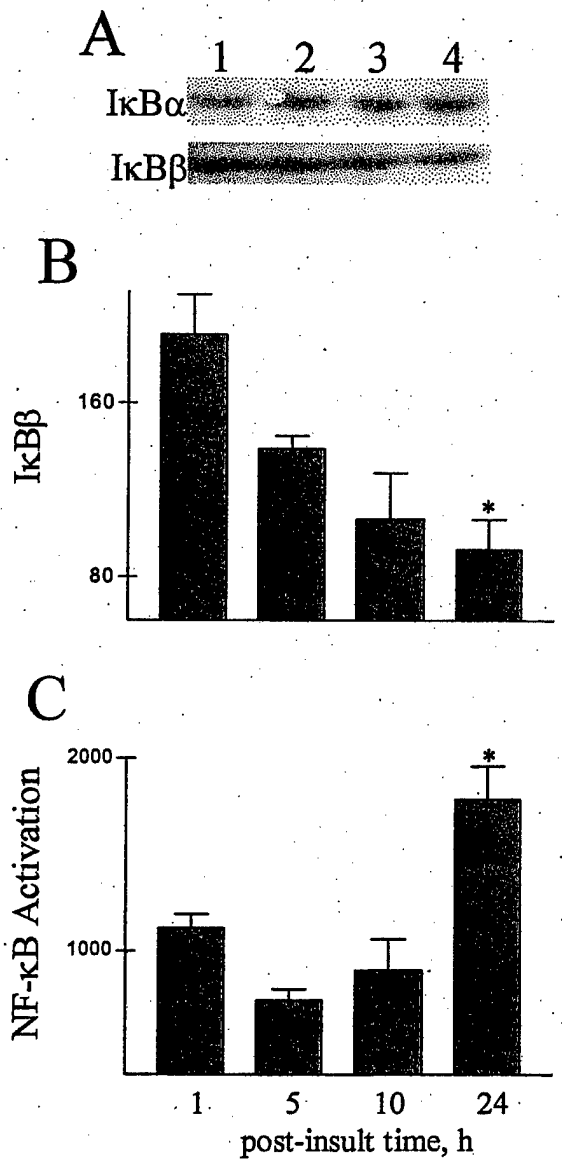

A
control NMDA
NF-κB
control NMDA
SP-1

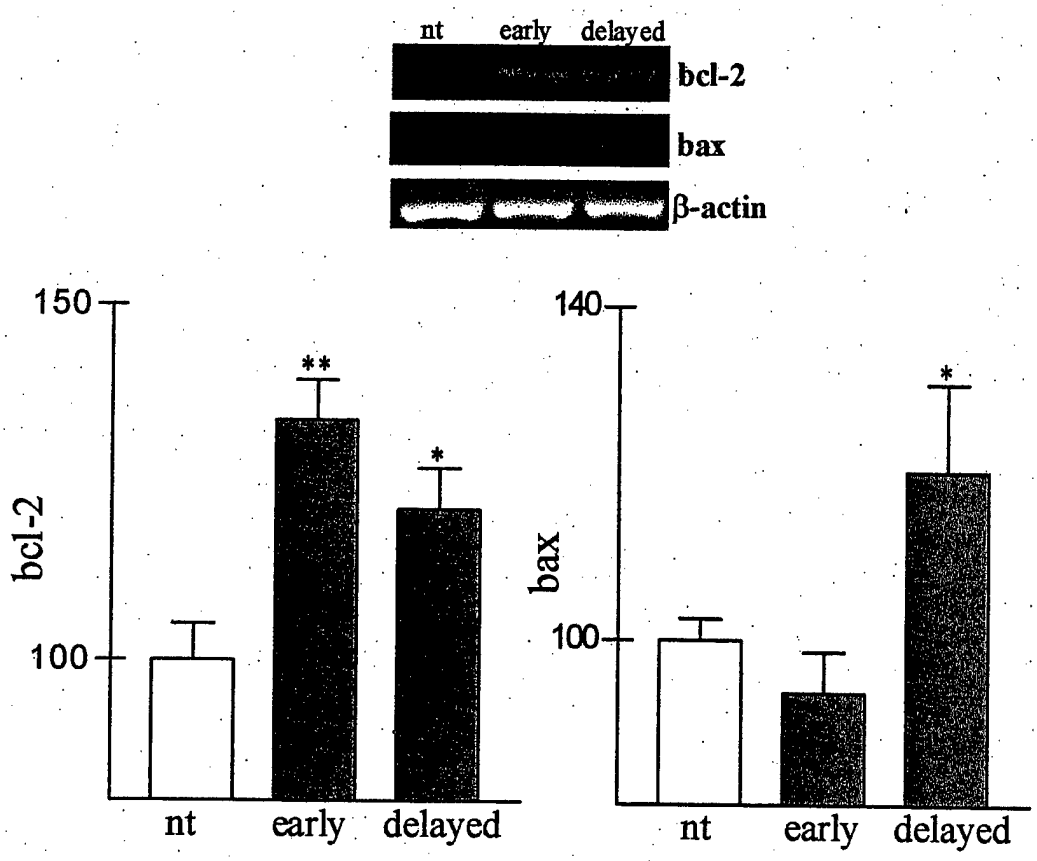

B
+ anti-p50
+ anti-p65
control NMDA NMDA NMDA


nt
1
5
10
24
(h)


A
EARLY
control
NMDA
NMDA
+AP5
B
DELAYED
control
NMDA
NMDA
+AP5
NF-κB


A
1
2
160
80
IκBα
B
1
2
160
80
*
IκBβ
C
1500
1000
500
0
***
NF-κB Activation
nt
0.5
post-insult time, h


A
1
2
3
4
IκBα
IκBβ
B
IκBβ
160
80
*
C
NF-κB Activation
2000
1000
*
1
5
10
24
post-insult time, h


nt
early
delayed
bcl-2
bax
β-actin
150
100
bcl-2
**
*
nt
early
delayed
140
100
bax
*
nt
early
delayed

June 30, 2003 Journal of Neurosci Research

Sulfate- and size-dependent polysaccharide modulation of AMPA receptor properties

Linda M. Chicoine [1,2], Vishnu Suppiramaniam[3], Thirumalini Vaithianathan [3], Gerald Gianutsos [1], and Ben A. Bahr [1,4]

[1] Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, CT 06269

[2] Cognetix, Inc., Salt Lake City, UT 84108

[3]Department of Biology, Tuskegee University, Tuskegee, AL 36088

[4]Center for Drug Discovery, University of Connecticut, Storrs, CT 06269

Correspondence to: Ben A. Bahr

Department of Pharmaceutical Sciences

University of Connecticut, Storrs, CT 06269-2092

Tel: 860-486-6043; Fax: 860-486-6858

E-mail: Bahr@uconn.edu

Supported by NIH grant 1R43NS38404-01 and U.S. Army Medical Research grant DAMD17-99-C9090.

Running title: Polysaccharide modulation of AMPA receptors

ABSTRACT

Previous work found evidence that α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors interact with and are functionally regulated by the glycosaminoglycan heparin. The present study tested whether dextran species affect ligand binding, channel kinetics, and calcium permeability of AMPA receptors. Dextran sulfate of 500 kDa dramatically reduced high affinity [^{3}H]AMPA binding in solubilized hippocampal membranes. In isolated receptors reconstituted in a lipid bilayer, the same dextran sulfate prolonged the lifetime of open states exhibited by AMPA-induced channel fluctuations. The large polysaccharide further changed the single channel kinetics by increasing the open channel probability 5-6 fold. Such modulation of channel activity corresponded with enhanced levels of calcium influx as shown in hippocampal neurons loaded with Fluo3AM dye. With an exposure time of <1 min, AMPA produced a dose-dependent increase in intracellular calcium that was blocked by CNQX. Dextran sulfate, at the same concentration range that modified ligand binding (EC_{50} of 5-10 nM), enhanced the AMPA-induced calcium influx by as much as 60%. The enhanced influx was blocked by CNQX, while unchanged by the NMDA receptor antagonist AP5. Confocal microscopy showed that the increase in calcium occurred in neuronal cell bodies and their processes. Interestingly, smaller 5-8 kDa dextran sulfate and a non-sulfated dextran of 500 kDa had little or no effect on the binding, channel, and calcium permeability properties. Together, these findings suggest that synaptic polysaccharide species modulate hippocampal AMPA receptors in a sulfate- and size-dependent manner.

Key words: AMPA-type glutamate receptors, glycosaminoglycans, dextran sulfate, synaptic modulation

INTRODUCTION

AMPA receptors are involved in excitatory synaptic transmission in the brain, and their regulation is thought to be responsible for many types of neuronal functions. Most notable, AMPA receptors are thought to be involved in memory encoding (Staubli et al., 1994, 1996; Mahanty and Sah, 1998; Shapiro, 2001; Riedel et al., 2003), synaptogenesis (Launey et al., 1998; König et al., 2001), and neuronal maintenance (McKinney et al., 1999; Lauterborn et al., 2000; Bahr et al., 2002). The regulation of AMPA receptors

during these activity-dependent events leads to alterations in AMPA receptor properties and changes at the synapse. Perhaps providing insight into possible mechanisms of regulation, AMPA receptor binding and/or channel properties have been shown to be modulated by subunit phosphorylation (e.g., see Correia et al., 2003), interactions with certain lectins (Lin and Levitan, 1991; Wong and Mayer, 1993), adhesion responses (Lüthi et al., 1994; Bahr et al., 1997; Kramar et al., 2002), and polysaccharides (Hall et al., 1996; Hoffman et al., 1997). The latter is of particular interest since synapses are known to contain polysaccharides implicated in plasticity events and receptor regulation (see Lander et al., 1998; Bahr et al., 1999; Lauri et al., 1999).

Understanding polysaccharide modulation of plasticity events and receptor regulation is important since it provides a plausible explanation of how the environment of the synapse may alter synaptic function. As an example, polysialic acid-containing synaptic adhesion molecules are thought to play a role in hippocampal plasticity (Lüthi et al., 1994; Rønn et al., 1995, 1998; Staubli et al., 1998), and selective removal of the polysaccharide component of cell adhesion molecules disrupts different forms of synaptic regulation (Muller et al., 1996). Correspondingly, plasticity induction stimuli have been shown to increase the levels of polysaccharide-laden adhesion molecules in the hippocampus (Muller et al., 1996; Hoffman, 1998). Such activity-dependent regulation was found for another polysaccharide carrier protein, the syndecan-3 proteoglycan, which is expressed at the processes of pyramidal neurons (Lauri et al., 1999). This transmembrane proteoglycan is prominently expressed in adult hippocampus and contains glycosaminoglycans that interact with cell surface receptors and components of the extracellular matrix. Providing further evidence of polysaccharides that regulate activity-dependent events, it was found that mice deficient in select proteoglycan species display altered plasticity properties (Brakebusch et al., 2002; Kaksonen et al., 2002). Together, these reports suggest that polysaccharide-containing elements of central synapses have critical influences on synaptic function.

The present report focuses on the ability of glycosaminoglycans to modulate synaptic properties. The glycosaminoglycan component of proteoglycans is of interest since enzymatic removal of glycosaminoglycans prevented activity-induced synaptic

plasticity in hippocampal slices (Lauri et al., 1999). Surprisingly, the addition of heparin-type polysaccharides in the rat brain blocked plasticity events, and heparin has also been reported to influence high-affinity AMPA binding (Hall et al., 1996). However, while heparin negatively influenced binding affinity, it positively modulated AMPA channel kinetics to prolong the open state, possibly occluding subsequent regulation by plasticity processes (Hall et al., 1996; Sinnarajah et al., 1999). As AMPA receptors attach to heparin-agarose during purification steps (Bahr et al., 1992; Hall et al., 1996), the polysaccharide modulation appears to be through direct interaction.

In the experiments described here, dextran sulfate of 500 kDa was tested for its ability to modulate AMPA receptor properties. Dextran sulfate is a freely soluble polyanion that mimics natural mucopolysaccharides including chondroitin sulfate and heparan sulfate. The described results show that dextran sulfate alters binding, channel and calcium-permeability properties of AMPA receptors in a sulfate- and size-dependent manner.

MATERIALS and METHODS

Binding Assay

Using Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), hippocampal membrane preparations, solubilization, and [^{3}H]AMPA binding assays were conducted as described (Bahr et al., 1992; Hall et al., 1992). In brief, membranes were prepared by differential centrifugation and osmotic lysis, and suspended in a buffer of 50 mM Tris acetate, pH 7.2. Membranes were solubilized with 0.4% Triton X-100 and non-solubilized material was removed by centrifugation (48,000 *g* for 2 h). Samples were assayed for binding of 10 nM [^{3}H]AMPA in the absence or presence of various polysaccharides (Sigma, Inc., St. Louis, MO).

Purification of AMPA receptors and single channel recording

Brains from adult Sprague-Dawley rats were rapidly removed following anesthesia and decapitation, and membranes were prepared as previously described (Hall et al., 1992). The membranes were solubilized in ice-cold buffer A (30 mM HEPES, 5 mM EDTA, 1 mM EGTA, and 0.02% NaN_3, pH 7.4) with 1% (wt/vol) Triton X-100,

20% (wt/vol) glycerol, and 80 mM KSCN, at a detergent:protein ratio of 3:2. Particulate matter was removed by centrifugation at 30,000 *g* for 3 h. The supernatant was diluted with an equal volume of buffer A and applied to a diethylaminoethyl-Sepharose column equilibrated at 4° C with buffer A containing 1% *n*-octylglucoside (Roche, Basil, Switzerland), and 0.05% phosphatidylcholine (buffer B). The column was washed with 4 column volumes of buffer B and eluted with a KSCN gradient. AMPA receptors were then reconstituted as previously reported in small "tip-dip" bilayers (Bahr et al., 1992; Vodyanoy et al., 1993). AMPA (Tocris, Inc., Ellisville, MO) was delivered to the *cis* side of the bilayer in the absence or presence of dextran sulfate of different sizes and sulfate content. The AMPA specific agonist, 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, Tocris), was also delivered in the presence of AMPA and DS-L. Single channel events were recorded with a VCR system and subjected to computer analysis of amplitude and time distributions (pClamp, Axon Instruments, Inc., Foster City, CA). Recorded signals were filtered at 1-5 kHz and digitized between 5-25 kHz, after which they were reduced to series of datasets each containing 2,000-125,000 data points. The minimum detectable dwell time (0.1 msec) was calibrated by detection of brief events.

Calcium influx in primary hippocampal cell culture

Hippocampi were obtained from postnatal day 9 Sprague-Dawley rat pups and enzymatically dissociated using 1.8% (v/v) papain, 15 mM $CaCl_2$, 100 nM cysteine, and 5 mM EDTA. Cultures were seeded on poly-D-lysine-coated coverslips or 96-well tissue culture panels at 25,000 cells/200 □l, using a TomTec Quadra96 fluid-handling robot (TomTec Inc., Hamden, CT). Cultures were grown in a 5% CO_2 environment with DMEM containing B-27 and F-12 supplements (Gibco/BRL), 10% fetal bovine serum, 20 mM glucose and 1x penicillin/streptomycin (Gibco/BRL), and used for experiments on culture days 8-10. They were then rinsed and incubated in Fluo3AM calcium dye (Molecular Probes, Inc., Eugene, OR) for 35 min at 37° C and excess dye was removed. For confocal studies, dye-loaded hippocampal cultures on coverslips were sequentially perfused at approximately 0.5 ml/min with an AMPA solution, followed by AMPA plus DS-L in the presence or absence of CNQX. In the 96-well plate experiments, a baseline reading of dye-loaded cells was measured with a fluorimeter at 485 nm emission/530 nm

excitation (Perseptives Biosystems, Inc., Stafford, TX). Then cells were treated with AMPA alone or with various polysaccharide agents, and the experimental reading was taken immediately. In the 96-well plates, 8 wells were used for each treatment and individual responses from the eight wells were averaged to represent the value for a single experiment. Plate experiments were repeated to obtain an n of 3-13. Total time for reading a single 96-well plate was 45 sec, and the calcium-dye complex was stable for at least 5 min. Raw data was collected and percent changes in fluorescence were graphed using the Origin macro-analysis program. Statistical analysis was done using one-way ANOVAs and Tukey's post-tests.

RESULTS

Initial experiments tested whether dextran sulfate reduces high-affinity AMPA binding as previously found for heparin (Hall et al., 1996; Sinnarajah et al., 1999). Figure 1 shows the effect of a large, 500-kDa dextran sulfate (DS-L) on [^{3}H]AMPA binding using a low ligand concentration below the receptor's high-affinity K_D. DS-L decreased the high-affinity [^{3}H]AMPA binding in solubilized hippocampal membranes with an IC_{50} of approximately 10 nM. Sulfate-free dextran of the same 500 kDa size had no effect on the binding at up to 30 μM in concentration (Fig. 1). This indicates that the polysaccharide modulation of AMPA binding is sulfate-dependent. In addition, the sulfates must be an integral part of the polysaccharide since up to 50 mM sodium sulfate had no detectable influence on the high-affinity [^{3}H]AMPA binding (see Table 1). Scatchard analysis confirmed that the DS-L effect was due to a decrease in the apparent affinity of AMPA binding sites, as found previously for another sulfated polysaccharide (Hall et al., 1996). In the absence of DS-L, a single high-affinity state was evident by linear regression ($r=0.98$). The B_{max} of this state was reduced by 84% in the presence of 10 nM DS-L, along with a concomitant exposure of a low-affinity state (K_D=380 nM) that accounts for almost all of the lost binding sites.

Other polysaccharide molecules were also examined for their potential to influence AMPA receptor binding affinity (Table 1). As expected, non-sulfated dextrans of three different sizes all had no effect. Heparin also lost its ability to affect binding when sulfates were removed, as was the case for de-N-sulfate heparin. Interestingly, a

small 5-8 kDa version of dextran sulfate (DS-S) exhibited much less effectiveness at modifying the high-affinity binding (IC_{50}□800 nM) as compared to DS-L. A smaller monomeric species, glucosamine 2,3-disulfate, was completely ineffective (Table 1), further suggesting that a size factor is associated with the sulfate factor of polysaccharide modulation. Across the several agents tested, DS-L was most potent at altering AMPA binding.

As found in previous studies (Hall et al., 1996; Sinnarajah et al., 1999), polysaccharide modulation of AMPA binding was accompanied by modulated channel kinetics. Figure 2 shows portions of recordings from patch bilayers containing reconstituted AMPA receptors exposed to AMPA agonist (trace B; compare to control trace A) with the voltage clamped at –72 mV. The DS-L-modified channel activity (trace E) was completely blocked by the AMPA receptor antagonist CNQX (trace F; also see Fig. 3B). Thus, DS-L does not appear to have an effect on the antagonism of the active site of AMPA receptors. Figure 3 shows that DS-L causes a twofold increase in the open channel time ($p<0.001$; graph A) and nearly a sixfold increase in the open state probability ($p<0.001$; graph B). While DS-L shows positive modulation of the channel kinetics, the smaller DS-S polysaccharide and sulfate-free dextran (see respective traces in Fig. 2) had no effect on AMPA-induced current fluctuations. Together with previous work showing no effect on channel kinetics by monomeric glucosamine 2,3-disulfate, the results indicate that the channel modulation is similar to binding changes with regard to sulfate- and size-dependence.

To test if polysaccharide modulation of the channel activity affects calcium influx through AMPA receptors, primary hippocampal cultures were pre-loaded with Fluo3AM calcium ion dye. Results in Figure 4A show that AMPA produced a dose-dependent increase in fluorescence that was quenched by CNQX. In addition, time-lapse confocal movies revealed background fluorescence in dye-loaded neurons treated with saline (Fig. 4B), a subsequent increase in cellular fluorescent signal upon addition of AMPA (Fig. 4C), and quenching of the AMPA-mediated signal by the specific antagonist (Fig. 4D). The AMPA effect also was pronounced in neuronal processes, and there was evidence of increased calcium at synaptic contacts (Fig. 5B).

Figure 6B shows that DS-L potentiates the calcium signal in cell bodies and processes as compared to AMPA alone (Fig. 6A). DS-L had no effect on the few glial cells that exhibit a calcium response (1.8% of total cells), and neurons made up the majority of AMPA responders. By measuring the percent change in AMPA-induced calcium-Fluo3AM fluorescence, the DS-L effect was found to be dose-dependent (Fig. 7), causing up to a 60% increase in the response to 60 □M AMPA. As indicated in Figures 6C and 8, DS-L modulation of the calcium influx was blocked by CNQX. In control experiments the AMPA response and the DS-L enhancement of the AMPA response were not affected by the NMDA receptor antagonist AP5 (Fig. 8), and were not due to the release of internal calcium stores (data not shown). Experiments using an L-type voltage-gated calcium channel inhibitor, nifedopine (1 μM), showed that voltage-gated calcium channels do not contribute to the DS-L-mediated increase in the AMPA response (-4.6% ± 7.1% change by nifedopine). These data indicate that the modulated calcium signal involves calcium influx through stimulated AMPA receptors.

As with the modulation of AMPA binding and channel kinetics, DS-L's effect on calcium influx also exhibited sulfate- and size-dependencies. While 10 nM DS-L markedly increased AMPA-induced calcium influx ($p<0.02$), 1 □M of the small DS-S species and 1 □M sulfate-free dextran had little to no effect (see Fig. 7). Also with no influence on the AMPA response were 10 □M glucosamine 2,3-disulfate (-0.9% ± 2% change) and 1 mM sodium sulfate (-4% ± 6% change), adding further to the common specificities across the binding, channel kinetics, and calcium influx properties.

DISCUSSION

The present report indicates that the glycosaminoglycan component of proteoglycans has the potential to modulate multiple AMPA receptor properties. Here, a related polysaccharide, dextran sulfate, was found to lower the binding affinity of purified AMPA receptors, and to increase the single channel open time and open channel probability of reconstituted AMPA receptors. In addition, the potentiated channel activity was associated with an enhancement of the AMPA-mediated calcium influx in hippocampal neurons. These findings strongly indicate that synaptic proteoglycans and

polysaccharides are appropriately positioned to interact with and regulate AMPA-type glutamate receptors. Although the exact mechanisms for these events are not completely understood, polysaccharide modulation of neurotransmitter receptors may help explain the relationships between synaptic structure and function.

In this study, dextran sulfate was shown to stabilize the open conformation of isolated AMPA channels, along with a receptor state that favors a low-affinity ligand interaction. Previous reports have shown that other polysaccharides promote similar changes in channel kinetics and binding properties (Hall et al., 1996; Hoffman et al., 1997; Sinnarajah et al., 1999), perhaps acting through an allosteric regulatory site on AMPA receptors (e.g., see Kessler et al., 1998; Bahr et al., 1999; Arai et al., 2000; Nagarajan et al., 2001; Suppiramaniam et al., 2001). Thus, it is possible that the dextran sulfate-induced changes in AMPA receptor binding and channel properties are due to a direct interaction between the polysaccharide and the receptor. Another possibility is that dextran sulfate indirectly influences AMPA receptors by interacting with or altering interactions between polysaccharides and polysaccharide carrier proteins of the synaptic environment and the surrounding extracellular matrix. We believe this is unlikely based on the fact that dextran sulfate altered AMPA receptor binding in a solubilized system where organized matrices are completely disrupted, and modified the channel kinetics of isolated AMPA receptors that were reconstituted in pure lipid bilayers.

The polysaccharide modulation exhibited sulfate- and size-dependencies with respect to all AMPA receptor properties monitored in the present study. Ligand binding, channel kinetics, and calcium influx through AMPA receptors were effectively modulated by a large dextran sulfate species, while little effect was produced by smaller dextran sulfate or sulfate-free dextran. Such findings may be related to the fact that the biological activity of glycosaminoglycans is based on specific sugar residues, size of the polysaccharides, and the number of sulfates they possess (see Rouslahti, 1988, 1989). Increasing the length of the polymer chain adds increasing numbers of negatively charged sulfates giving the larger dextran sulfate more charge, and possibly stronger interactions with the AMPA receptor. It is known that polysaccharides, through a high degree of specificity, induce protein-protein or protein-carbohydrate interactions that may alter

receptor structure and function (Lam et al., 1998; Hilgenberg et al., 1999; Capila and Linhardt, 2002). Thus, it is possible that the highly charged, 500-kDa dextran sulfate promotes receptor-receptor interactions that influence receptor function.

Clustering of neurotransmitter receptors, indeed, has been found to modulate the functional properties of the receptor (Takagi et al., 1992; Chen et al., 2001). Receptor clustering has been implicated in synaptic transmission (Xia et al., 1999; Ango et al., 2002) and synapse formation (O'Brien et al., 2002; Cottrell et al., 2000), and polysaccharides and proteoglycans are known to regulate receptor clustering (Zhou et al., 1997; Moransard et al., 2003). Interestingly, polysaccharide modulation has been shown to facilitate clustered AMPA channel responses exhibiting a remarkable degree of cooperativity (Hall et al., 1996). Thus, the large dextran sulfate in the present study may affect AMPA receptor properties by facilitating both the physical and functional clustering of the receptors. Synaptic polysaccharides, then, may modulate the basal activity of the synapse promoting cooperative activation of AMPA receptors. Further understanding of polysaccharide-receptor interactions at the molecular level may provide insight into the regulation of activity-dependent events such as synaptogenesis and synaptic plasticity.

ACKNOWLEDGEMENTS

We would like to thank Mr. Brian Kawasaki for excellent technical assistance.

REFERENCES

1. Ango F, Robbe D, Tu JC, Xiao B, Worley PF, Pin JP, Bockaery J, Fagni L. 2002. Homer-dependent cell surface expression of metabotropic glutamate receptor type 5 neurons. Mol Cell Neurosci 20:323-329.
2. Arai AC, Kessler M, Rogers G, Lynch G. 2000. Effects of the potent Ampakine CX614 on hippocampal recombinant AMPA receptors: interactions with cyclothiazide and GYKI 52466. Mol Pharmacol 58:802-813.

3. Bahr BA, Bendiske J, Brown QB, Munirathinam S, Caba E, Rudin M, Urwyler S, Sauter A, Rogers G. 2002. Survival signaling and selective neuroprotection through glutamatergic transmission. Exp Neurol 174:37-47.
4. Bahr BA, Solomon Y, Suppiramaniam V. 1999. Structural chemistries underlying synaptic signaling and regulation. J Mol Biol Biotech 1:1-15.
5. Bahr BA, Staubli U, Xiao P, Chun D, Ji Z, Esteban ET, Lynch G. 1997. Arg-gly-asp-ser-selective adhesion and the stabilization of long-term potentiation: pharmacological studies and the characterization of a candidate matrix receptor. J Neurosci 17:1320-1329.
6. Bahr BA, Vodyanoy V, Hall RA, Suppiramaniam V, Kessler M, Sumikawa K, Lynch G. 1992. Functional reconstitution of α-amino-3-hydroxy-5-methylisoxazole-4 - propionate (AMPA) receptors from rat brain. J. Neurochem. 59:1979-1982.
7. Brakebusch C, Seidenbecher CI, Asztely F, Rauch U, Matthies H, Meyer H, Krug M, Bockers TM, Zhou X, Kreutz MR, Montag D, Gundelfinger ED, Fassler R. 2002. Brevican-deficient mice display impaired hippocampal CA1 long-term potentiation but show no obvious deficits in learning and memory. Mol Cell Biol 22:7417-7427.
8. Capila I, Linhardt RJ. 2002. Heparin-protein interactions. Angew Chem Int Ed Engl 41:391-412.
9. Chen L, Wang H, Vicini S, Olsen RW. 2001. The gamma-aminobutyric acid type A (GABAA) receptor associated protein (GABARAP) promotes GABAA receptor clustering and modulates the channel kinetics. Proc Natl Acad Sci 97:11557-11562.
10. Correia SS, Duarte CB, Faro CJ, Pires EV, Carvalho AL. 2003. Protein kinase C gamma associates directly with the GluR4 alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subunit. Effect on receptor phosphorylation. J Biol Chem 278:6307-6313.
11. Cottrell JR., Dube GR., Egles C, Liu G. 2000. Distribution, density, and clustering of functional glutamate receptors before and after synaptogenesis in hippocampal neurons. J Neurophysiol 84:1573-1587.
12. Hall RA, Kessler M, Lynch G. 1992. Evidence that high- and low-affinity DL-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) binding sites reflect membrane-dependent states of a single receptor. J Neurochem 59:1997-2004.

13. Hall RA, Vodyanoy V, Quan A, Sinnarajah S, Suppiramaniam V, Kessler M., Bahr BA. 1996. Effects of heparin on the properties of solubilized and reconstituted rat brain AMPA receptors. Neurosci Lett 217:179-183.
14. Hilgenberg LG, Hoover CL, Smith MA. 1999. Evidence of an agrin receptor in cortical neurons. J Neurosci 19:7384-7393.
15. Hoffman KB, 1998. The relationship between adhesion molecules and neuronal plasticity. Cell Mol Neurobiol 18:461-475.
16. Hoffman KB, Kessler M, Lynch G. 1997. Sialic acid residues indirectly modulate the binding properties of AMPA-type glutamate receptors. Brain Res 753:309-314.
17. Kaksonen M, Pavlov I, Voikar V, Lauri SE, Hienola A, Riekki R, Lakso M, Taira T, Rauvala H. 2002. Syndecan-3-deficient mice exhibit enhanced LTP and impaired hippocampus-dependent memory. Mol Cell Neurosci 21:158-172.
18. Kessler M, Mutaneja MS, Rogers G, Lynch G. 1998. Regional preferences of AMPA receptor modulators determined through agonist binding autoradiography. Brain Res 783:121-126.
19. König N, Poluch S, Estabel J, Durand M, Drian MJ, Exbrayat JM. 2001. Synaptic and non-synaptic AMPA receptors permeable to calcium. Jpn J Pharmacol 86:1-17.
20. Kramar EA, Bernard JA, Gall CM, Lynch G. 2002. Alpha3 integrin receptors contribute to the consolidation of long-term potentiation. Neuroscience 110:29-39.
21. Lam K, Rao VS, Qasba PK. 1998. Molecular modeling studies on binding of bFGF to heparin and its receptor FGFR1. J Biomol Struct Dyn 15:1009-1027.
22. Lander C, Zhang H, Hockfield S. 1998. Neurons produce a neuronal cell surface-associated chondroitin sulfate proteoglycan. J Neurosci 18:174-183.
23. Launey T, Ivanov A, Ferrand N, Gueritaud JP. 1998. Developing rat brainstem motoneurons in organotypic culture express calcium permeable AMPA-gated receptors. Brain Res 781:148-158.
24. Lauri SE, Kaukinen S, Kinnunen T., Ylinen A, Imai S, Kaila K, Taira T, Rauvala H. 1999. Regulatory role and molecular interactions of a cell-surface heparan sulfate proteoglycan (N-syndecan) in hippocampal long-term potentiation. J Neurosci 19:1226-1235.

25. Lauterborn JC, Lynch G, Vanderklish P, Arai A, Gall CM. 2000. Positive modulation of AMPA receptors increases neurotrophin expression by hippocampal and cortical neurons. J Neurosci 20:8-21

26. Lin SS, Levitan IB. 1991. Concanavalin A: a tool to investigate neuronal plasticity. Trends Neurosci 14:273-277.

27. Lüthi A, Laurent JP, Figurov A, Muller D, Schachner M. 1994. Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. Nature 372:777-779.

28. Mahanty NK, Sah P. 1998. Calcium-permeable AMPA receptors mediate long-term potentiation in interneurons in the amygdala. Nature 394:683-687.

29. McKinney RA, Capogna M, Durr R, Gahwiler BH. 1999. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat Neurosci 2:44-49.

30. Moransard M, Borges LS, Willmann R, Marangi PA, Brenner HR, Ferns MJ, Fuhrer C. 2003. Agrin regulates rapsyn interaction with surface acetylcholine receptors, and this underlies cytoskeletal anchoring and clustering. J Biol Chem 278:7350-7359.

31. Muller D, Wang C, Skibo G, Toni H, Cremer H, Calaora V, Rougon G, Kiss JZ. 1996. PSA-NCAM is required for activity-induced synaptic plasticity. Neuron 17:413-422.

32. Nagarajan N, Quast C, Boxall AR, Shahid M, Rosenmund C. 2001. Mechanism and impact of allosteric AMPA receptor modulation by the Ampakine™ CX546. Neuropharm 41:650-663.

33. O'Brien R, Xu D, Mi R, Tang X, Hopf C, Worley P. 2002. Synaptically targeted narp plays an essential role in the aggregation of AMPA receptors at excitatory synapses in cultured spinal neurons. J Neurosci 22:4487-4498.

34. Riedel G, Platt B, Micheau J. 2003. Glutamate receptor function in learning and memory. Behav Brain Res 140:1-47.

35. Rønn LCB, Hartz BP, Bock E. 1998. The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system. Exp Gerontology. 33:853-864.

36. Rønn LC, Bock E, Linnemann D, Jahnsen H. 1995. NCAM-antibodies modulate induction of long-term potentiation in rat hippocampal CA1. Brain Res 677:145-151.

37. Rouslhati E. 1988. Structure and biology of proteoglycans. Ann Rev Cell Biol 4:229-255.

38. Rouslhati E. 1989. Proteoglycans in cell regulation. J Biol Chem 264:13369-13372.
39. Shapiro M. 2001. Plasticity, hippocampal place cells, and cognitive maps. Arch Neurol 58:874-881
40. Sinnarajah S, Suppiramaniam V, Kumar KP, Hall RA, Bahr BA, Vodyanoy V. 1999. Heparin modulates the single channel kinetics of reconstituted AMPA receptors from rat brain. Synapse 31:203-209.
41. Staubli U, Chun D, Lynch G. 1998. Time-dependent reversal of long-term potentiation by an integrin antagonist. J Neurosci 18:3460-3469.
42. Staubli U, Izrael Z, Xu F. 1996. Remembrance of odors past: enhancement by central facilitation of AMPA receptors. Behav Neurosci 110:1067-1073
43. Staubli U, Rogers G, Lynch G. 1994. Facilitation of glutamate receptors enhances memory. Proc Natl Acad Sci 91:777-781.
44. Suppiramaniam V, Bahr BA, Sinnarajah S, Owens K, Rogers G, Yilma S, Vodyanoy V. 2001. Member of the Ampakine class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. Synapse 40:154-158.
45. Takagi T, Pribilla I, Kirsch J, Betz H. 1992. Coexpression of the receptor-associated protein gephyrin changes the ligand binding affinities of $\square_2$ glycine receptors. FEBS Lett 303:178-180.
46. Vodyanoy V, Bahr BA, Suppiramaniam V, Hall RA, Baudry M, Lynch G. 1993. Single channel recordings of reconstituted AMPA receptors reveal low and high conductance states. Neurosci Lett 150:80-84.
47. Wong LA, Mayer ML. 1993. Differential modulation by cyclothiazide and concanavalin A of desensitization at native alpha-amino-3-hydroxy-5-methyl-isoxazolepropionic acid- and kainate-preferring glutamate receptors. Mol Pharmacol 44:504-510.
48. Xia J, Zhang X, Staudinger J, Huganir RL. 1999. Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron 22:179-187.
49. Zhou H, Muramatsu T, Halfter W, Tsim KW, Peng HB. 1997. A role of midkine in the development of the neuromuscular junction. Mol Cell Neurosci 10:56-70.

Table I. Relative potencies of various species on high-affinity [^{3}H]AMPA binding.

Agent	**Decreased high-affinity [^{3}H]AMPA binding (approximate IC_{50})**
DS-L (500 kDa)	10 nM
fucoidan	150 nM
DS-S (5-8 kDa)	800 nM
heparin	2 □M
hyaluronate	> 30 □M
de-N-sulfate heparin	>> 30 □M
dextran (18 kDa)	>> 30 □M
dextran (260 kDa)	>> 30 □M
dextran (500 kDa)	>> 30 □M
glucosamine 2,3-disulfate	> 10 mM
Na_2SO_4	> 50 mM

Figure 1. Inhibition by DS-L of [^{3}H]AMPA binding to detergent-solubilized AMPA receptors. The [^{3}H]AMPA concentration used (10 nM) was below the K_D value of 30 nM in order to assess primarily high-affinity binding sites. The percentage of control [^{3}H]AMPA binding remaining in the presence of increasing concentrations of DS-L and sulfate-free dextran is shown. The data represent the mean $\pm$ SEM for three experiments.

Figure 2. Effects of DS-L on AMPA channel activity. Channel fluctuations were elicited by 150 nM AMPA in isolated AMPA receptors reconstituted in a lipid bilayer and voltage clamped at –72mV. The closed state (c) was predominantly in the control trace **(A)**, while numerous opening events (o) were induced by AMPA in the absence **(B)** or presence of 5 nM dextran **(C)** or 5 nM DS-S **(D)**. Increased channel open time occurred with the addition of 5 nM DS-L **(E)**, and the modulated channels were blocked by 1 □M CNQX **(F)**. calibration bar: 2.3 pA, 960 msec.

Figure 3. Effects of polysaccharides on AMPA-elicited single channel open time **(A)** and open channel probability **(B)**. From bilayer experiments in Figure 2, AMPA-induced single channel open time was found to be increased only by DS-L (ANOVA=$p<0.0001$). The open time is the mean dwell time □. Similarly, only in the presence of DS-L was there an increase in open channel probability ($p<0.0001$). The probability of open channels is expressed as a percentage of all open and closed channels.

Figure 4. AMPA induces an increase in calcium influx. AMPA-induced fluorescence was measured from Fluo3AM-loaded hippocampal cultures and background was subtracted. Increasing AMPA concentrations cause an increase in fluorescent signal ($p<0.0001$; closed squares). Time-lapse confocal images from the same field of dye-loaded hippocampal cells show the background fluorescence in non-treated cells **(B)**, the subsequent increase in fluorescent signal when exposed to 60 μM AMPA **(C)**, and blockage of the AMPA-mediated increase in fluorescent signal by 30 □M CNQX **(D)**.

FIGURE 1

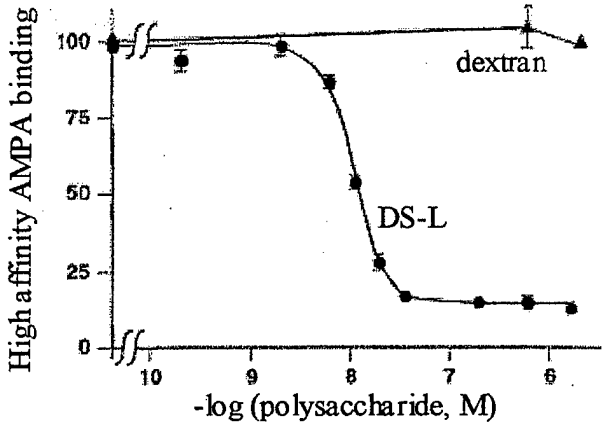

High affinity AMPA binding
100
75
50
25
0
dextran
DS-L
10
9
8
7
6
-log (polysaccharide, M)

Figure 5. AMPA increases calcium concentrations in the cell bodies and processes of hippocampal neurons, and at synaptic contacts along dendritic processes. Time-lapse confocal movies were made from dye-loaded hippocampal cells. Confocal images show saline control **(A)**, and the subsequent increase in fluorescence intensity upon exposure to 60 □M AMPA **(B)**. Arrows point to increases in intracellular calcium along cellular processes and the asterisk indicates evidence of synaptic contacts where AMPA mediated an increase in calcium influx.

Figure 6. DS-L enhances the AMPA-mediated calcium influx in primary hippocampal cell cultures. Still images from confocal movies from a single neuronal field show the Fluo3AM signal obtained by the 60 μM AMPA-mediated calcium influx **(A)**. The fluorescent signal in the field is intensified with the addition of 10 nM DS-L, indicating an enhancement of the AMPA-mediated calcium influx **(B)**. The addition of 30 μM CNQX quenches the fluorescence intensity generated by DS-L enhancement of the AMPA-mediated calcium influx **(C)**.

Figure 7. Dose-dependent effects of DS-L on the AMPA response in Fluo3AM-loaded hippocampal cells. Fluorimetry experiments were assessed for percent change in AMPA-mediated calcium response by different dextran species. Results show a dose-dependent increase in calcium influx by DS-L (ANOVA=$p<0.0001$), with little or no change in the AMPA-mediated calcium influx by high concentrations of DS-S and sulfate-free dextran.

Figure 8. DS-L modulation of calcium influx is specific for AMPA receptors. Changes in AMPA-induced fluorescent signals in Fluo3AM-loaded neurons are shown without and with the presence of 10 nM DS-L. The 60 □M AMPA-mediated calcium influx and the enhancement of this response by DS-L were blocked by 30 □M CNQX, but not by 50 □M AP5.

FIGURE 2

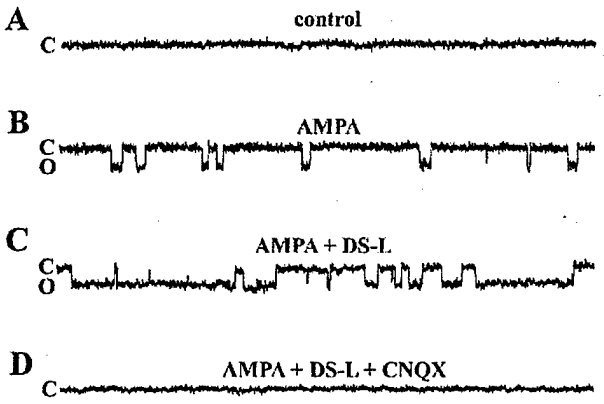


FIGURE 3

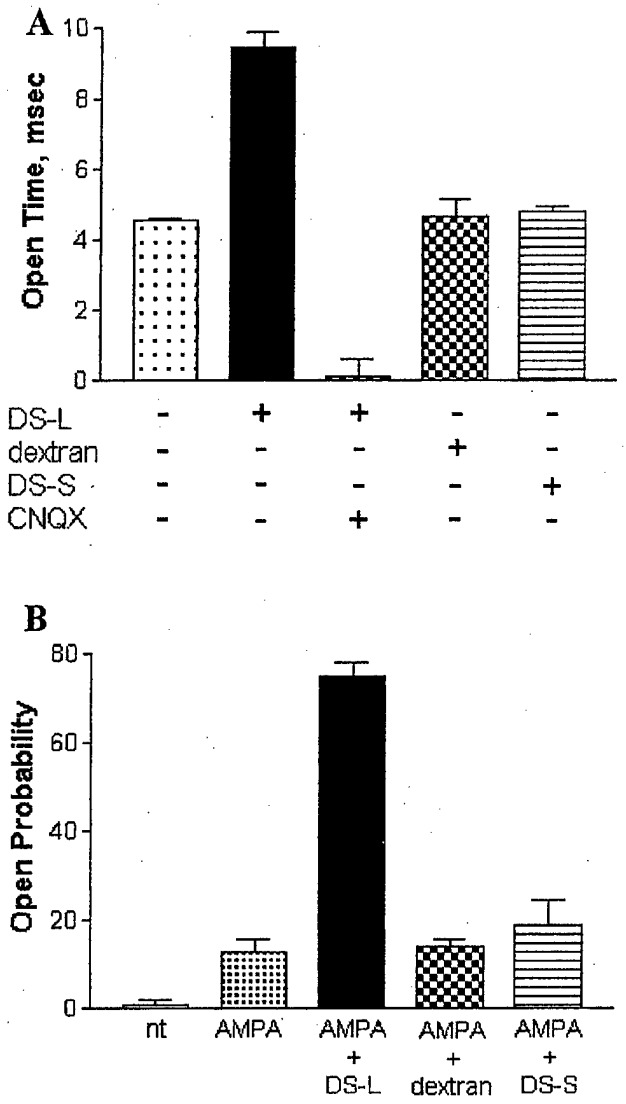
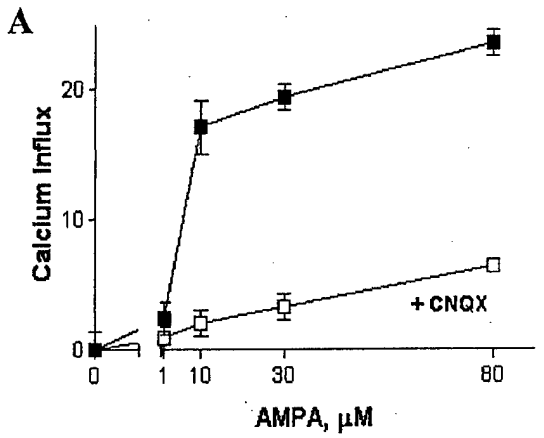

A
Open Time, msec
10
8
6
4
2
0
DS-L - + + - -
dextran - - - + -
DS-S - - - - +
CNQX - - + - -
B
Open Probability
80
60
40
20
0
nt
AMPA
AMPA + DS-L
AMPA + dextran
AMPA + DS-S

FIGURE 4



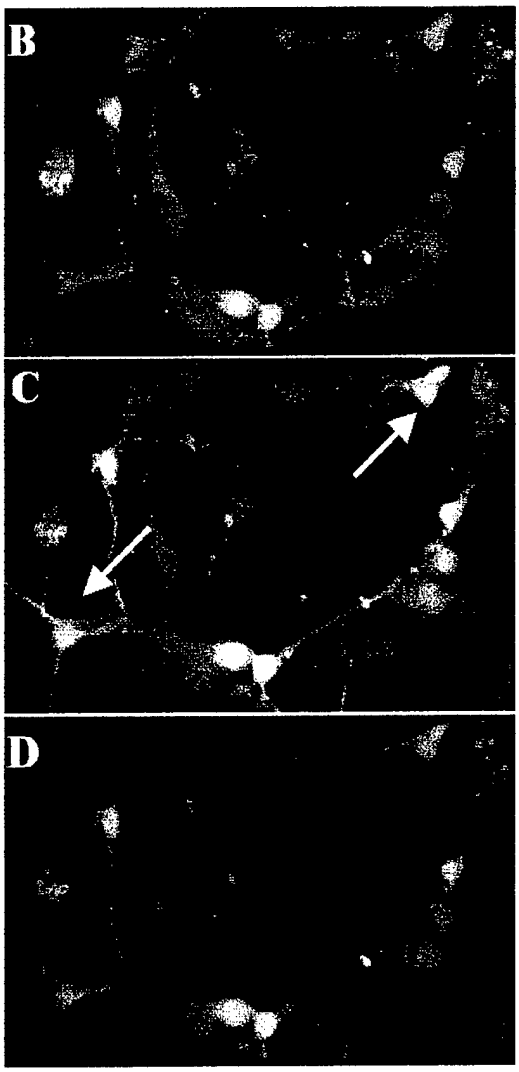


FIGURE 5

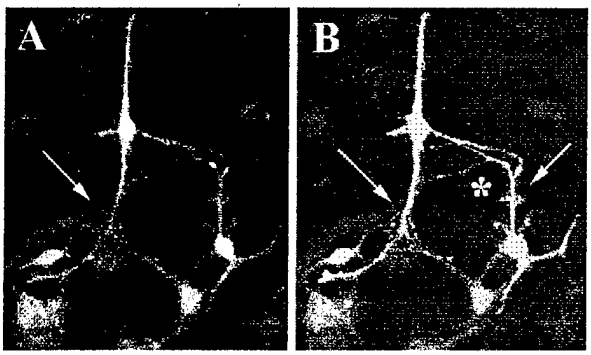


FIGURE 6

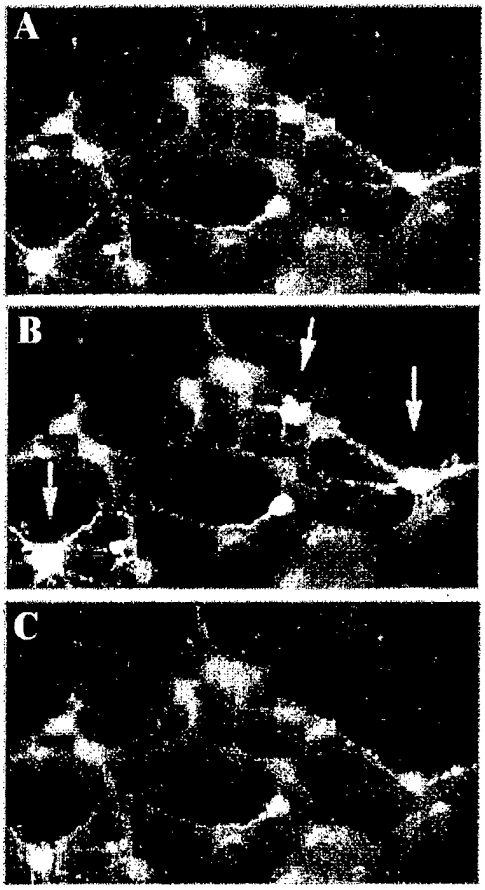


FIGURE 7

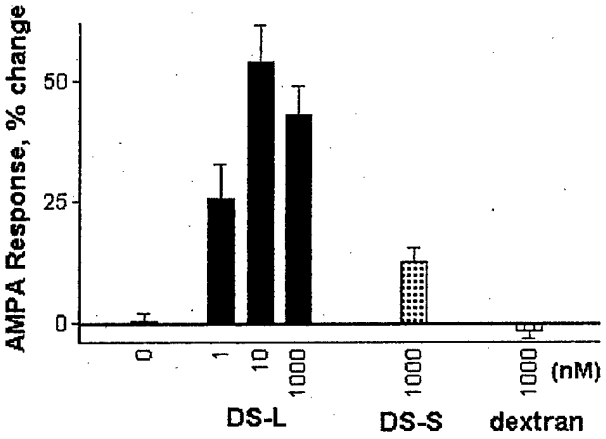
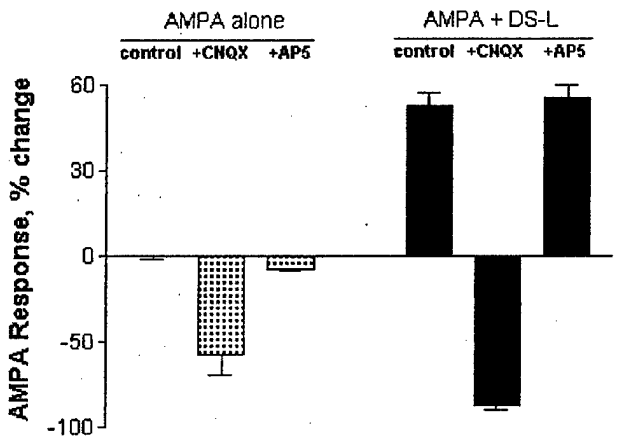

AMPA Response, % change
50
25
0
0
1
10
1000
1000
1000
(nM)
DS-L
DS-S
dextran

FIGURE 8


AMPA alone
control +CNQX +AP5
AMPA + DS-L
control +CNQX +AP5
AMPA Response, % change
60
30
0
-50
-100

Journal of Neurochemistry, 2003, **86**, 1223–1232 doi:10.1046/j.1471-4159.2003.01931.x

Both apoptosis and necrosis occur early after intracerebral grafting of ventral mesencephalic tissue: a role for protease activation

M. Emgård,*[,1] U. Hallin,† J. Karlsson,*[,2] B. A. Bahr,‡ P. Brundin* and K. Blomgren†[,§]

*Section for Neuronal Survival, Wallenberg Neuroscience Center, Lund University, Sweden
†Perinatal Center, Institute of Physiology and Pharmacology, Göteborg University, Sweden
‡Department of Pharmaceutical Sciences and the Neuroscience Program, University of Connecticut, USA
§Department of Pediatrics, The Queen Silvia Children's Hospital, Göteborg, Sweden

Abstract

Neural transplantation is an experimental treatment for Parkinson's disease. Widespread clinical application of the grafting technique is hampered by a relatively poor survival (around 10%) of implanted embryonic dopamine neurones. Earlier animal studies have indicated that a large proportion of the grafted cells die during graft tissue preparation and within the first few days after intracerebral implantation. The present study was designed to reveal the prevalence of cell death in rat intrastriatal grafts at 90 min, 1, 3, 6 and 42 days after implantation. We examined apoptotic cell death using semithin and paraffin sections stained with methylene blue and an antibody against activated caspase 3, respectively. We identified abundant apoptotic cell death up to 3 days after transplantation. In addition, we studied calpain activation using an antibody specific for calpain-cleaved fodrin. We report a peak in calpain activity 90 min after grafting. Surprisingly, we did not observe any significant difference in the number of dopaminergic neurones over time. The present results imply that grafted cells may be victims of either an early necrotic or a later apoptotic cell death and that there is substantial cell death as early as 90 min after implantation.

Keywords: apoptosis, calpain, necrosis, neural transplantation, Parkinson's disease, substantia nigra.
J. Neurochem. (2003) **86**, 1223–1232.

Neural transplantation is an exciting experimental therapy for Parkinson's disease. In the best cases, intracerebral transplants of embryonic dopaminergic neurones can give rise to dramatic, long-term improvement in patients with advanced disease (Piccini *et al.* 1999; Lindvall and Hagell 2000). Functional recovery appears to be dependent on an adequate number of dopamine neurones surviving in the graft tissue (Hagell and Brundin 2001) and sufficient integration into the host striatum (Brundin and Hagell 2001; Redmond 2002). Both animal and clinical data indicate that the survival of grafted dopamine neurones is only around 1–20% (Brundin *et al.* 2000). Due to the low survival rate, tissue from multiple embryos is needed for each patient (Hagell and Brundin 2001). In spite of observations of marked clinical benefit in some patients, only relatively few patients have been operated due to difficulties in obtaining tissue from an adequate number of donor embryos on a given day of surgery (Brundin and Hagell 2001; Redmond 2002).

In experimental animals, the majority of grafted nigral dopamine neurones die before or during the first week after implantation (Mahalik *et al.* 1994; Duan *et al.* 1995, 2000; Barker *et al.* 1996; Zawada *et al.* 1998; Emgård *et al.* 1999, 2002; Schierle *et al.* 1999a; Sortwell *et al.* 2000, 2001; Helt *et al.* 2001). While many studies have examined the cell death that occurs in nigral transplants after the first 4–7 days following surgery, relatively few reports describe events that take place in the grafts sooner after implantation (Zawada *et al.* 1998; Sortwell *et al.* 2000; Helt *et al.* 2001).

Received March 21, 2003; revised manuscript received May 14, 2003; accepted May 19, 2003.

Address correspondence and reprint requests to M. Emgård, Department of Veterinary Morphophysiology and Animal Production (DIMORFIPA), University of Bologna, Ozzano dell'Emilia, Italy.
E-mail: mia@emgard.com
[1]The present address of M. Emgård is the Department of Veterinary Morphophysiology and Animal Production (DIMORFIPA), University of Bologna, Ozzano dell'Emilia, Italy.
[2]The present address of J. Karlsson is the Community Environmental Health Program, University of New Mexico Health Sciences Center, Albuquerque, NM, USA.

Abbreviations used: FBDP, fodrin breakdown product; TH, tyrosine hydroxylase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labelling.

Expressed as a percentage of the number of dopamine neurones in the harvested donor tissue, the highest survival rates achieved so far have only been in the range of 40–50% (Hansson *et al.* 2000; Clarke and Branton 2002; Sortwell 2003). Several studies have focused on limiting cell death by counteracting apoptosis (for review see Brundin *et al.* 2000 and Castilho *et al.* 2000; Duan *et al.* 2000; Espejo *et al.* 2000; Hansson *et al.* 2000; Helt *et al.* 2001; Zawada *et al.* 2001; Cicchetti *et al.* 2002), while other modes of cell death have been addressed less systematically (Cicchetti *et al.* 2002; Zietlow *et al.* 2002).

Caspases and calpains are two groups of cysteine proteases known to participate in mechanisms of cell death. Caspases are considered major executioners of apoptosis, whereas pathological activation of calpains is considered more important in necrosis. Apoptosis is a fundamentally important process whereby non-functional cells can be eliminated with a minimum of inflammation. Caspases are at the heart of the apoptotic pathways and have been named in order of publication (Nicholson and Thornberry 1997; Wolf *et al.* 1999), at present caspase 1 to 14. Treatment with high concentrations of the caspase inhibitor acetyl-Tyr-Val-Ala-Asp-chloro methylketone has been shown to increase the survival of grafted dopaminergic neurones (Schierle *et al.* 1999b; Hansson *et al.* 2000). However, at the concentrations employed, acetyl-Tyr-Val-Ala-Asp-chloro methylketone is likely to have also inhibited other proteases in the graft tissue (Hansson *et al.* 2000; Gray *et al.* 2001). Calpains are a group of Ca^{2+}-activated neutral cysteine proteases present in all mammalian cells (Croall and DeMartino 1991; Saido *et al.* 1994; Vanderklish and Bahr 2000). Calpain activity is strictly regulated by cytosolic calcium and interaction with, e.g. the endogenous inhibitor protein calpastatin and membrane phospholipids. Under pathological conditions, calpains have mainly been implicated in excitotoxic neuronal injury (Huang and Wang 2001). *In vivo* administration of calpain inhibitors is neuroprotective in the adult rodent brain (Lee *et al.* 1991; Rami and Krieglstein 1993; Bartus *et al.* 1994; Hong *et al.* 1994; Saatman *et al.* 1996). Calpains are activated early (minutes to hours) in situations of energy depletion and/or increased calcium influx and may trigger other downstream events leading to neurodegeneration. Caspases, on the other hand, are generally activated much later (hours to days) following an insult. Furthermore, functional connections between calpains and caspases have recently been identified (Wang 2000; Blomgren *et al.* 2001).

The aims of the present study were to investigate cell death in intrastriatal grafts shortly after transplantation surgery and to shed further light on the mechanisms underlying the cell death process. Specifically, we monitored the occurrence of degenerating neurones as soon as 90 min after graft injection and followed the implants for up to 6 weeks. Markers of caspase and calpain activation, as well as methylene blue-stained semi-thin sections, were followed throughout the time course to monitor the demise of cells in the tissue cell graft and in the host brain following transplantation.

Materials and methods

Animals and chemicals

Intrastriatal transplantations of embryonic ventral mesencephalic tissue were performed as described previously (Emgård *et al.* 1999). All animals were purchased from B & K Universal (Sollentuna, Sweden). The experimental procedure was ethically approved and animals were handled according to the animal protection act of the Swedish Government. All chemicals were purchased from Sigma (Stockholm, Sweden) if not otherwise stated.

Preparation of donor tissue

Embryonic dopaminergic neurones were obtained from the ventral mesencephalon of 14-day-old (crown-to-rump length 12 mm) rat embryos, where the day after mating was considered as day zero. The pregnant rats were killed with sodium pentobarbitone (200 mg/kg body weight i.p.) and the uterus removed by Caesarean section. The embryos were dissected under sterile conditions in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Gibco, Life Technologies, Täby, Sweden) and the tissue pieces were incubated with 0.1% trypsin (Worthington, Lakewood, NJ, USA)/0.05% DNase in Hank's balanced salt solution for 20 min at 37°C. After incubation, the ventral mesencephalon tissue pieces were washed four times in 0.05% DNase in Hank's balanced salt solution and mechanically dissociated using a fire-polished Pasteur pipette. The resulting cell suspension, with a volume equal to one ventral mesencephalon in 6 μL, contained a mixture of single cells and small tissue aggregates.

Intrastriatal transplantations

Adult female Sprague–Dawley rats (weighing 220 g at the start of the experiment) were anaesthetized with a mixture of Hypnorm (fentanyl citrate and fluanisome, 0.213 mg and 6.75 mg/kg bodyweight, respectively; Janssen Pharmaceutical, Beerse, Belgium) and Dormicum (midazolam, 3.375 mg/kg bodyweight; Hoffman-La Roche, Basel, Switzerland). The rats were placed in a Kopf stereotactic apparatus and tissue injections were made with the following coordinates (in mm, with reference to the bregma and dura): rostral, −1.0; lateral, −3.0; ventral, −5.0 and 4.5; with tooth-bar, 0. Injections were performed at a rate of 1 μL/min using a 10-μL Hamilton syringe, fitted with a cannula with a 0.26-mm inner diameter and 0.46 mm outer diameter. Each rat received a total of 3 μL cell suspension, equivalent to one half ventral mesencephalon. The cannula was left in place for 2 min between deposits and an additional 4 min before withdrawal after the last implantation. To evaluate damage in the host striatum caused by the needle injection, in a separate set of animals, sham-transplantations were made with injections of 3 μL of Hank's balanced salt solution. Temgesic (0.3 mg/mL Buprenorphine solution; 0.17 mg/kg body weight s.c.; Reckitt and Colman, Hull, UK) was administered immediately after surgery for analgesia.

Tissue preparation

The survival times for both tissue-transplanted and sham-grafted rats were 90 min, 1, 3, 6 or 42 days. Rats were deeply anaesthetized with sodium pentobarbitone (200 mg/kg body weight, i.p.) and

perfused with 4% paraformaldehyde as previously described (Emgård *et al.* 1999). The animals (tissue or sham grafted) that were further prepared for semi-thin sections were perfused with 2% glutaraldehyde. The brains were post-fixed for 1 day in the same fixative.

Semi-thin sections

For the semi-thin sections, a vertical tube of the transplanted hemisphere was dissected out and further fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer. The tissue blocks were rinsed, dehydrated in graded concentrations of ethanol and rinsed in propylenoxide. The tissue blocks were further incubated overnight in a 1 : 1 mixture of propylenoxide and Agar 100 resin solution (consisting of Agar 100 epoxy resin, dodecnylsuccinic anhydride, methyl Nadic anhydride and benzyldimethylamine; Agar Link Nordiska AB, Upplands Vasby, Sweden), followed by an incubation for 6 h in pure Agar 100 epoxy resin. Finally, the tissue blocks were allowed to polymerize for 3 days at 60°C. The plastic-embedded tissue blocks were sectioned saggitally into semi-thin, i.e. 1–2 μm, sections. Azur (1%) and methylene blue (1% in 1% borax) were used as Nissl stain and the cell morphology of the grafted neurones was studied.

Paraffin sections

After dehydration with graded ethanol concentrations and xylene, the brains were paraffin embedded and cut into 5-μm coronal sections using a microtome. The sections were divided into 10 series. Each series constituted immediate adjacent sections, with 12–15 sections per brain and series. Before immunohistochemical staining, the sections were incubated for 30 min at 65°C and deparaffinized with xylene and graded series of ethanol. Antigen retrieval was performed by boiling the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min.

Immunohistochemistry

Tyrosine hydroxylase immunohistochemistry

Endogenous peroxidase activity was quenched with 3% H_2O_2 followed by incubation with 2% normal rabbit serum (ICN Biochemicals, Aurora, OH, USA) in 1% Triton X-100 for 1 h. A monoclonal mouse anti-tyrosine hydroxylase (TH; Chemicon, Hampshire, UK) was added onto the sections (1 : 2000) and incubated in a humidified box overnight at 4°C, followed by a Cy3-conjugated rabbit anti-mouse secondary antibody (1 : 50; Jackson Immuno-Research, West Grove, PA, USA) for 1 h at room temperature. Sections were mounted with poly vinyl-alcohol in diaza-bicyclo-octane (ICN Biochemicals).

Fodrin breakdown product immunohistochemistry

The sections were pre-incubated for 30 min in 4% normal goat serum followed by incubation in a humidified box at 4°C overnight with a polyclonal antibody against the 150 kDa calpain-specific fodrin breakdown product (FBDP, 1 : 50; Bahr *et al.* 1995). A biotinylated goat anti-rabbit IgG (1 : 136; Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody. Sections were incubated for 1 h at room temperature, followed by 10 min with 3% H_2O_2 in phosphate-buffered saline. After rinsing, the sections were incubated with avidin–biotin complex (Vectastain Elite; Vector Laboratories) for 1 h at room temperature. Chromogen development was performed with nickel-enhanced diamino benzidine. Sections were mounted with Histofix (Histolab, Göteborg, Sweden).

Active caspase 3 immunohistochemistry

The sections were pre-treated with 10 μg/mL proteinase K (Roche Diagnostics, Mannheim, Germany) for 10 min at room temperature before incubation with 4% normal goat serum for 1 h. The polyclonal primary antibody against active caspase 3 (1 : 50; PharMingen, Becton Dickinson AB, Stockholm, Sweden) was incubated in a humidified box at 4°C overnight, followed by a 1 h incubation with a biotinylated secondary goat anti-rabbit antibody (1 : 500; Vector Laboratories). Quenching of endogenous peroxidase activity and development with avidin–biotin complex and diamino benzidine was performed as described above.

Double staining

Two different double stainings were carried out on one representative brain per time point (90 min, 1, 3 and 6 days), combining TH and FBDP or TH and caspase 3. Staining procedures were initiated with staining for TH (as described above) with a red fluorescent goat anti-mouse secondary antibody (1 : 100; Alexa Fluor 594; Molecular Probes, Eugene, OR, USA). Staining for FBDP and active caspase 3 was performed as described above with a green fluorescent goat anti-rabbit secondary antibody (1 : 400; Alexa Fluor 488). Sections were mounted with fluorescent mounting medium (DAKO Corporation, Carpinteria, CA, USA).

Microscopic evaluation

The TH-, FBDP- and caspase 3-positive cells within the grafts were counted manually and the total number of cells was calculated according to Abercrombie (1946). In the sections stained for TH, positively stained cells without condensed chromatin or granular appearance were counted. The graft–host border in the FBDP- and caspase 3-stained sections was distinguished by comparing with the immediately adjacent TH-stained sections. The morphology of the grafted cells and the presence of erythrocytes were assessed in the semi-thin sections. Apoptotic cells were considered to be cells with highly condensed chromatin. Brain sections were evaluated using a BX60 microscope (Olympus, MicroMacro AB, Göteborg, Sweden) and photographs taken with a DP50 cooled digital camera (Olympus). Double-stained sections were evaluated in a fluorescence microscope and the number of cells double stained for TH and FBDP or TH and caspase 3 was counted in all the sections in the series.

Statistics

All statistical analyses were performed using STATVIEW (version 5.0). One factor analysis of variance (ANOVA) was used to compare several different groups and was followed by Scheffé's post-hoc test to evaluate individual group differences.

Results

Semi-thin sections through transplants

Cells with apoptotic morphology, i.e. cells with highly condensed chromatin, were observed within the grafts at the

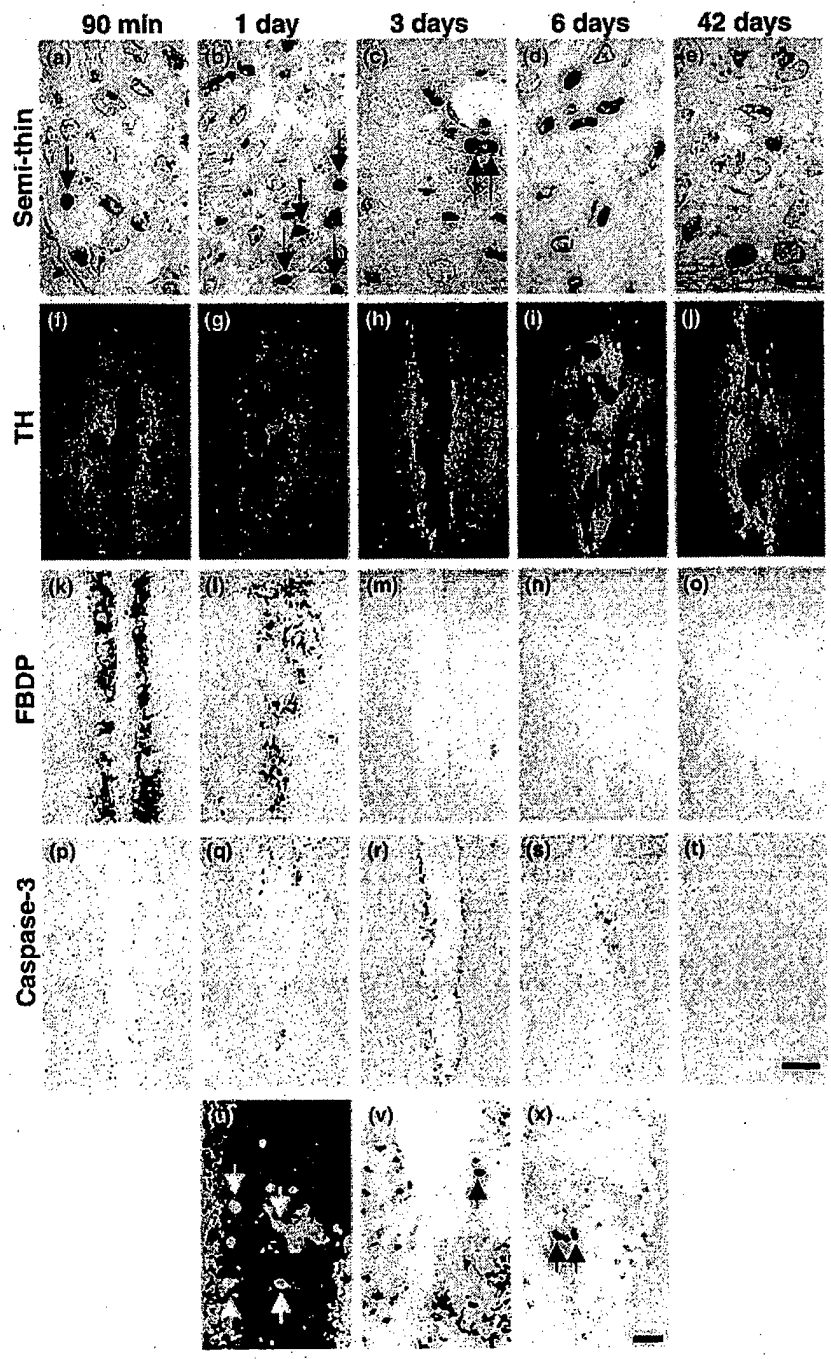


Fig. 1 (a–e) Photographs of semi-thin sections showing clearly viable and apoptotic cells, including numerous erythrocytes. Arrows indicate cells with highly condensed chromatin. Photographs of paraffin sections stained for (f–j) tyrosine hydroxylase (TH), (k–o) fodrin breakdown product (FBDP) or (p–t) active caspase 3. (u) Numerous TH-positive neurones (arrows) were observed at all time points with a weaker expression at the earlier time points. (v) Staining for FBDP (arrow) revealed extensive calpain activity at 90 min and 1 day post-implantation whereas (x) caspase 3-positive cells were primarily detected at 1 and 3 days (arrow). Magnification: (a–e). 600× (scale bar 10 μm); (f–t), 100× (scale bar 200 μm) and (u–x), 400× (scale bar 20 μm).

four earliest time points (Figs 1a–e). Evaluation of the number of apoptotic cells per section revealed a significant peak at 1 day after implantation compared with the other time points ($F(4,9) = 12.2$, $p = 0.0011$) (Fig. 2a). We also observed numerous neutrophils and erythrocytes that were characterized according to their morphologies in the oedematous core of the transplants, which largely consisted of a fluid-filled cavity, up to 6 days after transplantation. In brain sections from animals that received sham transplants we observed few scattered apoptotic cells in the striatum adjacent to the implant, primarily at the 90 min and 1 day time points (data not shown). The vehicle sham injections caused tissue damage and oedema in the host cortex and striatum along the needle tract, similar to tissue implantation.

Tyrosine hydroxylase immunohistochemistry

Every tenth section was stained for TH. We observed TH-positive neurones at all time points examined, i.e.

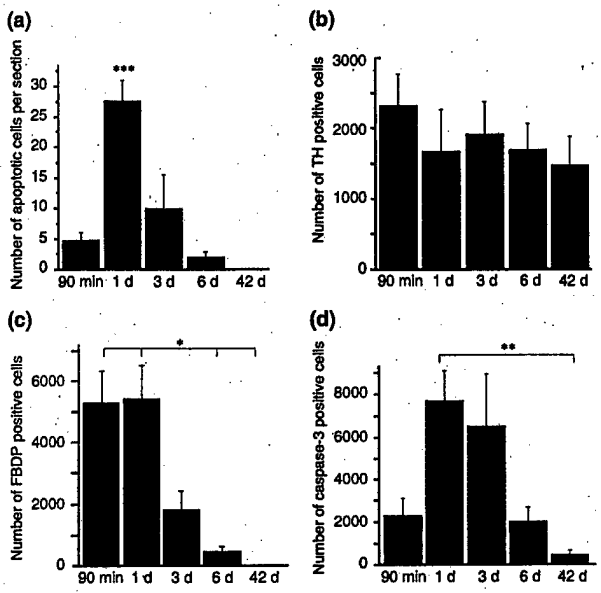


Fig. 2 (a) Number of cells with condensed chromatin in the methylene blue-stained semi-thin sections and number of cells stained for (b) tyrosine hydroxylase (TH), (c) fodrin breakdown product (FBDP) or (d) active caspase 3. There was a significant difference in the number of cells with apoptotic morphology over time in the semi-thin sections, expressed as number of cells per brain section. However, there was no significant difference over time in the number of TH-positive neurones. The number of FBDP- and caspase 3-positive cells decreased significantly over time. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.0011$.

90 min, 1, 3, 6 and 42 days after implantation (Figs 1f–j and u). There was no significant difference over time in the number of TH-positive neurones ($F(4,14) = 0.42$, $p > 0.05$) (Fig. 2b). Staining for TH appeared to be weaker at early time points after grafting and gradually increased in intensity at later times (Figs 1f–j). At 90 min and 1 day after surgery, the cell bodies were smaller in size with few, if any, neurites (Fig. 1u).

Fodrin breakdown product immunohistochemistry

We observed numerous FBDP-immunopositive cells in grafts early after transplantation (Figs 1k–o and v). Statistical analysis revealed a significant difference over time regarding the number of cells positive for FBDP in the grafts ($F(4,14) = 12.9$, $p = 0.0001$) (Fig. 2c). Post-hoc analysis with Scheffé's test showed no difference between the 90 min and 1 day time points ($p > 0.05$) but there was a difference between these two early time points compared with both 6 and 42 days after surgery ($p < 0.05$). The 3-day group differed from the 1-day group ($p < 0.05$) but not from the 90 min time point ($p > 0.05$). The magnitude of FBDP staining in the recipient brains surrounding the graft site was similar to that observed in sham-injected animals (data not shown). Thus, in addition to the numerous cells labelled in the grafts, there was also a rim of FBDP-positive cells in the striatum adjacent to the graft and in the cortex along the cannula tract.

Active caspase 3 immunohistochemistry

Cells positive for caspase 3 were observed from 90 min to 6 days after transplantation (Figs 1p–t and x). Scheffé's post-hoc test revealed no difference between 1 day and either 90 min, 3 or 6 days post implantation ($p > 0.05$) but there was a significant difference between 1 and 42 days ($F(4,14) = 5.0$, $p = 0.01$) (Fig. 2d). Similar to semi-thin sections and sections stained for FBDP, there were some degenerating cells (positive for caspase 3) along the needle tract up to 3 days after grafting in the sham-operated animals (data not shown).

Double stainings

Cells positive for both TH and FBDP or TH and caspase 3 were observed up to 6 days after grafting (Fig. 3). The number of TH-positive cells that were also positive for FBDP or caspase 3 changed over time, similar to the changes in the total number of FBDP- and caspase 3-positive cells, i.e. largest at 90 min and 1 day, respectively. The number of TH-positive cells that were also positive for FBDP decreased from 64.2% at 90 min to 53% at 1 day, 10% at 3 days and 15.8% at 6 days. The percentage of TH-positive cells double-positive for caspase 3 was initially 11.9%, increased to

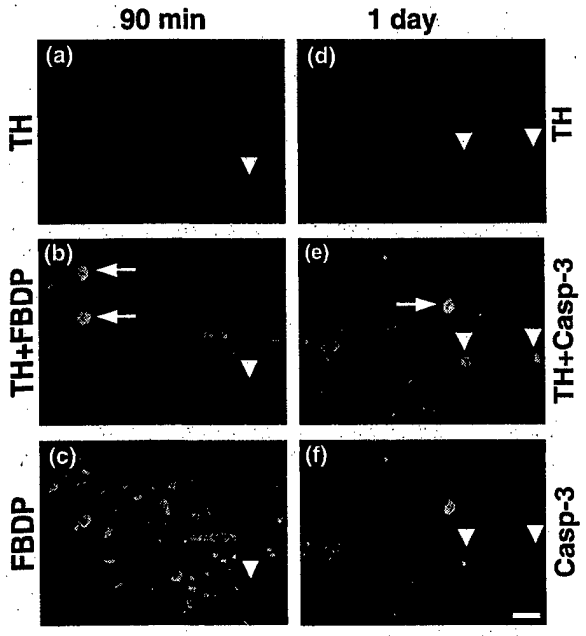


Fig. 3 Double staining for (a and d) tyrosine hydroxylase (TH) (arrowheads) and (c) fodrin breakdown product (FBDP) or (f) active caspase 3. Photographs were taken at 90 min (a–c) or 1 day after implantation (d–f) with cells positive for both TH and FBDP (b) or caspase 3 (e). Double-positive cells are indicated with arrows. Magnification: 600× (scale bar 20 μm).

23.6% 1 day after implantation, 28.4% after 3 days and decreased after 6 days to 6.1%.

Discussion

In the present study we evaluated cell death in embryonic nigral transplants at different time points between 90 min and 6 weeks after implantation into the striatum of adult rats. Initially, we examined grafts that had been embedded in epoxy plastic, cut into semi-thin sections and stained with azur and methylene blue. This technique provided two advantages. First, the internal structure of the brain sections was well preserved. In contrast, previous studies, e.g. using free-floating frozen sections (Duan *et al.* 1995; Emgård *et al.* 1999) or paraffin-embedded sections (Sortwell *et al.* 2000), describe that during the first few days after tissue implantation large portions of the graft tissue seem to be missing and fall out from the sections. Second, in semi-thin sections labelled with routine stains it is possible to discern, e.g. detailed nuclear morphology in individual cells. Thereby, apoptotic profiles can be observed and information obtained regarding ongoing cell death in the grafts. At 90 min after surgery, the earliest time point examined, the central parts of the grafts typically were devoid of nucleated cells. Instead, we found fluid-filled cavities that contained scattered erythrocytes, reminiscent of the description of implanted strands of embryonic nigral tissue provided by Zawada *et al.* (1998). Thus, previous reports, describing that tissue sections fall apart in the striatum if brains are sectioned soon after transplantation surgery (Duan *et al.* 1995; Emgård *et al.* 1999; Sortwell *et al.* 2000), may have partly misinterpreted their findings. What was earlier described as an artifact of tissue preparation probably represents an oedematous area in the host striatum, secondary to the injection of a significant volume of vehicle together with the grafted cells. In agreement with Zawada *et al.* (1998), we found that during the first day after surgery the grafted cells adhered to the walls of the fluid-filled cavity. Several earlier studies have pointed out that in mature nigral grafts the dopaminergic neurones tend to be located around the periphery of the transplant tissue (see, e.g. Duan *et al.* 1995; Emgård *et al.* 1999). This has been suggested to be due to selective cell death of dopaminergic neurones in the centre of the grafts due to poor trophic support or migration of the dopaminergic neurones from central portions to the periphery (Emgård *et al.* 1999). The current study does not exclude such events but suggests that the core of the graft site may initially be completely devoid of neural cells.

We have previously described the temporal evolution of cell death in nigral transplants between 6 and 42 days after implantation surgery using Fluoro-Jade as a marker for dying/dead cells (Emgård *et al.* 1999). We found that Fluoro-Jade-labelled cells were most frequent at the earliest, 6-day time point. The semi-thin sections in the present study provided us with evidence for widespread cell death in the transplants much sooner after transplantation. We observed numerous cells with condensed nuclei at 1 and 3 days after surgery and some degenerating cells were also apparent at 6 days. These observations agree with several previous reports that have described cells positive for terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labelling (TUNEL) (Zawada *et al.* 1998; Schierle *et al.* 1999a; Duan *et al.* 2000; Sortwell *et al.* 2000; Helt *et al.* 2001) between 1 and 4 days after surgery. The observations of cell death soon after graft implantation stimulated us to further characterize the type of cell death occurring in the nigral grafts by using specific cell death markers.

Caspase 3 is one of the major executor caspases in the cell death cascade. Caspase substrates are typically structural proteins or proteins involved in cellular homeostasis and repair (for review see Chan and Mattson 1999). The immature brain retains its apoptotic machinery to a larger extent than the adult brain, at least as judged by the presence of caspase 3 (Ni *et al.* 1998; Blomgren *et al.* 2001). Immature grafted neurones may, therefore, be more prone to apoptotic death while terminally differentiated neurones are more likely to die by necrosis (Hu *et al.* 2000). In the semi-thin sections, we observed a peak in apoptotic cell death 1 day after transplantation. In addition, apoptotic cell death was detected using immunohistochemisty for active caspase 3, showing marked staining at 1 and 3 days after grafting. We obtained similar results (data not shown) using a hairpin probe oligonucleotide, which has been shown to correlate with caspase 3 activation (Zhu *et al.* 2000), as an alternative marker for apoptotic neurones. Several different triggers may have initiated this apoptotic cell death. For cells dying soon after implantation, it is likely that the graft tissue preparation itself has triggered a delayed apoptosis (Emgård *et al.* 2002). This may either be due to severe mechanical stress (Fawcett *et al.* 1995) and metabolic perturbation (Castilho *et al.* 2000) during tissue dissociation or to loss of specific cell contacts, known to induce special forms of programmed cell death (anoikis) in other cell systems (Sakai *et al.* 2000; Ling *et al.* 2001). At later time points, the triggering of apoptosis may have been generated by the host brain environment (Sinclair *et al.* 1999; Karlsson *et al.* 2000; Zietlow *et al.* 2002). The presence of toxic cytokines and an unfavourable ionic milieu in the traumatized striatum, as well as an absence of appropriate neurotrophic factors, are all conceivable triggers of apoptosis.

Our results obtained using the antibody against active caspase 3 are essentially in agreement with earlier findings demonstrating apoptotic cells in grafts during the first 1–2 weeks after transplantation and a decline in their numbers over time (Zawada *et al.* 1998; Sortwell *et al.* 2000, 2001). Earlier studies on apoptosis in nigral transplants have used TUNEL as the primary marker for dying cells (Mahalik *et al.* 1994; Zawada *et al.* 1998; Schierle *et al.*

1999a; Duan *et al.* 2000; Sortwell *et al.* 2000, 2001; Helt *et al.* 2001). For example, Sortwell *et al.* (2000) found significantly more TUNEL-positive cells at 4 days than at 7 and 28 days. Similarly, Schierle *et al.* (1999a) and Duan *et al.* (2000) reported relatively low numbers of TUNEL-positive cells in nigral grafts at 2 and 4 days after surgery, respectively. There is an extensive loss of dopaminergic neurones in nigral grafts (Brundin *et al.* 2000) and yet relatively low numbers of cells are positive for either TUNEL or caspase 3. This can be interpreted in many different ways. First, a large proportion of the dopaminergic neuroblasts may have died during the graft tissue preparation or before 90 min after implantation. Indeed, earlier work suggests that there is significant cell death, including apoptosis, already during the tissue preparation steps (for review see Brundin *et al.* 2000; Emgård *et al.* 2002). Second, the time it takes for a cell to undergo apoptosis, from the onset of nuclear DNA fragmentation until it is cleared by a phagocyte, has been estimated to be as short as 1–3 h (Gavrieli *et al.* 1992). Therefore, only small numbers of cells would be expected to be caspase 3- or TUNEL-positive at any given time, even if apoptosis is a major contributor to cell death. Third, it is possible that apoptosis is not the major route of cell death followed by degenerating cells in mesencephalic transplants (Hurelbrink *et al.* 2001) and that necrosis contributes significantly to the demise of grafted cells. Indeed, even though several earlier studies have reported increased survival by inhibition of apoptosis, the protection is not complete. For this reason we were interested in studying markers of cell death not primarily associated with apoptosis.

Calpains have been implicated in excitotoxic neuronal injury and necrosis (Siman and Noszek 1988; Seubert *et al.* 1989; Saido *et al.* 1994; Blomgren *et al.* 1995) and pharmacological inhibitors of calpains exert a cerebroprotective effect (Lee *et al.* 1991; Rami and Krieglstein 1993; Bartus *et al.* 1994). Fodrin is a well-known calpain substrate and detection of specific FBDPs has been widely used to detect calpain activity (Siman *et al.* 1984; Nixon 1986; Roberts-Lewis *et al.* 1994; Saido *et al.* 1994; Bahr *et al.* 1995; Blomgren *et al.* 1999, 2001; Emgård *et al.* 2002). Calpain activity is high in the developing brain (Blomgren and Karlsson 1989; Blomgren *et al.* 1995) but FBDP is not detectable in normal adult brain tissue (e.g. Seubert *et al.* 1989).

The number of FBDP-positive cells in the graft was already high 90 min after transplantation and remained at this level for at least 24 h. After 3 days the number was dramatically reduced and FBDP-positive cells were virtually non-existent after 42 days. This suggests that calpain activation is primarily involved in the death of grafted cells during the first 24 h after transplantation. The coexistence of FBDP- and caspase 3-positive cells at the early time points may either indicate that necrotic and apoptotic processes take place in parallel or that calpain activation triggers the activation of caspase 3 (Nakagawa and Yuan 2000; Blomgren *et al.* 2001). A growing body of literature suggests functional connections between calpains and caspases (Wang 2000). Common substrate proteins have been identified, such as fodrin (Nath *et al.* 1996; Vanags *et al.* 1996; Jänicke *et al.* 1998; Wang *et al.* 1998) and calpastatin (Pörn-Ares *et al.* 1998; Wang *et al.* 1998). In a model of neonatal, cerebral hypoxia–ischaemia, two phases of fodrin degradation have been observed, where the early phase entailed mainly production of the calpain-specific 150-kDa FBDP and the later phase also included the caspase 3-specific 120-kDa FBDP (Wang *et al.* 2001). Hence, the initially simple classification of calpains being involved in cytotoxic and caspases in apoptotic cell death has proven to be far more complex. It can, therefore, be speculated that pharmacological calpain inhibition during tissue preparation and during the first 24 h following grafting would greatly improve graft cell survival.

The number of TH-positive cells in the grafts did not change between 90 min and 42 days after transplantation. We have previously observed that a large proportion of cells in the mesencephalic cell suspensions undergoes degenerative changes before implantation (Emgård *et al.* 2002) and, therefore, a major portion of the death of TH neurones may be complete by the time they are injected into the host brain. However, there may be a discrepancy between the apparent numbers of immunopositive dopamine neurones and the numbers of neurones carrying the TH gene, from 90 min after graft injection and beyond. Although it is known that, at embryonic day 14, rat nigral neurones express TH at the time of grafting and are still TH immunoreactive after they have been subjected to tissue dissociation (Sauer and Brundin 1991), it is possible that TH expression is down-regulated during the first days after grafting, as previously suggested (Zawada *et al.* 1998). Indeed, in the present study we observed many weakly stained TH neurones with few neurites up to 6 days after surgery, suggesting that the expression of TH is relatively low during the first days after implantation. Therefore, by using immunohistochemical detection, the number of dopaminergic neurones early after transplantation may be underestimated. Furthermore, some dopaminergic neurones may not have fully expressed their phenotype at the time of grafting and their continued development and expression of TH *de novo* could compensate for the gradual death of grafted dopaminergic neurones. A similar alternative explanation for the constant numbers of TH-positive neurones in the grafts despite an ongoing cell death is that neurogenesis may take place in parallel and exactly balance the cell loss. Speaking strongly against this possibility are the observations of Sinclair *et al.* (1999), indicating that genesis of transplanted nigral dopamine is complete in rats by embryonic day 14.

Recent reports suggest that calpains or caspase 3 may not only be activated during cell death but can also be stimulated

during cellular repair and cytoskeletal remodelling. The early calpain activation may not merely indicate necrotic cell death but can be evidence of neurones experiencing stress with resultant cyoskeletal damage (Bahr *et al.* 2002). It has been suggested by Bahr *et al.* (2002) that neurones can survive even though they have been subjected to a transient calpain activation. Moreover, activation of caspase 3 was recently proposed to be involved in the neuroprotection seen by preconditioning of brain ischaemia (McLaughlin *et al.* 2003). There is a possibility that some of the cells in the present report which stained positive for both TH and FBDP or caspase 3 may actually survive. This could, to some degree, explain why we observed a relatively high number of double-positive cells but without a decrease in the total number of dopaminergic cells over time.

In conclusion, the survival and morphology of nigral grafts have not previously been studied in detail in tissue sections as early as 90 min after transplantation. Using plastic or paraffin embedding we were able to successfully monitor cell death in embryonic nigral tissue from 90 min until 6 weeks after grafting. Application of specific cell death markers provided further insight into mechanisms of cell death and our results suggest that not only the apoptotic demise of neurones is important in nigral transplants. Calpain activation also appeared to contribute to the death of grafted cells during the first 24 h following transplantation and, therefore, pharmacological inhibition of calpain activity may prove a fruitful strategy to improve cell survival in nigral grafts.

Acknowledgements

We gratefully acknowledge the assistance from Birgit Haraldsson and Britt Lindberg at the Section for Neuronal Survival at Lund University and Ingrid Hallberg at the hand surgery clinic in Malmö, Sweden. This work was supported by the Swedish Research Council (PB and KB), the Swedish Parkinson's Disease Foundation, the National Society for Neurologically Disabled, the Åhlén Foundation, the Wilhelmi and Martina Lundgren Foundation, the Sven Jerring Foundation, the Magnus Bergvall Foundation, the Frimurare Barnhus Foundation, the Göteborg Medical Society, the Swedish Society for Medical Research and the Swedish Society of Medicine.

References

Abercrombie M. (1946) Estimation of nuclear population from microtome sections. *Anat. Rec.* **94**, 239–247.

Bahr B., Tirivedhi S., Park G. and Lynch G. (1995) Induction of calpain-mediated spectrin fragments by pathogenic treatments in long-term hippocampal slices. *J. Pharmacol. Exp. Ther.* **273**, 902–908.

Bahr B. A., Bendiske J., Brown Q. B., Munirathinam S., Caba E., Rudin M., Urwyler S., Sauter A. and Rogers G. (2002) Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp. Neurol.* **174**, 37–47.

Barker R. A., Dunnett S. B., Faissner A. and Fawcett J. W. (1996) The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. *Exp. Neurol.* **141**, 79–93.

Bartus R. T., Hayward N. J., Elliott P. J., Sawyer S. D., Baker K. L., Dean R. L., Akiyama A., Straub J. A., Harbeson S. L., Li Z. *et al.* (1994) Calpain inhibitor AK295 protects neurons from focal brain ischemia. Effects of post-occlusion intra-arterial administration. *Stroke* **25**, 2265–2270.

Blomgren K. and Karlsson J. O. (1989) Developmental changes of calpain and calpastatin in rabbit brain. *Neurochem. Res.* **14**, 1149–1152.

Blomgren K., Kawashima S., Saido T. C., Karlsson J. O., Elmered A. and Hagberg H. (1995) Fodrin degradation and subcellular distribution of calpains after neonatal rat cerebral hypoxic-ischemia. *Brain Res.* **684**, 143–149.

Blomgren K., Hallin U., Andersson A. L., Puka-Sundvall M., Bahr B. A., McRae A., Saido T. C., Kawashima S. and Hagberg H. (1999) Calpastatin is up-regulated in response to hypoxia and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. *J. Biol. Chem.* **274**, 14 046–14 052.

Blomgren K., Zhu C., Wang X., Karlsson J. O., Leverin A. L., Bahr B. A., Mallard C. and Hagberg H. (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia. A mechanism of 'pathological apoptosis'? *J. Biol. Chem.* **276**, 10 191–10 198.

Brundin P. and Hagell P. (2001) The neurobiology of cell transplantation in Parkinson's disease. *Clin. Neurosci. Res.* **1**, 507–520.

Brundin P., Karlsson J., Emgård M., Schierle G. S., Hansson O., Petersen A. and Castilho R. F. (2000) Improving the survival of grafted dopaminergic neurons: a review over current approaches. *Cell Transplant.* **9**, 179–195.

Castilho R. F., Hansson O. and Brundin P. (2000) Improving the survival of grafted embryonic dopamine neurons in rodent models of Parkinson's disease. *Prog. Brain Res.* **127**, 203–231.

Chan S. L. and Mattson M. P. (1999) Caspase and calpain substrates: roles in synaptic plasticity and cell death. *J. Neurosci. Res.* **58**, 167–190.

Cicchetti F., Costantini L., Belizaire R., Burton W., Isacson O. and Fodor W. (2002) Combined inhibition of apoptosis and complement improves neural graft survival of embryonic rat and porcine mesencephalon in the rat brain. *Exp. Neurol.* **177**, 376–384.

Clarke D. J. and Branton R. L. (2002) A role for tumor necrosis factor alpha in death of dopaminergic neurons following neural transplantation. *Exp. Neurol.* **176**, 154–162.

Croall D. E. and DeMartino G. N. (1991) Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol. Rev.* **71**, 813–847.

Duan W. M., Widner H. and Brundin P. (1995) Temporal pattern of host responses against intrastriatal grafts of syngeneic, allogeneic or xenogeneic embryonic neuronal tissue in rats. *Exp. Brain Res.* **104**, 227–242.

Duan W. M., Zhao L. R., Westerman M., Lovick D., Furcht L. T., McCarthy J. B. and Low W. C. (2000) Enhancement of nigral graft survival in rat brain with the systemic administration of synthetic fibronectin peptide V. *Neuroscience* **100**, 521–530.

Emgård M., Karlsson J., Hansson O. and Brundin P. (1999) Patterns of cell death and dopaminergic neuron survival in intrastriatal nigral grafts. *Exp. Neurol.* **160**, 279–288.

Emgård M., Blomgren K. and Brundin P. (2002) Characterization of cell damage and death in embryonic mesencephalic tissue: a study on ultrastructure, vital stains and protease activity. *Neuroscience* **115**, 1177–1187.

Espejo M., Cutillas B., Arenas T. E. and Ambrosio S. (2000) Increased survival of dopaminergic neurons in striatal grafts of fetal ventral

mesencephalic cells exposed to neurotrophin-3 or glial cell line-derived neurotrophic factor. *Cell Transplant.* **9**, 45–53.

Fawcett J. W., Barker R. A. and Dunnett S. B. (1995) Dopaminergic neuronal survival and the effects of bFGF in explant, three dimensional and monolayer cultures of embryonic rat ventral mesencephalon. *Exp. Brain Res.* **106**, 275–282.

Gavrieli Y., Sherman Y. and Ben-Sasson S. A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493–501.

Gray J., Haran M. M., Schneider K., Vesce S., Ray A. M., Owen D., White I. R., Cutler P. and Davis J. B. (2001) Evidence that inhibition of cathepsin-B contributes to the neuroprotective properties of caspase inhibitor Tyr-Val-Ala-Asp-chloromethyl ketone. *J. Biol. Chem.* **276**, 32 750–32 755.

Hagell P. and Brundin P. (2001) Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease. *J. Neuropathol. Exp. Neurol.* **60**, 741–752.

Hansson O., Castilho R. F., Kaminski Schierle G. S., Karlsson J., Nicotera P., Leist M. and Brundin P. (2000) Additive effects of caspase inhibitor and lazaroid on the survival of transplanted rat and human embryonic dopamine neurons. *Exp. Neurol.* **164**, 102–111.

Helt C. E., Hoernig G. R., Albeck D. S., Gerhardt G. A., Ickes B., Reyland M. E., Quissell D. O., Stromberg I. and Granholm A. C. (2001) Neuroprotection of grafted neurons with a GDNF/caspase inhibitor cocktail. *Exp. Neurol.* **170**, 258–269.

Hong S. C., Goto Y., Lanzino G., Soleau S., Kassell N. F. and Lee K. S. (1994) Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. *Stroke* **25**, 663–669.

Hu B. R., Liu C. L., Ouyang Y., Blomgren K. and Siesjo B. K. (2000) Involvement of caspase-3 in cell death after hypoxia-ischemia declines during maturation. *J. Cereb. Blood Flow Metab.* **20**, 1294–1300.

Huang Y. and Wang K. K. (2001) The calpain family and human disease. *Trends Mol. Med.* **7**, 355–362.

Hurelbrink C. B., Armstrong R. J., Tuheshi L. M., Dunnett S. B., Rosser A. E. and Barker R. A. (2001) Death of dopaminergic neurons in vitro and in nigral grafts: reevaluating the role of caspase activation. *Exp. Neurol.* **171**, 46–58.

Jänicke R., Ng P., Sprengart M. and Porter A. (1998) Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J. Biol. Chem.* **273**, 15 540–15 545.

Karlsson J., Emgård M., Gidö G., Wieloch T. and Brundin P. (2000) Increased survival of embryonic nigral neurons when grafted to hypothermic rats. *Neuroreport* **11**, 1665–1668.

Lee K. S., Frank S., Vanderklish P., Arai A. and Lynch G. (1991) Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc. Natl Acad. Sci. USA* **88**, 7233–7237.

Lindvall O. and Hagell P. (2000) Clinical observations after neural transplantation in Parkinson's disease. *Prog. Brain Res.* **127**, 299–320.

Ling Y., Zhong Y. and Perez-Soler R. (2001) Disruption of cell adhesion and caspase-mediated proteolysis of beta- and gamma-catenins and APC protein in paclitaxel-induced apoptosis. *Mol. Pharmacol.* **59**, 593–603.

Mahalik T. J., Hahn W. E., Clayton G. H. and Owens G. P. (1994) Programmed cell death in developing grafts of fetal substantia nigra. *Exp. Neurol.* **129**, 27–36.

McLaughlin B., Hartnett K. A., Erhardt J. A., Legos J. J., White R. F., Barone F. C. and Aizenman E. (2003) Caspase 3 activation is essential for neuroprotection in preconditioning. *Proc. Natl Acad. Sci. USA* **100**, 715–720.

Nakagawa T. and Yuan J. (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell. Biol.* **150**, 887–894.

Nath R., Raser K. J., Stafford D., Hajimohammadreza I., Posner A., Allen H., Talanian R. V., Yuen P., Gilbertsen R. B. and Wang K. K. (1996) Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. *Biochem. J.* **319**, 683–690.

Ni B., Wu X., Su Y., Stephenson D., Smalstig E. B., Clemens J. and Paul S. M. (1998) Transient global forebrain ischemia induces a prolonged expression of the caspase-3 mRNA in rat hippocampal CA1 pyramidal neurons. *J. Cereb. Blood Flow Metab.* **18**, 248–256.

Nicholson D. W. and Thornberry N. A. (1997) Caspases: killer proteases. *Trends Biochem. Sci.* **22**, 299–306.

Nixon R. A. (1986) Fodrin degradation by calcium-activated neutral proteinase (CANP) in retinal ganglion cell neurons and optic glia: preferential localization of CANP activities in neurons. *J. Neurosci.* **6**, 1264–1271.

Piccini P., Brooks D. J., Björklund A., Gunn R. N., Grasby P. M., Rimoldi O., Brundin P., Hagell P., Rehncrona S., Widner H. *et al.* (1999) Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. *Nature Neurosci.* **2**, 1137–1140.

Pörn-Ares M. I., Samali A. and Orrenius S. (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. *Cell Death Differ.* **5**, 1028–1033.

Rami A. and Krieglstein J. (1993) Protective effects of calpain inhibitors against neuronal damage caused by cytotoxic hypoxia in vitro and ischemia in vivo. *Brain Res.* **609**, 67–70.

Redmond D. E. (2002) Cellular replacement therapy for Parkinson's disease — where we are today? *Neuroscientist* **8**, 457–488.

Roberts-Lewis J., Savage M., Marcy V., Pinsker L. and Siman R. (1994) Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J. Neurosci.* **14**, 3934–3944.

Saatman K. E., Murai H., Bartus R. T., Smith D. H., Hayward N. J., Perri B. R. and McIntosh T. K. (1996) Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. *Proc. Natl Acad. Sci. USA* **93**, 3428–3433.

Saido T., Sorimachi H. and Suzuki K. (1994) Calpain: new perspectives in molecular diversity and physiological pathological involvement. *FASEB J.* **8**, 814–822.

Sakai H., Kobayashi Y., Sakai E., Shibata M. and Kato Y. (2000) Cell adhesion is a prerequisite for osteoclast survival. *Biochem. Biophys. Res. Commun.* **270**, 550–556.

Sauer H. and Brundin P. (1991) Effects of cool storage on survival and function of intrastriatal ventral mesencephalic grafts. *Rest. Neurol. Neurosci.* **2**, 123–135.

Schierle G. S., Hansson O., Leist M., Nicotera P., Widner H. and Brundin P. (1999a) Caspase inhibition reduces apoptosis and increases survival of nigral transplants. *Nature Med.* **5**, 97–100.

Schierle G. S., Leist M., Martinou J. C., Widner H., Nicotera P. and Brundin P. (1999b) Differential effects of Bcl-2 overexpression on fibre outgrowth and survival of embryonic dopaminergic neurons in intracerebral transplants. *Eur. J. Neurosci.* **11**, 3073–3081.

Seubert P., Lee K. and Lynch G. (1989) Ischemia triggers NMDA receptor-linked cytoskeletal proteolysis in hippocampus. *Brain Res.* **492**, 366–370.

Siman R. and Noszek J. (1988) Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo. *Neuron* **1**, 279–287.

Siman R., Baudry H. and Lynch G. (1984) Brain fodrin: substrate for calpain I, an endogenous calcium-activated protease. *Proc. Natl Acad. Sci. USA* **81**, 3572–3576.

Sinclair S. R., Fawcett J. W. and Dunnett S. B. (1999) Delayed implantation of nigral grafts improves survival of dopamine neurones and rate of functional recovery. *Neuroreport* **10**, 1263–1267.

Sortwell C. E. (2003) Strategies for the augmentation of grafted dopamine neuron survival. *Front. Biosci.* **8**, 522–532.

Sortwell C. E., Daley B. F., Pitzer M. R., McGuire S. O., Sladek J. R. and Collier T. J. (2000) Oligodendrocyte-type 2 astrocyte-derived trophic factors increase survival of developing dopamine neurons through the inhibition of apoptotic cell death. *J. Comp. Neurol.* **426**, 143–153.

Sortwell C. E., Camargo M. D., Pitzer M. R., Gyawali S. and Collier T. J. (2001) Diminished survival of mesencephalic dopamine neurons grafted into aged hosts occurs during the immediate postgrafting interval. *Exp. Neurol.* **169**, 23–29.

Vanags D., Pörn-Ares M., Coppola S., Burgess D. and Orrenius S. (1996) Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J. Biol. Chem* **271**, 31 075–31 085.

Vanderklish P. W. and Bahr B. A. (2000) The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. *Int. J. Exp. Pathol.* **81**, 323–339.

Wang K., Posmantur R., Nath R., McGinnis K., Whitton M., Talanian R., Glantz S. and Morrow J. (1998) Simultaneous degradation of alphaII- and betaII-spectrin by caspase 3 (cpp32) in apoptotic cells. *J. Biol. Chem.* **273**, 22 490–22 497.

Wang K. K. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci.* **23**, 20–26.

Wang X., Karlsson J. O., Zhu C., Bahr B. A., Hagberg H. and Blomgren K. (2001) Caspase-3 activation after neonatal rat cerebral hypoxia-ischemia. *Biol. Neonate* **3–4**, 172–179.

Wolf B. B., Schuler M., Echeverri F. and Green D. R. (1999) Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. *J. Biol. Chem.* **274**, 30 651–30 656.

Zawada W. M., Zastrow D. J., Clarkson E. D., Adams F. S., Bell K. P. and Freed C. R. (1998) Growth factors improve immediate survival of embryonic dopamine neurons after transplantation into rats. *Brain Res.* **786**, 96–103.

Zawada W. M., Meintzer M. K., Rao P., Marotti J., Wang X., Esplen J. E., Clarkson E. D., Freed C. R. and Heidenreich K. A. (2001) Inhibitors of p38 MAP kinase increase the survival of transplanted dopamine neurons. *Brain Res.* **891**, 185–196.

Zhu C., Wang X., Hagberg H. and Blomgren K. (2000) Correlation between caspase-3 activation and three different markers of DNA damage in neonatal cerebral hypoxia-ischemia. *J. Neurochem.* **75**, 819–829.

Zietlow R., Sinclair S. R., Schwiening C. J., Dunnett S. B. and Fawcettt J. W. (2002) The release of excitatory amino acids, dopamine, and potassium following transplantation of embryonic mesencephalic dopaminergic grafts to the rat striatum, and their effects on dopaminergic neuronal survival in vitro. *Cell Transplant.* **11**, 637–652.

Journal of Neurochemistry, 2003, **85**, 1431–1442 doi:10.1046/j.1471-4159.2003.01794.x

Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome *c* and caspase-3 activation in neurons exposed to transient hypoglycemia

Michel Ferrand-Drake,*,† Changlian Zhu,‡ Gunilla Gidö,* Anker J. Hansen,§ Jan-Olof Karlsson,¶ Ben A. Bahr,** Naoufal Zamzami,†† Guido Kroemer,†† Pak H. Chan,† Tadeusz Wieloch* and Klas Blomgren‡‡,§§

**Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University Hospital, Lund, Sweden*

†Department of Neurosurgery, Department of Neurology and Neurological Sciences and Program in Neurosciences, Stanford University School of Medicine, Stanford, California, USA

‡Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China

§Department of Pharmacology, Novo Nordisk A/S Maaloev, Denmark

¶Institute of Anatomy and Cell Biology, Göteborg University, Göteborg, Sweden

***Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut, USA*

††Institut Gustave Roussy, Villejuif, France

‡‡Perinatal Center, Department of Physiology, Göteborg University, Göteborg, Sweden

§§Perinatal Center, Department of Pediatrics, Göteborg University, The Queen Silvia Children's Hospital, Göteborg, Sweden

Abstract

Blockade of mitochondrial permeability transition protects against hypoglycemic brain damage. To study the mechanisms downstream from mitochondria that may cause neuronal death, we investigated the effects of cyclosporin A on subcellular localization of apoptosis-inducing factor and cytochrome *c*, activation of the cysteine proteases calpain and caspase-3, as well as its effect on brain extracellular calcium concentrations. Redistribution of cytochrome *c* occurred at 30 min of iso-electricity, whereas translocation of apoptosis-inducing factor to nuclei occurred at 30 min of recovery following 30 min of iso-electricity. Active caspase-3 and calpain-induced fodrin breakdown products were barely detectable in the dentate gyrus and CA1 region of the hippocampus of rat brain exposed to 30 or 60 min of insulin-induced hypoglycemia. However, 30 min or 3 h after recovery of blood glucose levels, fodrin breakdown products and active caspase-3 markedly increased, concomitant with a twofold increase in caspase-3-like enzymatic activity. When rats were treated with neuroprotective doses of cyclosporin A, but not with FK 506, the redistribution of apoptosis-inducing factor and cytochrome *c* was reduced and fodrin breakdown products and active caspase-3 immuno-reactivity was diminished whereas the extracellular calcium concentration was unaffected. We conclude that hypoglycemia leads to mitochondrial permeability transition which, upon recovery of energy metabolism, mediates the activation of caspase-3 and calpains, promoting cell death.

Keywords: apoptosis-inducing factor, calpain, caspase-3, hypoglycemia mitochondrial permeability transition.

J. Neurochem. (2003) **85**, 1431–1442.

Received December 19, 2002; revised manuscript received March 3, 2003; accepted March 3, 2003.

Address correspondence and reprint requests to Michel Ferrand-Drake, Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University Hospital, 221 85, Lund, Sweden. E-mail: michelf@stanford.edu

Abbreviations used: Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin; AIF, apoptosis-inducing factor; CsA, cyclosporin A; DG, dentate gyrus; FBDP, fodrin breakdown product; MPT, mitochondrial permeability transition; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

Mitochondria are key regulators in the process of cell death through their capacity to release a number of pro-apoptotic proteins from their intermembrane space, such as cytochrome *c* and apoptosis-inducing factor (AIF) (Liu *et al.* 1996; Susin *et al.* 1996). One mechanism of mitochondrial membrane permeabilization involves opening of the Ca^{2+}-stimulated, cyclosporin A (CsA)-inhibitable mitochondrial permeability transition (MPT) pore (Haworth and Hunter 1980; Crompton *et al.* 1988; Broekemeier *et al.* 1989; Crompton and Costi 1990; Nicolli *et al.* 1996). During MPT, mitochondria undergo large amplitude swelling, the outer membrane disrupts and proteins residing in the intermembrane space are released (Jacotot *et al.* 1999). Release of cytochrome *c* from the mitochondria to the cytosol and subsequent complex formation among cytochrome *c*, dATP, apoptosis-activating factor-1 and procaspase-9 induces autolytic cleavage of caspase-9, which activates caspase-3 (Li *et al.* 1997). It has been confirmed that AIF can mediate neuronal cell death (Braun *et al.* 2002; Cregan *et al.* 2002; Klein *et al.* 2002; Yu *et al.* 2002; Zhang *et al.* 2002). Unlike cytochrome *c*, AIF acts in a caspase-independent manner (Susin *et al.* 1999).

Caspases have been identified as executors of apoptosis (Nicholson *et al.* 1995). Caspase-3 is the major downstream effector caspase in the brain, particularly in the immature brain (Blomgren *et al.* 2001). Calpains, another family of cysteine proteases, are calcium activated and proposed to participate in intracellular signal transduction (Saido *et al.* 1994). Calpains have mainly been implicated in excitotoxic neuronal injury and necrosis (Wang 2000). Pharmacological inhibition of calpains (Lee *et al.* 1991; Rami and Krieglstein 1993; Bartus *et al.* 1994; Hong *et al.* 1994) or caspases (Hara *et al.* 1997; Cheng *et al.* 1998; Endres *et al.* 1998) provides neuroprotection following cerebral ischemia. Calpains are strictly calcium dependent but the activity is tightly regulated by cofactors, such as calpastatin and phospholipids (Huang and Wang 2001). Furthermore, interactions between calpains and caspases have been demonstrated. Calpains have been shown to promote activation of caspase-3 (Nakagawa and Yuan 2000; Blomgren *et al.* 2001) and caspase-3 can cleave the endogenous calpain inhibitor, calpastatin (Porn-Ares *et al.* 1998; Wang *et al.* 1998; Blomgren *et al.* 1999).

Insulin-induced hypoglycemia, which constitutes an ever-present threat in the management of type I diabetes, leads to an axon-sparing dendro-somatic lesion, causing selective neurodegeneration of subpopulations in the brain (Auer *et al.* 1984b). Glutamate has been implicated in this process (Wieloch 1985). During the insult, the ionic gradients can no longer be maintained, leading to influx of Ca^{2+}, Na^+, Cl^- and H_2O (Siesjo and Bengtsson 1989), resulting in dendritic swelling concurrent with mitochondrial swelling (Auer *et al.* 1985a). We have demonstrated that mitochondrial swelling could be prevented by CsA, providing neuroprotection (Friberg *et al.* 1998). This suggests that mitochondria-dependent cell death processes are activated during hypoglycemia.

We hypothesized that influx of calcium and release of apoptogenic proteins through MPT would lead to activation of calpains and caspase-3 during and following hypoglycemia. Our aim was to characterize the protease activation and evaluate the effects of MPT inhibition by CsA.

Materials and methods

Surgical procedures

The hypoglycemia model described by Auer *et al.* (1984a) was used. For a detailed description of the surgical procedures, see Friberg *et al.* (1998). The ethical committee at the University of Lund approved all the animal experiments. Adult male Wistar rats from Møllegaard avlslaboratorium (Copenhagen, Denmark), weighing 300–340 g, were used. The animals were fasted overnight with access to water. Concomitant with the start of surgery, the rats received an i.p. injection of insulin (2 IU/kg; Actrapid; Novo A/S, Copenhagen, Denmark). Two s.c. biparietal needle electrodes were used to record a bipolar electro-encephalogram, which was monitored continuously. Isoelectric was defined as the time point when the electro-encephalogram turned isoelectric. At the onset of cerebral isoelectricity the blood pressure increased, which was compensated for by exsanguination, keeping the blood pressure between 140 and 160 mmHg. A bolus injection of 0.2 mL of 10% glucose solution, followed by infusion at a rate of 1.5 mL/h, terminated the period of isoelectricity. When the animals recovered spontaneous respiration, they were extubated and transferred to a cage with access to food and water.

Extracellular Ca^{2+} measurement

Double-barreled glass microelectrodes with a tip diameter of 5–7 μm were used for recording extracellular Ca^{2+} concentration and DC potential shift. One barrel was filled with calcium ionophore I (Cocktail A 21048; Fluka AG, Buchs, Switzerland) and 100 mM $CaCl_2$ and the other with 150 mM NaCl. Each barrel was connected with Ag-AgCl wires to a high input resistance amplifier. An Ag-AgCl wire was inserted s.c. into the neck for connection of the animal to the ground. The calcium electrode was calibrated in solutions containing 0.1, 0.5, 1.0 and 5.0 mM $CaCl_2$ before and after the experiment (Hansen and Zeuthen 1981; Gido *et al.* 1994). The animal was placed in a stereotaxic instrument and a burr hole was made in the parietal cortex at the following coordinates: bregma −3.6, lateral 0.9 mm for electrode placement into the dentate gyrus (DG). The microelectrode was placed at the brain surface and lowered 3.6 mm to reach the tip of the DG. Extracellular Ca^{2+} concentrations and DC potential shifts were recorded during 30 min of isoelectricity, followed by 15 min of recovery in the animals treated with CsA ($n = 6$) or vehicle ($n = 4$).

Immunohistochemistry

Animals were deeply anesthetized and perfusion fixed, following 30 or 60 min of isoelectricity or following 30 min of isoelectricity plus 30 min or 3 h of recovery, with 4% paraformaldehyde in 0.1 M

phosphate-buffered saline (PBS). The brains were rapidly removed and immersion fixed at 4°C overnight. After dehydration with graded ethanol and xylene, the brains were paraffin embedded and cut into 5-μm sections which were deparaffinized in xylene and rehydrated in graded ethanol before staining. All secondary antibodies were from Vector Laboratories (Burlingame, CA, USA). Each experimental group consisted of at least three animals.

Active caspase-3

Sections were pre-treated with proteinase K (Roche Diagnostics, Indianapolis, IN, USA), 10 μg/mL in PBS, for 10 min at ambient temperature. Antigen recovery was performed by boiling the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min and subsequent cooling for 30 min. Non-specific binding was blocked for 30 min with 4% goat serum in PBS. Anti-active caspase-3 (no. 280, against the p17 fragment, residues 176–277; a kind gift from Dr Donald W. Nicholson, Merck Frosst Center for Therapeutic Research, Quebec, Canada) was applied diluted 1 : 500 in PBS and incubated for 60 min at room temperature, followed by biotinylated goat anti-rabbit IgG (6 μg/mL in PBS) for 60 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS for 5 min. Visualization was performed using the VECTASTAIN ABC Elite kit (Vector Laboratories) with 0.5 mg/mL 3,3′-diaminobenzidine enhanced with 15 mg/mL ammonium nickel sulfate, 2 mg/mL beta-D glucose, 0.4 mg/mL ammonium chloride and 0.01 mg/mL beta-glucose oxidase (Sigma, St Louis, MO, USA). Negative controls, where the primary antibody was omitted, were completely blank. Pre-absorption of the primary antibody with an excess recombinant active caspase-3 (MBL, Nagoya, Japan) also yielded blank stainings. The active caspase-3 used for pre-absorption was not a pure preparation, but a mixture of proteins, and the molar concentration of caspase-3 was unknown. However, the total protein content was known and, calculating as if all the protein was actually active caspase-3, we used a molar ratio of 200 : 1 for pre-absorption. Hence, the surplus of caspase-3 to antibody was less than 200-fold but the precise ratio is unknown.

Apoptosis-inducing-factor

Antigen recovery and blocking were performed as above. Anti-AIF (Susin *et al.* 1999) was applied diluted 1 : 150 in Tris-buffered saline (TBS) containing 1% bovine serum albumin and 0.1% Triton X-100 and incubated for 60 min at room temperature, followed by a biotinylated goat anti-rabbit antibody (6 μg/mL in PBS) for 60 min. Peroxidase blocking and visualization were performed as above. Negative controls, where the primary antibody was omitted, were completely blank. The staining obtained with this AIF antibody and the D-20 antibody (2 μg/mL, sc-9416; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also virtually indistinguishable and pre-absorption with the AIF peptide abolished staining.

Calpain-specific fodrin breakdown product

Antigen recovery and blocking were performed as above. The anti-fodrin breakdown product (FBDP) (Bahr *et al.* 1995) was applied diluted 1 : 50 in PBS containing 0.2% Triton X-100 and incubated for 60 min at room temperature, followed by biotinylated goat anti-rabbit IgG (11 μg/mL in PBS) for 60 min. Peroxidase blocking and visualization were the same as above. Negative controls, where the primary antibody was omitted or where the primary antibody was incubated for 1 h at room temperature with a 20-fold excess of the peptide used to raise the antibody, were completely blank.

Cytochrome *c*

Antigen recovery was performed as above. Non-specific binding was blocked with 4% horse serum in PBS for 30 min. Anti-cytochrome *c* (clone 7H8.2C12; BD Pharmingen, San Diego, CA, USA), diluted 1 : 500 (2 μg/mL) in PBS, was incubated for 60 min at room temperature, followed by 60 min with a biotinylated horse anti-mouse antibody (2 μg/mL) diluted with PBS. Peroxidase blocking and visualization were the same as above. When the primary antibody was incubated with a 20-fold molar excess of cytochrome *c* from bovine heart (Sigma), the staining was abolished.

Preparation of samples for measurement of caspase-3 activity or western blotting

Animals were decapitated and the brains ($n = 5$) were quickly removed and frozen on dry ice. The region of choice was dissected at −18°C and homogenized by sonication in 50 mM Tris-HCl (pH 7.3), 5 mM EDTA, 100 mM NaCl and stored at −80°C until further processing. The protein concentration of whole cell homogenates was determined (Karlsson *et al.* 1994). For western blotting, samples were mixed with an equal volume of 3 × Laemmli buffer and heated at 96°C for 5 min.

Caspase-3-like activity assay

Samples of homogenate (50 μL) were mixed with 50 μL of extraction buffer, containing 50 mM Tris-HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail (Sigma) and 0.2% CHAPS, on a microtiter plate (Microfluor; Dynatech, Chantilly, VA, USA). After incubation for 15 min at room temperature, 100 μL of peptide substrate, 50 μM Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC; Peptide Institute, Osaka, Japan) in extraction buffer without inhibitors or CHAPS but with 4 mM dithiothreitol, was added. Caspase-3-like activity was measured by the appearance of the fluorescent cleavage product, AMC, detected using a Spectramax Gemini fluorometer (Molecular Devices, Sunnyvale, CA, USA). The excitation and emission wavelengths were 380 and 460 nm. Data were collected during the linear phase of degradation and expressed as pmol AMC formed/μg protein/min.

Western blotting

Samples containing 20 μg protein were separated on 8–16% Novex (Invitrogen, San Diego, CA, USA) Tris-glycine gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were washed in TBS-T (10 mM Tris, pH 7.4, 150 mM NaCl and 0.05% Tween 20) and blocked with 5% milk in TBS-T. Subsequently, the membranes were incubated with the FBDP antibody (1 : 50) for 1 h at room temperature or overnight at 4°C. Visualization was performed using a peroxidase-conjugated anti-rabbit secondary antibody, Super Signal chemiluminescent substrates (Pierce, Rockford, IL, USA) and RX film (Fuji Photo Film Co., Tokyo, Japan).

Cyclosporin A treatment

Animals were injected i.v. with a single dose of 50 mg/kg CsA (Sandimmun®; Sandoz, Basel, Switzerland) ($n = 3$) or vehicle

(7.5% ethanol and 0.85% Tween 80 in saline) ($n = 3$) approximately 30 min before the onset of isoelectricity. Subsequently, the animals were perfusion fixed with 4% paraformaldehyde and further processed for immunohistochemistry. Animals were killed at 30 min or 2 days of recovery following 30 min of isoelectricity.

FK 506 (Tacrolimus) treatment

To control for the immunosuppressive effects of CsA, i.e. inhibition of calcineurin, a series of animals were treated with FK 506 ($n = 3$). Animals were injected i.v. with 2 mg/kg as a single dose and killed at 2 days of recovery following 30 min of isoelectricity.

Results

Extracellular Ca^{2+} concentration during and following hypoglycemia

Figure 1 shows typical extracellular Ca^{2+} registrations in a vehicle-treated (Fig. 1a) and a CsA-treated (Fig. 1b) animal. In both CsA- and vehicle-treated animals the resting concentrations of extracellular Ca^{2+} were about 1 mM. At the beginning of isoelectricity and up to 10 min after, extracellular Ca^{2+} levels started to fall in both groups. Neither depolarization time nor the lowest extracellular Ca^{2+} concentration during isoelectricity differed between the groups. In animals treated with CsA or the vehicle the lowest extracellular Ca^{2+} concentration was 0.24 ± 0.19 and 0.12 ± 0.03 mM within a period of 19.2 ± 5.4 and 19.5 ± 9.0 min of depolarization, respectively. When hypoglycemia was terminated with glucose infusion, the extracellular Ca^{2+} concentration increased to about 75% of the initial concentration within 15 min (data not shown). The changes in extracellular Ca^{2+} concentrations are reflected in the change in the DC potential.

Redistribution of apoptosis-inducing factor and cytochrome *c* during and following hypoglycemia

Apoptosis-inducing factor staining was weak and diffuse in the medial CA1 region of the hippocampus and in the crest of the DG on sections from the control animals (Fig. 2a). After

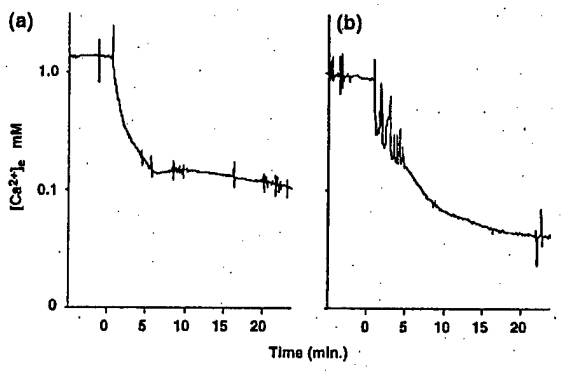


Fig. 1 Typical recordings of the extracellular calcium concentration in the dentate gyrus in hypoglycemic rats treated with (a) vehicle or (b) cyclosporin A. Time zero indicates the onset of isoelectricity.

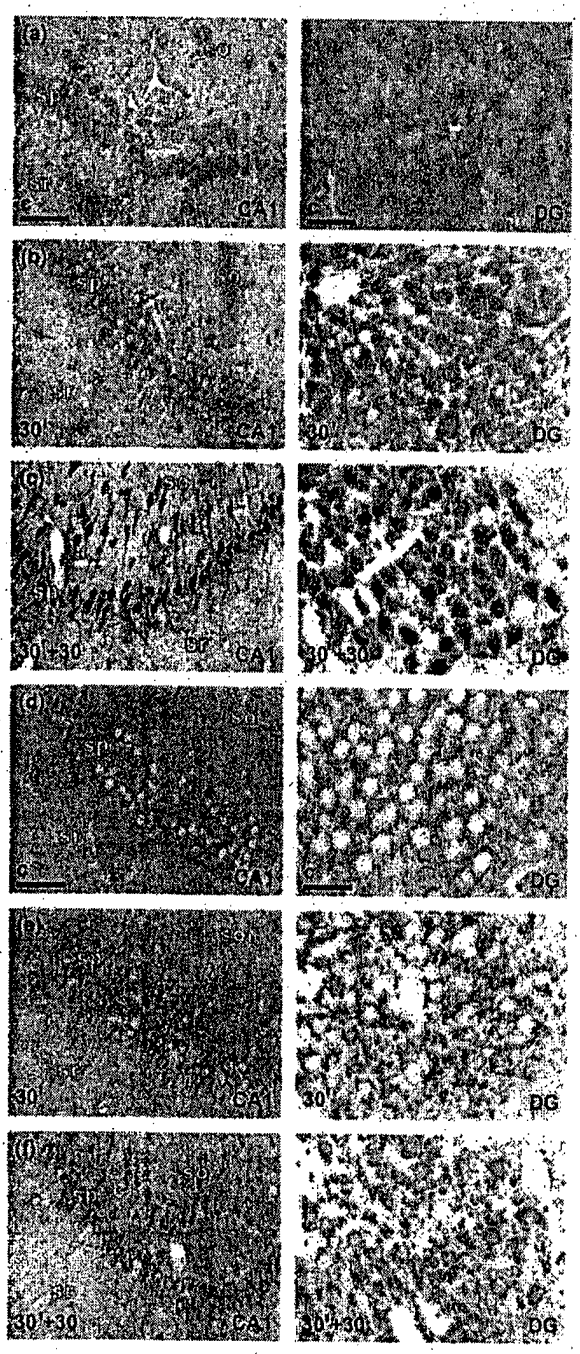


Fig. 2 Photomicrographs showing (a–c) apoptosis-inducing factor or (d–f) cytochrome *c* staining in the hippocampal CA1 region and in the crest of the dentate gyrus (DG). Sections from the control (a and d), 30 min of isoelectricity (b and e) and 30 min of isoelectricity plus 30 min of recovery (c and f). c, Control; 30′, 30 min of isoelectricity; 30′ + 30, 30 min of isoelectricity + 30 min of recovery; sp, stratum pyramidale; so, stratum oriens; sr, stratum radiatum. Scale bars, CA1, 80 μm; DG, 20 μm.

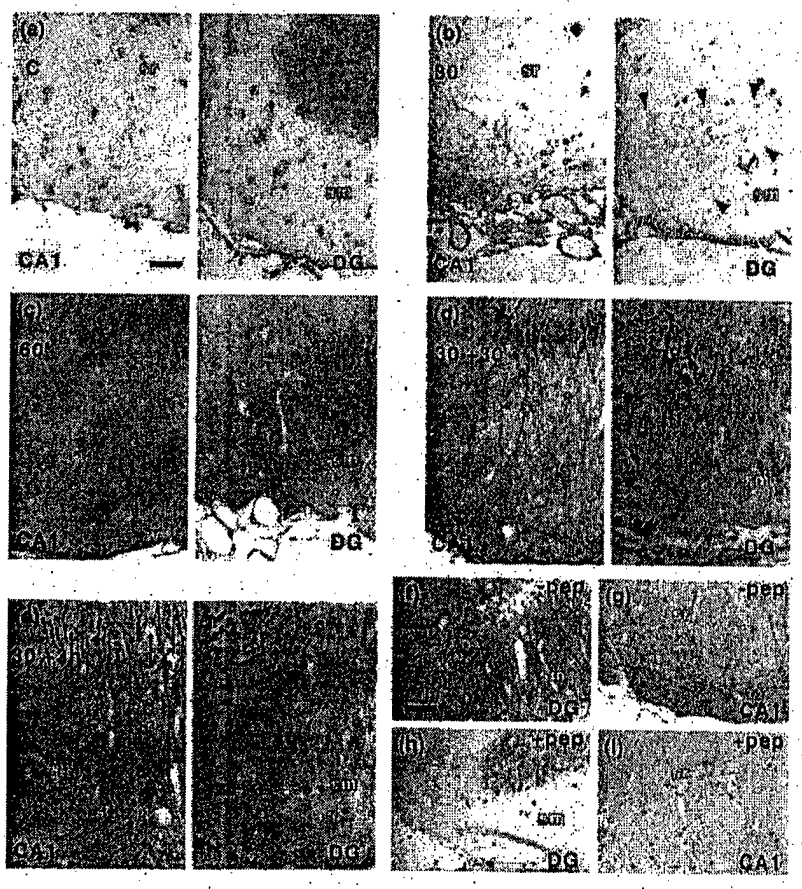


Fig. 3 Photomicrographs from stratum moleculare (sm) of the dentate gyrus (DG) and stratum radiatum (sr) of the hippocampal CA1 region showing immunoreactivity against fodrin breakdown product (FBDP). Sections from (a) control, (b) 30 min of isoelectricity, (c) 60 min of isoelectricity, (d) 30 min of recovery following 30 min of isoelectricity and (e) 3 h of recovery following 30 min of isoelectricity. Sections from animals subjected to 30 min of isoelectricity and 30 min of recovery treated with antibody against FBDP, without antibody pre-absorption (f and g) and parallel sections treated with pre-absorbed antibody against FBDP (h and i). c, Control; 30′, 30 min of isoelectricity; 30′ + 30, 30 min of isoelectricity + 30 min of recovery; 60′, 60 min of isoelectricity; 30′ + 3 h, 30 min of isoelectricity + 3 h of recovery; –pep/+pep, without and with pre-absorption, respectively. Arrow heads demarcate weak FBDP immunoreactivity. Scale bars, (a–e), 35 μm; (f–i), 50 μm.

30 min of isoelectricity a slight increase in staining could be discerned in cells in both the CA1 region and in the DG, frequently localized to one pole of the nucleus (Fig. 2b). At 30 min of recovery following 30 min of isoelectricity a marked increase in staining could be seen, localized mainly to the nuclei but also to dendrites and perinuclear cytoplasm (Fig. 2c). After 60 min of isoelectricity the staining was similar to that seen after 30 min of isoelectricity (data not shown). Relatively weak, punctate staining of cytochrome *c* was seen in the medial CA1 region and in the DG in control sections (Fig. 2d). At the end of 30 min of isoelectricity a distinct staining in cells in the CA1 region and in the crest of the DG was seen, appearing in clusters around the nuclei (Fig. 2e). A similar staining pattern was detected at 30 min of recovery following 30 min of isoelectricity in both regions (Fig. 2f). Likewise, after 60 min of isoelectricity the staining looked like that after 30 min of isoelectricity (data not shown).

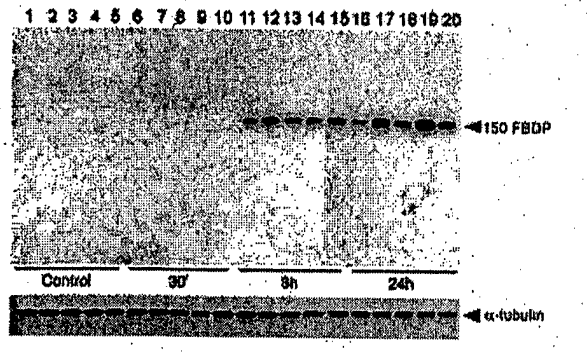


Fig. 4 An immunoblot of homogenates from the vulnerable part of the hippocampus stained with the antibody against fodrin breakdown product (FBDP). Five samples from each control and time point are shown. Lanes: 1–5, controls; 6–10, 30 min of isoelectricity; 11–15, 3 h or 16–20, 24 h of recovery following 30 min of isoelectricity. The apparent molecular mass of the FBDP is indicated (150 kDa). The membranes were stripped and probed with a α-tubulin antibody as a loading control.

Calpain activation during and following hypoglycemia

Fodrin breakdown product immunoreactivity was not seen in the stratum moleculare of the DG or stratum radiatum or the stratum moleculare of the hippocampal CA1 region in the control sections (Fig. 3a). Following 30 or 60 min of isoelectricity, weak immunoreactivity could be discerned in all these regions (Figs 3b and c, respectively). At 30 min of recovery following 30 min of isoelectricity, intense FBDP staining was seen in the DG and in the CA1 region (Fig. 3d). Conspicuous dendritic staining was still seen in the CA1

region and the DG at 3 h of recovery following 30 min of isoelectricity (Fig. 3e). The immunoreactivity seen at 30 min of isoelectricity followed by 30 min of recovery in the DG and in the CA1 region (Figs 3f and g, respectively) was completely abolished by antibody pre-absorption (Figs 3h and i). The immunohistochemical staining was confirmed on western blots stained with the FBDP antibody (Fig. 4). No bands were detected in controls or animals subjected to only 30 min of isoelectricity, whereas a prominent, single band with an apparent molecular weight of 150 kDa was detected in all samples from animals subjected to 30 min of isoelectricity followed by 3 or 24 h of recovery.

Caspase-3 activation during and following hypoglycemia

Using an antibody specific for activated caspase-3, we found no immunoreactivity in the hippocampal CA1 region or the crest of the DG in control sections, except for some scattered cells in the hilar region of the DG (Fig. 5a). Following 30 or 60 min of isoelectricity, caspase-3 staining was discernible but modest in the CA1 region and in the crest of the DG (Figs 5b and c, respectively). After 30 min of isoelectricity and 30 min of recovery widespread caspase-3 staining was seen in the pyramidal cell layer of the CA1 region and in the crest of the DG (Fig. 5d). The staining was even stronger after 3 h of recovery (Fig. 5e). The staining was abolished by antibody pre-absorption with active caspase-3 (Figs 5f and g). Caspase-3-like activity (DEVD-AMC cleavage) was examined in the hippocampus (Fig. 6). In the control group the mean activity was 2.2 ± 0.8 pmol/μg protein/min. At 3 h of recovery there was a 2.3-fold, significant ($p < 0.05$) increase in activity compared with the controls. By 24 h of recovery the activity had returned to control levels.

Cyclosporin A treatment

After 30 min of isoelectricity and 30 min of recovery, the AIF staining of vehicle-treated animals (Fig. 7a and b) was the same as in untreated animals (see Fig. 2c). Animals treated with CsA displayed substantially reduced AIF staining in the crest of the DG (Fig. 7c), whereas staining in the CA1 region was less affected (Fig. 7d). Similarly, cytochrome *c* staining was virtually identical in untreated (see Fig. 2f) and vehicle-treated animals (Figs 7e and f), typically appearing in clusters surrounding the nuclei or polarized to one side of the nuclei (Figs 7e and f). However, in animals treated with CsA, the staining was less intense and

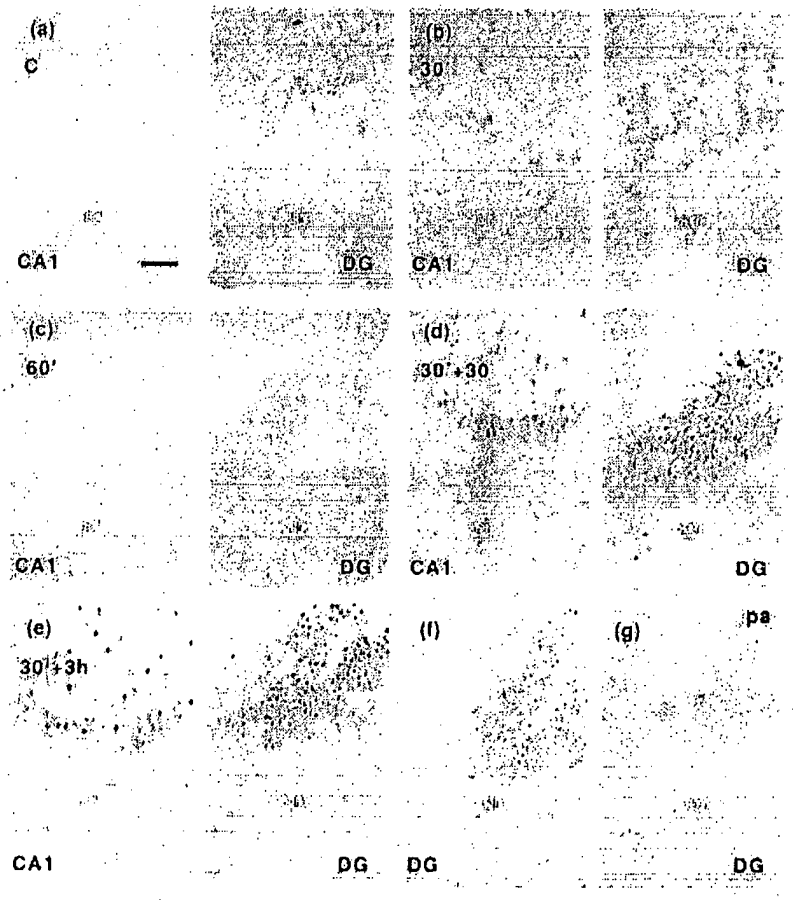


Fig. 5 Photomicrographs from sections of the hippocampal CA1 region and the dentate gyrus (DG) of the hippocampal formation which have been stained for caspase-3. Sections from (a) control, (b) 30 min of isoelectricity, (c) 60 min of isoelectricity, (d) 30 min of recovery following 30 min of isoelectricity and (e) 3 h of recovery following 30 min of isoelectricity. Sections from the DG following 3 h of recovery and 30 min of isoelectricity which have been stained for (f) caspase-3 antibody or (g) caspase-3 antibody pre-absorbed with active caspase-3. c, Control; 30', 30 min of isoelectricity; 30' + 30, 30 min of isoelectricity + 30 min of recovery; 60', 60 min of isoelectricity; 30' + 3 h, 30 min of isoelectricity + 3 h of recovery; sm, stratum moleculare; sr, stratum radiatum; pa, pre-absorption. Scale bar, (a–g), 45 μm.

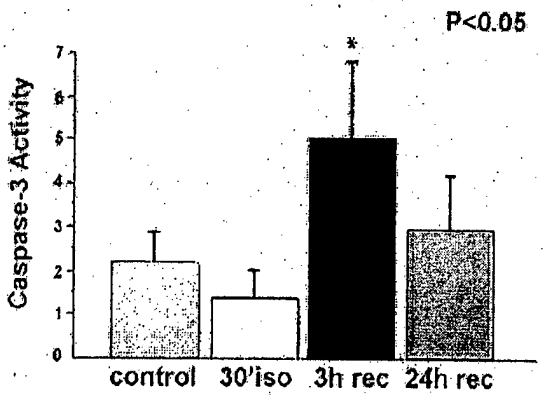


Fig. 6 Caspase-3 activity in whole cell homogenates of the vulnerable part of the hippocampus, from control brains or brains from animals subjected to 30 min of isoelectricity followed by 0, 3 or 24 h of recovery. The data are expressed in pmol AMC formed/min/μg protein and as means ± SD ($n = 5$). *$p < 0.05$ denotes Mann–Whitney U and Bonferroni correction. 30′ iso, 30 min of isoelectricity; 3 and 24 h rec, 3 and 24 h of recovery following 30 min of isoelectricity, respectively.

considerably less clustering of cytochrome *c* staining was seen in the crest of the DG (Fig. 7g), whereas the changes in cytochrome *c* staining in the CA1 region were less pronounced (Fig. 7h).

The intense FBDP staining, indicating calpain activation, in the DG and the CA1 region at 30 min of recovery was also seen in the vehicle-treated animals (Fig. 8a and b). Interestingly, the FBDP staining was completely abolished after CsA treatment in both the DG and the CA1 region (Figs 8c and d). Similarly, the caspase-3 staining in both the DG and the CA1 region (Figs 8e and f) was abolished by CsA treatment (Figs 8g and h).

FK 506 treatment

After 30 min of isoelectricity and 2 days of recovery, most cells in the crest of the DG and in the medial CA1 region showed signs of neurodegeneration, similar to previous investigations (Auer *et al.* 1985b; Friberg *et al.* 1998). Vehicle-treated animals displayed scattered, more or less pyknotic, AIF- and cytochrome *c*-positive cells in both the CA1 region and the DG (Figs 9a, b, m and n, respectively). The number of cells was lower than at 30 min of recovery. Treatment with FK 506 produced a modest decrease in the number of AIF- and cytochrome *c*-positive cells (Figs 9i, j, u and v, respectively), whereas CsA treatment completely abolished them (Figs 9e, f, q and r, respectively). The number of FBDP-positive cells in the vehicle-treated animals at 2 days of recovery was also lower than at 30 min of recovery and the conspicuous dendrite staining was much reduced (Figs 9c and o). FK 506 treatment partly reduced the FBDP staining in some animals (Figs 9k and w), whereas CsA treatment completely abolished it (Figs 9g and s). The number of cells positive for active caspase-3 in the vehicle-

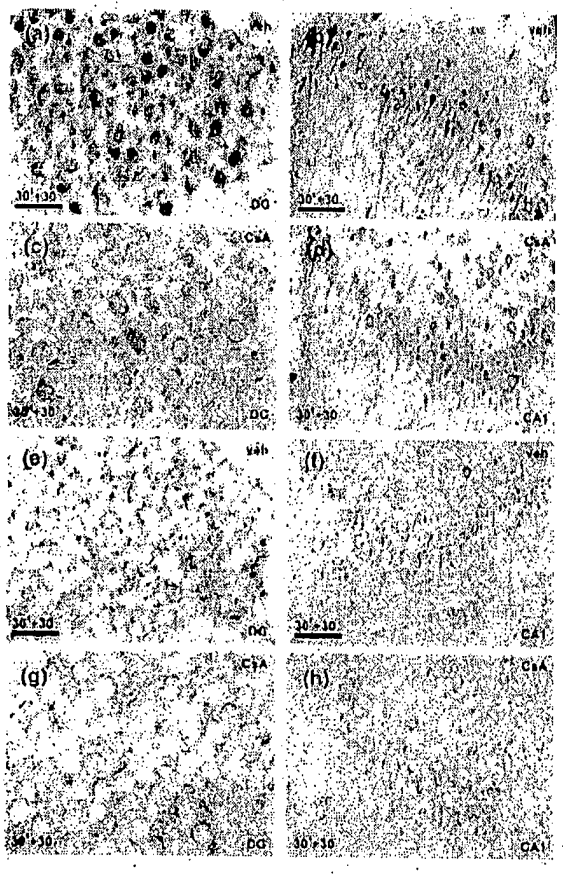


Fig. 7 Photomicrographs of sections from the crest of the dentate gyrus (DG) and the medial hippocampal CA1 region stained with the apoptosis-inducing-factor (a–d) or the cytochrome *c* (e–h) antibody. Animals were treated with vehicle solution (veh) (a, b, e and f) or cyclosporin A (CsA) (c, d, g and h), subjected to 30 min of isoelectricity and allowed 30 min of recovery. 30′ + 30, 30 min of isoelectricity + 30 min of recovery; sp, stratum pyramidale; so, stratum oriens; sr, stratum radiatum. Scale bars, CA1, 20 μm; DG, 80 μm.

treated animals was similar to or even higher than at 30 min of recovery (Figs 9d and p), supporting earlier findings that activation of caspase-3 is delayed and sustained (Blomgren *et al.* 2001). As in the case of the other markers, FK 506 treatment only slightly reduced the caspase-3 staining (Figs 9l and x), whereas little or no caspase-3 immunoreactivity was seen after CsA treatment (Figs 9h and t).

Discussion

We demonstrated previously that CsA, but not the immunosuppressor and calcineurin inhibitor FK 506, completely prevented cell death in the crest of the DG (Friberg *et al.* 1998). Concomitantly, mitochondrial, but not dendritic, swelling was prevented. This strongly implicates a critical role for MPT in the neuronal mitochondrial swelling seen during severe hypoglycemia and for the development of

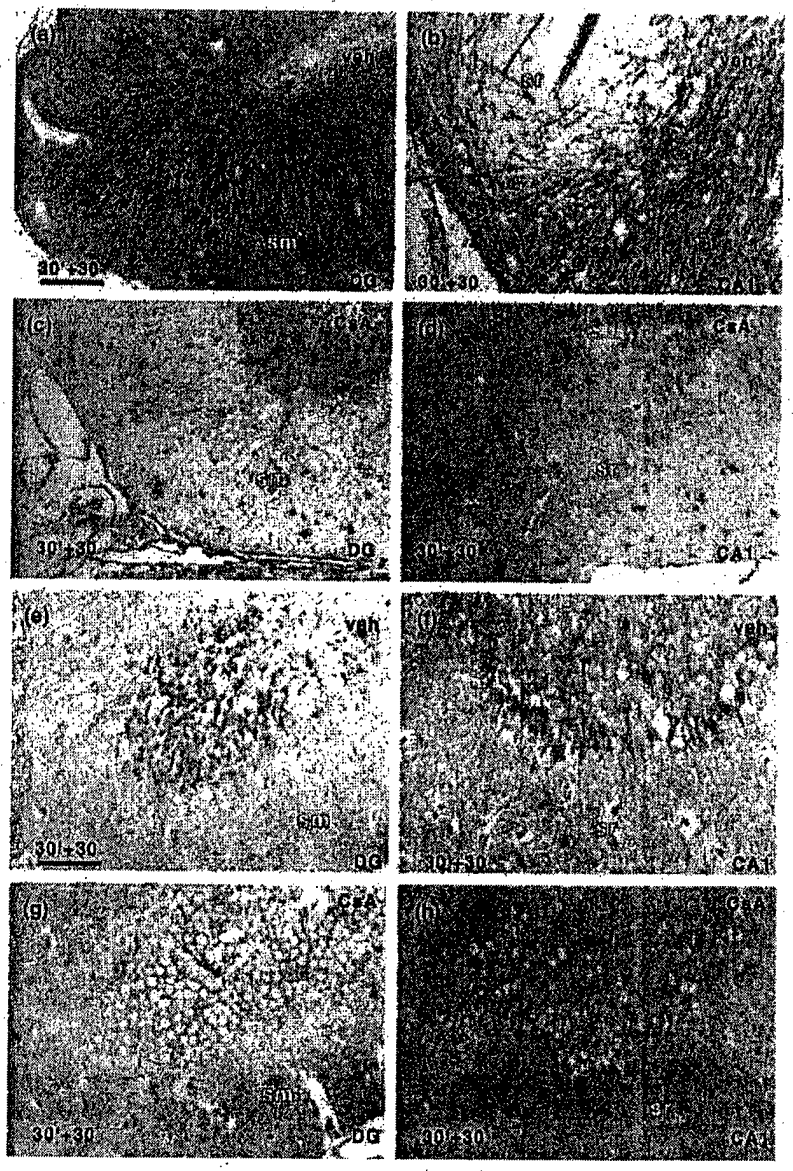


Fig. 8 Photomicrographs of sections from stratum moleculare (sm) and the dentate granule cells of the dentate gyrus (DG) and stratum radiatum (sr) of the hippocampal CA1 region stained with (a–d) the fodrin breakdown product or (e–h) the caspase-3 antibody. Immunostaining following 30 min of isoelectricity and 30 min of recovery from animals treated with the vehicle (veh) (a, b, e and f) or cyclosporin A (CsA) (c, d, g and h). 30′ + 30, 30 min of isoelectricity + 30 min of recovery. Scale bars, 60 μm.

hypoglycemic neuronal damage. The dendritic swelling was unaffected by CsA (Friberg *et al.* 1998), demonstrating that the collapse of ion homeostasis across the plasma membrane still occurred, which includes calcium and sodium entry into cells concomitant with water (Siesjo and Bengtsson 1989). The decreased levels of extracellular calcium, and concomitant increased levels of intracellular calcium, which occur in the presence of CsA do not lead to cell damage or degeneration. It is, therefore, reasonable to assume that events immediately downstream from the mitochondria lead to cell death, including the activation of proteases. In this study we showed that redistribution of AIF and cytochrome *c* occurred and that calpains and caspase-3 were activated following hypoglycemia in cells that subsequently will succumb. This redistribution of mitochondrial, pro-apoptotic proteins and the activation of proteases was inhibited by CsA but not by FK 506 in the DG, a region which is completely protected by CsA treatment. In the hippocampal CA1 region, the neuroprotective effect of CsA is incomplete (Friberg *et al.* 1998), which fits with the finding that the effect of CsA on the redistribution of cytochrome *c* and AIF was less pronounced in this region. Taken together, this implies a strong coupling between MPT, protease activation and hypoglycemic cell death in the brain.

Calpain activation

We know from previous studies that, at the end of 30 min of isoelectricity, the mitochondria in the dentate granule cells are grossly swollen in the perikarya and along the dendrites. This swelling is presumably due to the dissipation of the

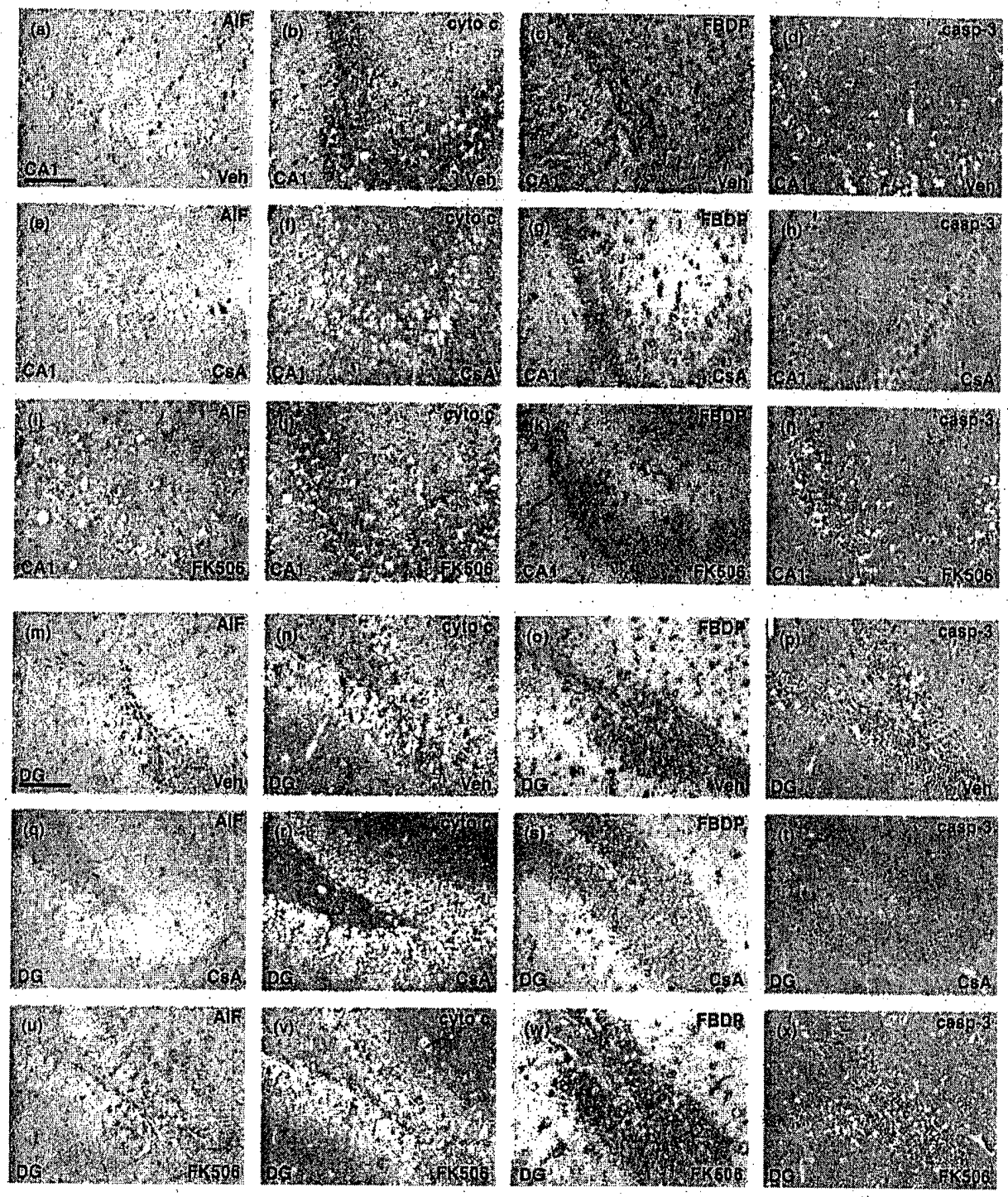


Fig. 9 Photomicrographs of sections from (a–l) the hippocampal CA1 region and (m–x) the dentate gyrus (DG) taken from animals treated with the vehicle (veh) (a–d and m–p), cyclosporin A (CsA) (e–h and q–t) or FK 506 (i–l and u–x) and subjected to 30 min of isoelectricity and allowed 48 h of recovery. Sections were stained for apoptosis-inducing factor (AIF) (a, e, i, m, q and u), cytochrome *c* (cyto c) (b, f, j, n, r and v), fodrin breakdown product (FBDP) (c, g, k, o, s and w) and active caspase-3 (casp-3) (d, h, l, p, t and x). Scale bars, 200 μm.

ionic gradients across the cell membrane, which leads to an influx of calcium, sodium ions and water into the cells (Harris *et al.* 1984). Glutamate receptor antagonists mitigate hypoglycemic damage in the striatum (Wieloch 1985), DG and CA1 region (Papagapiou and Auer 1990; Nellgard and Wieloch 1992). This led to the hypothesis of glutamate

toxicity in hypoglycemic damage (Wieloch 1985), which stated that calcium overload due to NMDA receptor activation contributes to cell death (Siesjo *et al.* 1995). Downstream from the increase in intracellular calcium levels, degradative processes, including proteolysis, have been implicated in neuronal cell death (Siman and Noszek 1988; Seubert *et al.* 1989). Given the fact that calpains have an absolute requirement for Ca^{2+} for activity (Huang and Wang 2001), it is somewhat surprising that only weak FBDP immunostaining was seen following 30 and 60 min of hypoglycemia, considering that the cells were flooded with Ca^{2+} (Siesjo and Bengtsson 1989). Apparently, it was not the total time spent with increased intracellular Ca^{2+} levels that determined the extent of calpain activation, because 60 min of isoelectricity produced little or no FBDP staining, whereas 30 min of isoelectricity plus 30 min of recovery produced massive FBDP staining. This strongly indicates that events other than Ca^{2+} influx during recovery are critical for calpain activation. Cysteine proteases, like calpains and caspases, require a reduced cysteine residue at their active site to maintain proteolytic activity. During hypoglycemia, the intracellular redox state is shifted towards oxidation, which is the opposite of what happens during ischemia (Siesjo 1988). The relatively low levels of FBDP during hypoglycemia, despite high levels of intracellular calcium, might be explained by oxidation of the active site cysteine, known to inhibit calpain activity (Di Cola and Sacchetta 1987; Guttmann and Johnson 1998). Once normoglycemia is restored, and the cellular redox state is normalized, calpains may become activated. This activation could be further amplified by the degradation of calpastatin, the endogenous inhibitor of calpain and a substrate of both calpains and caspase-3 (Emori *et al.* 1987; Porn-Ares *et al.* 1998; Wang *et al.* 1998; Blomgren *et al.* 1999). The fact that CsA, but not FK 506, prevented calpain activation strongly suggests that MPT somehow mediates the observed calpain activation following hypoglycemia. This mechanism is still elusive. It is possible that CsA enhances the mitochondrial uptake and retention of calcium and thereby prevents calpain activation during recovery, as indicated in a recent study where CsA prevented calpain activation following microcystin-induced MPT and cell death in hepatocytes (Ding *et al.* 2002). Alternatively, other factors may be released through MPT during hypoglycemia, which mediate calpain activation during recovery.

Caspase-3 activation

As mentioned earlier, it has been shown that caspase-3 is activated following MPT through the release of cytochrome *c* from the mitochondria and the subsequent formation of an 'apoptosome' protein complex, which transforms pro-caspase-3 to caspase-3. Furthermore, calpains may activate caspase-3, either directly or via caspase-12 (Nakagawa and Yuan 2000; Blomgren *et al.* 2001). dATP is an absolute requirement for the formation of the apoptosome complex (Li *et al.* 1997). In our investigation, caspase-3 activity, as judged by DEVD-AMC cleavage, was unaffected (or even somewhat reduced) during hypoglycemia, despite a modest increase in immunoreactivity for active caspase-3. This absence of caspase-3 activation during hypoglycemia under conditions which evidently lead to cytochrome *c* release could, therefore, be due to the low levels of ATP preventing apoptosome formation. This is also supported by the increased DEVD-AMC cleavage seen after 3 h of recovery, when the ATP levels are partially replenished (Agardh *et al.* 1978). Mitochondrial ATP-dependent potassium channels may also be involved, since activation of these has been demonstrated to protect against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome *c* release (Liu *et al.* 2002). The relatively oxidative environment during hypoglycemia, as mentioned above, may also serve to inhibit cysteine proteases like caspases.

In conclusion, we propose that mitochondria undergo MPT during hypoglycemia. Due to oxidative conditions in the neurons, calpains are not activated despite increased cellular calcium levels. The low levels of ATP during hypoglycemia prevent the formation of the apoptosome and the activation of caspase-3. When normoglycemia is restored, the intracellular redox environment and ATP levels are normalized, leading to calpain activation (fodrin degradation) concomitant with activation of caspase-3. Both calpain- and caspase-3-mediated cellular proteolysis may contribute to cell death induced by hypoglycemia.

Acknowledgements

This work was supported by the Swedish Research Council (J-OK, TW and KB), Juvenile Diabetes Foundation International, European Commission grant QLG1-1999-00739, Swedish Brain Foundation (Hjärnfonden), Bergendahl Foundation, Åhlén Foundation, Wilhelm and Martina Lundgren Foundation, Sven Jerring Foundation, Magnus Bergvall Foundation, Frimurare Barnhus Foundation, Swedish Wennergren Foundation and Vetenskapsrådet (Sweden). We thank Kerstin Beirup for excellent technical assistance, Beth Hoyte and Tomas Björklund for preparation of artwork and Cheryl Christensen for manuscript editing.

References

Agardh C. D., Folbergrova J. and Siesjo B. K. (1978) Cerebral metabolic changes in profound, insulin-induced hypoglycemia, and in the recovery period following glucose administration. *J. Neurochem.* **31**, 1135–1142.

Auer R. N., Olsson Y. and Siesjo B. K. (1984a) Hypoglycemic brain injury in the rat. Correlation of density of brain damage with the EEG isoelectric time: a quantitative study. *Diabetes* **33**, 1090–1098.

Auer R. N., Wieloch T., Olsson Y. and Siesjo B. K. (1984b) The distribution of hypoglycemic brain damage. *Acta Neuropathol.* **64**, 177–191.

Auer R., Kalimo H., Olsson Y. and Wieloch T. (1985a) The dentate gyrus in hypoglycemia: pathology implicating excitotoxin-mediated neuronal necrosis. *Acta Neuropathol.* **67**, 279–288.

Auer R. N., Kalimo H., Olsson Y. and Siesjo B. K. (1985b) The temporal evolution of hypoglycemic brain damage. II. Light- and electron-microscopic findings in the hippocampal gyrus and subiculum of the rat. *Acta Neuropathol.* **67**, 25–36.

Bahr B. A., Tiriveedhi S., Park G. Y. and Lynch G. (1995) Induction of calpain-mediated spectrin fragments by pathogenic treatments in long-term hippocampal slices. *J. Pharmacol. Exp. Ther.* **273**, 902–908.

Bartus R. T., Baker K. L., Heiser A. D., Sawyer S. D., Dean R. L., Elliott P. J. and Straub J. A. (1994) Postischemic administration of AK275, a calpain inhibitor, provides substantial protection against focal ischemic brain damage. *J. Cereb. Blood Flow Metab.* **14**, 537–544.

Blomgren K., Hallin U., Andersson A. L., Puka-Sundvall M., Bahr B. A., McRae A., Saido T. C., Kawashima S. and Hagberg H. (1999) Calpastatin is up-regulated in response to hypoxia and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. *J. Biol. Chem.* **274**, 14 046–14 052.

Blomgren K., Zhu C., Wang X., Karlsson J. O., Leverin A. L., Bahr B. A., Mallard C. and Hagberg H. (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mechanism of 'pathological apoptosis'? *J. Biol. Chem.* **276**, 10 191–10 198.

Braun J. S., Sublett J. E., Freyer D., Mitchell T. J., Cleveland J. L., Tuomanen E. I. and Weber J. R. (2002) Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. *J. Clin. Invest.* **109**, 19–27.

Broekemeier K. M., Dempsey M. E. and Pfeiffer D. R. (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J. Biol. Chem.* **264**, 7826–7830.

Cheng Y., Deshmukh M., D'Costa A., Demaro J. A., Gidday J. M., Shah A., Sun Y., Jacquin M. F., Johnson E. M. and Holtzman D. M. (1998) Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. *J. Clin. Invest.* **101**, 1992–1999.

Cregan S. P., Fortin A., MacLaurin J. G., Callaghan S. M., Cecconi F., Yu S. W., Dawson T. M., Dawson V. L., Park D. S., Kroemer G. and Slack R. S. (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J. Cell Biol.* **158**, 507–517.

Crompton M. and Costi A. (1990) A heart mitochondrial Ca2(+)-dependent pore of possible relevance to re-perfusion-induced injury. Evidence that ADP facilitates pore interconversion between the closed and open states. *Biochem. J.* **266**, 33–39.

Crompton M., Ellinger H. and Costi A. (1988) Inhibition by cyclosporin A of a Ca2−-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem. J.* **255**, 357–360.

Di Cola D. and Sacchetta P. (1987) Irreversible inactivation of calcium-dependent proteinases from rat liver by biological disulfides. *FEBS Lett.* **210**, 81–84.

Ding W. X., Shen H. M. and Ong C. N. (2002) Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes. *Biochem. Biophys. Res. Commun.* **291**, 321–331.

Emori Y., Kawasaki H., Imajoh S., Imahori K. and Suzuki K. (1987) Endogenous inhibitor for calcium-dependent cysteine protease contains four internal repeats that could be responsible for its multiple reactive sites. *Proc. Natl Acad. Sci. USA* **84**, 3590–3594.

Endres M., Namura S., Shimizu-Sasamata M., Waeber C., Zhang L., Gomez-Isla T., Hyman B. T. and Moskowitz M. A. (1998) Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. *J. Cereb. Blood Flow Metab.* **18**, 238–247.

Friberg H., Ferrand-Drake M., Bengtsson F., Halestrap A. P. and Wieloch T. (1998) Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. *J. Neurosci.* **18**, 5151–5159.

Gido G., Kristian T. and Siesjo B. K. (1994) Induced spreading depressions in energy-compromised neocortical tissue: calcium transients and histopathological correlates. *Neurobiol. Dis.* **1**, 31–41.

Guttmann R. P. and Johnson G. V. (1998) Oxidative stress inhibits calpain activity in situ. *J. Biol. Chem.* **273**, 13 331–13 338.

Hansen A. J. and Zeuthen T. (1981) Extracellular ion concentrations during spreading depression and ischemia in the rat brain cortex. *Acta Physiol. Scand.* **113**, 437–445.

Hara H., Friedlander R. M., Gagliardini V., Ayata C., Fink K., Huang Z., Shimizu-Sasamata M., Yuan J. and Moskowitz M. A. (1997) Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc. Natl Acad. Sci. USA* **94**, 2007–2012.

Harris R. J., Wieloch T., Symon L. and Siesjo B. K. (1984) Cerebral extracellular calcium activity in severe hypoglycemia: relation to extracellular potassium and energy state. *J. Cereb. Blood Flow Metab.* **4**, 187–193.

Haworth R. A. and Hunter D. R. (1980) Allosteric inhibition of the Ca2+-activated hydrophilic channel of the mitochondrial inner membrane by nucleotides. *J. Membr. Biol.* **54**, 231–236.

Hong S. C., Goto Y., Lanzino G., Soleau S., Kassell N. F. and Lee K. S. (1994) Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. *Stroke* **25**, 663–669.

Huang Y. and Wang K. K. (2001) The calpain family and human disease. *Trends Mol. Med.* **7**, 355–362.

Jacotot E., Costantini P., Laboureau E., Zamzami N., Susin S. A. and Kroemer G. (1999) Mitochondrial membrane permeabilization during the apoptotic process. *Ann. NY Acad. Sci.* **887**, 18–30.

Karlsson J. O., Ostwald K., Kabjorn C. and Andersson M. (1994) A method for protein assay in Laemmli buffer. *Anal. Biochem.* **219**, 144–146.

Klein J. A., Longo-Guess C. M., Rossmann M. P., Seburn K. L., Hurd R. E., Frankel W. N., Bronson R. T. and Ackerman S. L. (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. *Nature* **419**, 367–374.

Lee K. S., Frank S., Vanderklish P., Arai A. and Lynch G. (1991) Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc. Natl Acad. Sci. USA* **88**, 7233–7237.

Li P., Nijhawan D., Budihardjo I., Srinivasula S. M., Ahmad M., Alnemri E. S. and Wang X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479–489.

Liu D., Lu C., Wan R., Auyeung W. W. and Mattson M. P. (2002) Activation of mitochondrial ATP-dependent potassium channels protects neurons against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome c release. *J. Cereb. Blood Flow Metab.* **22**, 431–443.

Liu X., Kim C. N., Yang J., Jemmerson R. and Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147–157.

Nakagawa T. and Yuan J. (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.* **150**, 887–894.

Nellgard B. and Wieloch T. (1992) Cerebral protection by AMPA- and NMDA-receptor antagonists administered after severe insulin-induced hypoglycemia. *Exp. Brain Res.* **92**, 259–266.

Nicholson D. W., Ali A., Thornberry N. A., Vaillancourt J. P., Ding C. K., Gallant M., Gareau Y., Griffin P. R., Labelle M., Lazebnik Y. A. *et al.* (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37–43.

Nicolli A., Basso E., Petronilli V., Wenger R. M. and Bernardi P. (1996) Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel. *J. Biol. Chem.* **271**, 2185–2192.

Papagapiou M. P. and Auer R. N. (1990) Regional neuroprotective effects of the NMDA receptor antagonist MK-801 (dizocilpine) in hypoglycemic brain damage. *J. Cereb. Blood Flow Metab.* **10**, 270–276.

Porn-Ares M. I., Samali A. and Orrenius S. (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. *Cell Death Differ.* **5**, 1028–1033.

Rami A. and Krieglstein J. (1993) Protective effects of calpain inhibitors against neuronal damage caused by cytotoxic hypoxia in vitro and ischemia in vivo. *Brain Res.* **609**, 67–70.

Saido T. C., Sorimachi H. and Suzuki K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *Faseb J.* **8**, 814–822.

Seubert P., Nakagawa Y., Ivy G., Vanderklish P., Baudry M. and Lynch G. (1989) Intrahippocampal colchicine injection results in spectrin proteolysis. *Neuroscience* **31**, 195–202.

Siesjo B. K. (1988) Hypoglycemia, brain metabolism, and brain damage. *Diabetes Metab. Rev.* **4**, 113–144.

Siesjo B. K. and Bengtsson F. (1989) Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis. *J. Cereb. Blood Flow Metab.* **9**, 127–140.

Siesjo B. K., Katsura K., Zhao Q., Folbergrova J., Pahlmark K., Siesjo P. and Smith M. L. (1995) Mechanisms of secondary brain damage in global and focal ischemia: a speculative synthesis. *J. Neurotrauma* **12**, 943–956.

Siman R. and Noszek J. C. (1988) Excitatory amino acids activate calpain I and induce structural protein breakdown in vivo. *Neuron* **1**, 279–287.

Susin S. A., Zamzami N., Castedo M., Hirsch T., Marchetti P., Macho A., Daugas E., Geuskens M. and Kroemer G. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J. Exp Med.* **184**, 1331–1341.

Susin S. A., Lorenzo H. K., Zamzami N., Marzo I., Snow B. E., Brothers G. M., Mangion J., Jacotot E., Costantini P., Loeffler M. *et al.* (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441–446.

Wang K. K. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci.* **23**, 20–26.

Wang K. K., Posmantur R., Nadimpalli R., Nath R., Mohan P., Nixon R. A., Talanian R. V., Keegan M., Herzog L. and Allen H. (1998) Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Arch. Biochem. Biophys.* **356**, 187–196.

Wieloch T. (1985) Hypoglycemia-induced neuronal damage prevented by an N-methyl-D-aspartate antagonist. *Science* **230**, 681–683.

Yu S. W., Wang H., Poitras M. F., Coombs C., Bowers W. J., Federoff H. J., Poirier G. G., Dawson T. M. and Dawson V. L. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* **297**, 259–263.

Zhang X., Chen J., Du Graham S. H. L., Kochanek P. M., Draviam R., Guo F., Nathaniel P. D., Szabo C., Watkins S. C. and Clark R. S. (2002) Intranuclear localization of apoptosis-inducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. *J. Neurochem.* **82**, 181–191.

LYSOSOMAL ACTIVATION IS A COMPENSATORY RESPONSE AGAINST PROTEIN ACCUMULATION AND ASSOCIATED SYNAPTOPATHOGENESIS—AN APPROACH FOR SLOWING ALZHEIMER DISEASE?

JENNIFER BENDISKE, PHD AND BEN A. BAHR, PHD

Made in United States of America
Reprinted from JOURNAL OF NEUROPATHOLOGY AND EXPERIMENTAL NEUROLOGY
Vol. 62, No. 5, May 2003

Journal of Neuropathology and Experimental Neurology

Vol. 62, No. 5
May, 2003
pp. 451–463

Lysosomal Activation Is a Compensatory Response Against Protein Accumulation and Associated Synaptopathogenesis—An Approach for Slowing Alzheimer Disease?

JENNIFER BENDISKE, PHD AND BEN A. BAHR, PHD

Abstract. Previous reports suggest that age-related lysosomal disturbances contribute to Alzheimer-type accumulations of protein species, blockage of axonal/dendritic transport, and synaptic decline. Here, we tested the hypothesis that lysosomal enzymes are upregulated as a compensatory response to pathogenic protein accumulation. In the hippocampal slice model, tau deposits and amyloidogenic fragments induced by the lysosomal inhibitor chloroquine were accompanied by disrupted microtubule integrity and by corresponding declines in postsynaptic glutamate receptors and the presynaptic marker synaptophysin. In the same slices, cathepsins B, D, and L, β-glucuronidase, and elastase were upregulated by 70% to 135%. To address whether this selective activation of the lysosomal system represents compensatory signaling, N-Cbz-L-phenylalanyl-L-alanyl-diazomethylketone (PADK) was used to enhance the lysosome response, generating 4- to 8-fold increases in lysosomal enzymes. PADK-mediated lysosomal modulation was stable for weeks while synaptic components remained normal. When PADK and chloroquine were co-infused, chloroquine no longer increased cellular tau levels. To assess pre-existing pathology, chloroquine was applied for 6 days after which its removal resulted in continued degeneration. In contrast, enhancing lysosomal activation by replacing chloroquine after 6 days with PADK led to clearance of accumulated protein species and restored microtubule integrity. Transport processes lost during chloroquine exposure were consequently re-established, resulting in marked recovery of synaptic components. These data indicate that compensatory activation of lysosomes follows protein accumulation events, and that lysosomal modulation represents a novel approach for treating Alzheimer disease and other protein deposition diseases.

Key Words: Hippocampal slice culture; Lysosomal activation; Lysosomal modulation; Neuroprotection; N-Cbz-L-phenylalanyl-L-alanyl-diazomethylketone (PADK); Protein deposition disease; Synaptic recovery.

INTRODUCTION

Proteins with a propensity to aggregate or oligomerize are thought to be causative factors in a wide range of neurodegenerative disorders. Among age-related protein deposition diseases, altered processing and toxic accumulation of proteins have been reported to involve disturbances in the endosomal-lysosomal system (1). For example, several studies implicate lysosomal disruption in the development of amyloidogenic fragments (2–7) and tau deposits (8–12) within neurons. The Huntington disease huntingtin protein also may require lysosomal structures to form aggregates (13, 14). It is not surprising then that several pathologic features of age-related disorders are induced by the suppression of lysosomal function (1). Interestingly, many protein deposition disorders also exhibit activation of the lysosomal system, as evidenced by the appearance of numerous lysosomal vacuoles with increased enzyme content (3, 15–22). The Alzheimer Aβ1–42 peptide of the amyloid precursor protein (APP) causes an enzymatic upregulation consistent with lysosomal activation (23), and this occurs in conjunction with the peptide's facilitation of lysosomal damage, amyloidogenic processing (24–27), and associated synaptic decline (25). While elevated levels of lysosomes and their digestive enzymes are generally reflective of lysosomal disturbances, the role such increases play has been a subject of speculation. One idea is that protein accumulation events trigger the lysosomal activation response in order to slow the progressive neurodegeneration they elicit. The present study tested this idea.

Amplification of lysosomal hydrolases is a key indicator of lysosomal activation, and this type of cellular regulation may represent a compensatory response against age-related neurodegeneration as previously suggested (15, 16). Lysosomal activation appears to be a response to protein deposition and synaptic pathology as found in brain tissue exposed to lysosomal enzyme inhibitors or to acidotropic compounds that disrupt lysosomal pH (9). Such agents, indeed, have been used to induce age-related lysosomal dysfunction and to study the resultant neuropathogenesis (5, 6, 8–12). These and other studies (23, 25) further support the idea that upregulation of appropriate lysosomal enzymes is driven by signaling events in an attempt to offset the consequences of abnormal protein accumulation. If lysosomal activation represents compensatory signaling, positive modulation of the lysosomal response should lead to protection against protein accumulation and the associated synaptic pathology.

Protein accumulation/aggregation is a common pathogenic mechanism among age-related disorders including Alzheimer disease (AD). Thus, the present study made use of a model system that can reproduce AD-related

From the Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut.

Correspondence to: Ben A. Bahr, Dept. of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269-2092. E-mail: Bahr@uconn.edu

This work was supported by the University of Connecticut Research Foundation and the University of Connecticut Neurosciences Program.

protein accumulations as well as progressive neurodegeneration (9, 12). By experimentally inducing lysosomal dysfunction with chloroquine in hippocampal slice cultures, we were able to test the concept of compensatory activation of the lysosomal system. Of particular interest was the effect lysosomal activation has on cellular changes that lead to synaptopathogenesis in the slice cultures. Such changes appear to result in a dramatic decline in the number of synapses per neuron (1), a decline that is also evident in early-stage AD (28). Biochemical and morphological indicators suggest that synapses are the initial target of Alzheimer-type pathogenesis (20, 29–32), especially in the hippocampus (33–35). The hippocampal slice model exhibits a loss of postsynaptic glutamate receptors at the protein and message level (5, 12, 36), similar to that found in the AD hippocampus and entorhinal cortex (37–40). The presynaptic marker synaptophysin also declines in the model, and its loss in hippocampus and other regions correlates with the degree of cognitive decline in AD (29, 41, 42) and occurs early in some transgenic models of the disease (43, 44).

Using the slice model, we demonstrate here that lysosomal enzyme responses can be positively modulated to promote clearance of protein deposits and, in an associative manner, to restore cellular markers used to track chloroquine-induced synaptic decline. Recent findings suggest that protein accumulation blocks synaptic maintenance by disrupting microtubule-based transport, and that initial transport failure occurs before appreciable protein deposition (12). Correspondingly, experiments in the present report show that restoration of transport mechanisms and reversal of synaptopathogenesis follows lysosomal modulation, thus supporting the hypothesis that lysosomal activation represents compensatory signaling against the AD-type pathogenic cascade.

MATERIALS AND METHODS

Antibodies and Chemicals

Monoclonal antibodies against synaptophysin, tau, and α-acetylated tubulin were obtained from Boehringer Mannheim (Indianapolis, IN) and ICN Biomedicals (Costa Mesa, CA). Antibodies to actin were obtained from Sigma Chemical Company (St. Louis, MO), antibodies to cathepsin B from Upstate Biotechnology (Lake Placid, NY), antibodies to cathepsins D, L, and S and β-glucuronidase from Cortex Biochem (San Leandro, CA), anti-elastase from Biodesign International (Saco, ME), PHF-1 antibodies against human paired helical filaments (PHFs) from ICN Biomedicals, and affinity-purified antibodies to proteasome 20S C2 subunit were from Affinity BioReagents (Golden, CO). Antibodies that recognize carboxyl-terminal fragments of APP were from Boehringer Mannheim. Affinity-purified antibodies to the AMPA receptor subunit GluR1 were prepared as previously described (45). N-Cbz-L-phenylalanyl-L-alanyl-diazomethylketone (PADK), diazoacetyl-DL-2-aminohexanoic acid methyl ester, and glycyl-phenylalanyl-glycine-aldehyde semicarbazone were obtained from Bachem (King of Prussia, PA). Aβ1–42 and a scrambled version of the peptide were synthesized and characterized by electrospray mass spectroscopy (46), obtained from Dr. Charles Glabe (University of California, Irvine). All other cell culture supplies, chloroquine, and horseradish peroxidase (HRP) were obtained from Sigma Chemical Company. Protease inhibitors were obtained from Boehringer Mannheim. Nitrocellulose immunoblotting paper was obtained from Osmonics (Westborough, MA). Alkaline phosphatase-conjugated and HRP-conjugated antibodies and substrate kits were from Bio-Rad Laboratories (Richmond, CA) and Vector Laboratories (Burlingame, CA). RNase Easy and RNase-free DNase kits were supplied by Quiagen (Valencia, CA).

Organotypic Hippocampal Slice Cultures

Sprague-Dawley rat litters (Charles River Laboratories, Wilmington, MA) were housed in accordance with guidelines from the National Institutes of Health. Animals 11 to 12 days postnatal were subjected to isoflurane anesthesia (Baxter, Deerfield, IL) and immediate decapitation. Transverse slices of hippocampus (400 μm) were then quickly prepared, maintained on Millicell-CM inserts (Millipore Corporation, Bedford, MA), and periodically supplied with fresh media composed of 50% basal medium Eagle, 25% Earle's balanced salts, 25% horse serum, and defined supplements (5, 47). Slices were maintained at 37°C with a 5% CO_2-enriched atmosphere for 15 to 20 days before use in experiments. Synaptic function was assessed as previously described (5).

Slice cultures were incubated with media containing vehicle, 60 μM chloroquine, 17 μM Aβ peptides, 1 to 10 μM PADK, or combinations thereof. Treatments were staggered so that all slices were in culture for the same length of time. Chloroquine is a weak base that accumulates in lysosomes, leading to the disruption of protein degradation while having no direct effect on protein synthesis (5, 48, 49). The chloroquine dose used was shown to influence lysosomal processes without affecting glycosylation, tyrosine sulfation, protein synthesis and secretion, or neuronal properties underlying synaptic waveform. Chloroquine produces a characteristic delayed effect on protein processing and synaptic composition, nearly identical to that produced by cathepsin inhibitors and by an ATPase blocker that disrupts lysosomal pH (5, 8, 9, 11).

Immunoblot Analysis

Slices were removed with a soft brush and homogenized as samples of 6 to 8 slices each using ice-cold buffer containing 0.32 M sucrose, 5 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 μM okadaic acid, 10 nM calyculin A, and the protease inhibitors aprotinin, leupeptin, bestatin, E-64, pepstatin A (each at 2 μg/mL), and 4-(2-aminoethyl)benzenesulfonyl fluoride (0.3 mM). Protein content was determined with BSA standards and equal aliquots of protein (80 μg) were denatured in sample buffer for 5 min at 100°C, then separated by 4% to 16% SDS-PAGE and blotted to nitrocellulose. Blots were treated with primary antibodies overnight, and secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates. Color development used the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate system. The development of bands was terminated before maximal intensity was reached in

order to ensure a linear relationship with increasing amount of sample protein. Immunoreactive bands were scanned at high resolution and assessed for integrated density with BIOQUANT software (R&M Biometrics, Nashville, TN). Prior to staining for tau immunoreactivity, blots were treated with alkaline phosphatase at a concentration of 0.1 units/mL.

Immunocytochemistry

The slices were rinsed with 0.1 M phosphate buffer, pH 7.4 (PB) and fixed for 2 h in PB containing 4% paraformaldehyde. Slices were cryoprotected and carefully removed from the insert, then sectioned at 20-μm thickness. Sections were immunolabeled with PHF-1 antibodies using the avidin-biotin-peroxidase technique and 3,3′-diaminobenzidine as the chromogen. Image capture was made possible with the BIOQUANT image analysis system linked to an Olympus AX70 microscope.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cultured slices were harvested, RNA isolated, and corresponding cDNA generated as previously described (12). PCR was carried out using 200 ng cDNA in a total volume of 25 μL containing 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 mM primers, and 1.25 U/μL Taq DNA polymerase. Cathepsin D, GluR1, and house-keeping gene (HPRT) primers were designed according to GenBank sequences for the respective genes, and synthesized by the University of Connecticut Biotechnology Center. PCR products were electrophoretically separated on 2% agarose gels and their images captured using the Kodak EDAS120 system (Rochester, NY).

Transport Analysis

A small slurry was prepared with 20 mg of HRP in slice buffer containing 2 M sucrose. Using cultures on membrane inserts, HRP was administered to pyramidal neurons of control and treated hippocampal slices via a fine-tip electrode. After 2 h at 37°C, the treated slices were washed in PB and then fixed in cold PB containing 4% paraformaldehyde for 2 h. Slices were cryoprotected in a 20% sucrose solution, carefully removed from the inserts, and mounted on gelatin-coated slides. The slices were then stained for 5 to 10 min with 3-3′-diaminobenzidine, treated with ascending concentrations of ethanol and clearing agent, and coverslipped.

RESULTS

Hippocampal slice cultures were used since they possess native synaptic circuitries and exhibit cellular and synaptic changes consistent with early AD pathogenesis when treated with lysosomal disruptors (5, 24, 36). The acidotropic compound chloroquine caused a steady decline in the presynaptic vesicle protein synaptophysin (Fig. 1a) and in a postsynaptic marker (Fig. 2b), while other proteins remained the same (e.g. actin) or increased in concentration. Such synaptic decline is similar to that previously reported, and the selective reduction occurred over the same time period that the lysosomal inhibitor

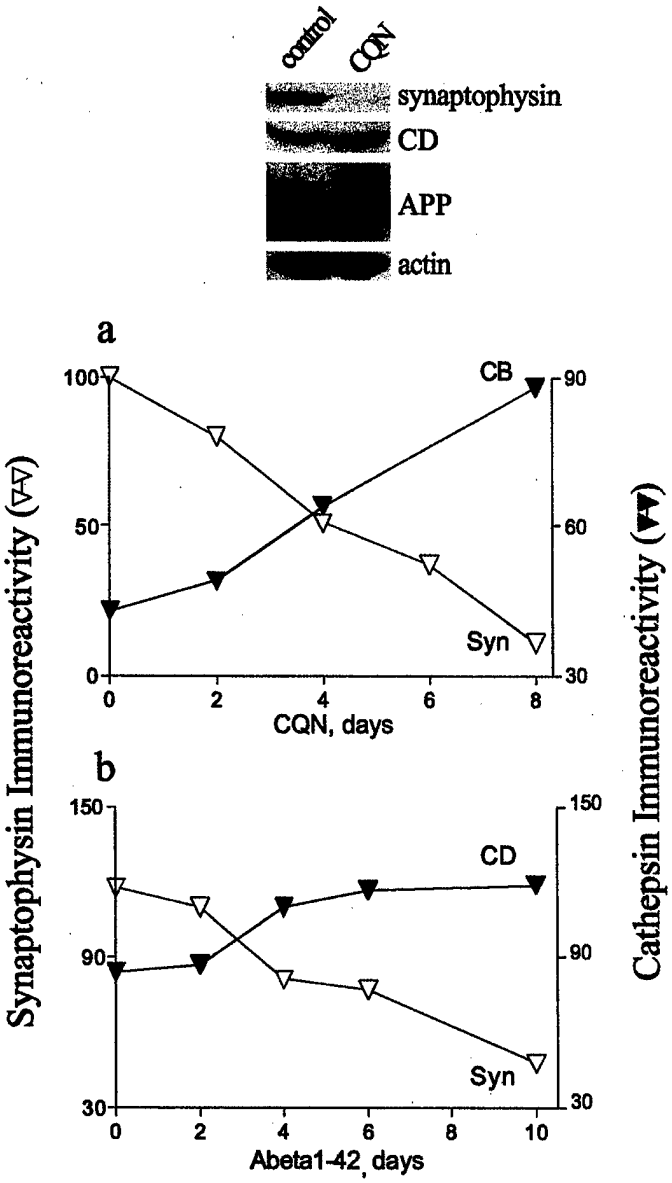


Fig. 1. Lysosomal activation correlates with synaptic decline. Hippocampal slice cultures were treated with chloroquine (CQN) **(a)** or Aβ1-42 **(b)** for the number of days indicated, then harvested in groups of 6 to 8 and prepared for immunoblot analysis. Blots were stained for synaptophysin (Syn), cathepsin B (CB), cathepsin D (CD), APP holoprotein, and actin as a control. The plotted data represent mean integrated optical densities determined from 3 separate groups of slices.

induced protein deposition, transport failure, and a corresponding reduction in evoked EPSPs (5, 12). Associated with the reduced expression of synaptic proteins was the upregulation of the lysosomal hydrolase cathepsin B (Fig. 1a); a similar response was expressed by related enzymes cathepsins D and L (Table; Fig. 1a). Besides members of the cathepsin family of hydrolases, other lysosomal enzymes were selectively upregulated, including

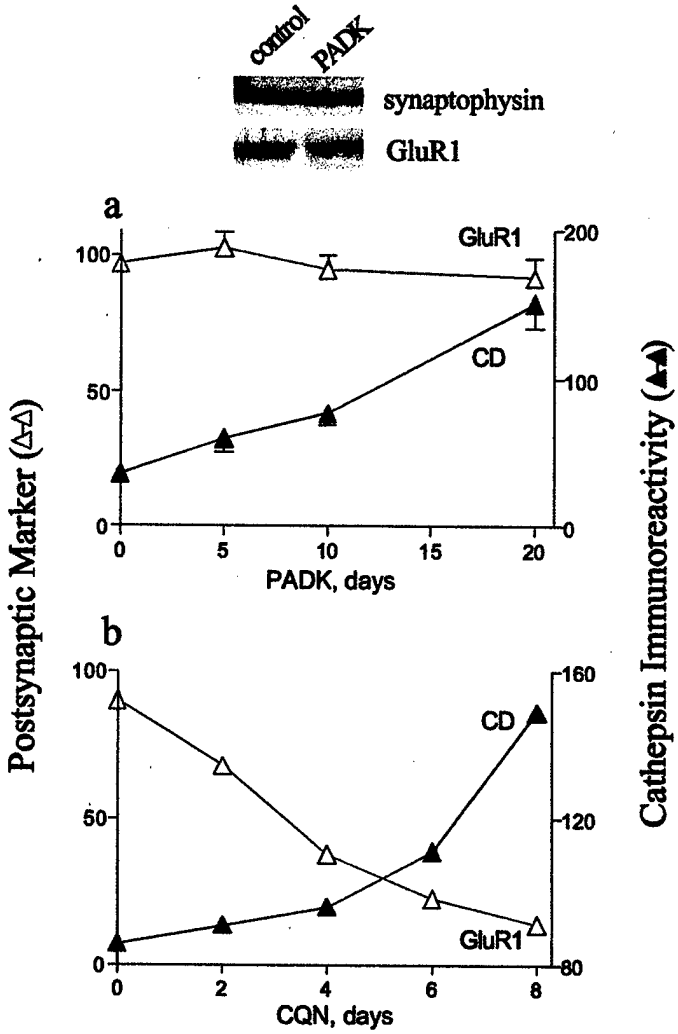


Fig. 2. PADK causes positive lysosomal modulation without affecting synaptic maintenance. Cultured slices were treated with PADK for 0 to 20 days (**a**) or with chloroquine (CQN) for up to 8 days (**b**) before being harvested and analyzed by immunoblot for synaptic markers and cathepsin D (CD). The blots shown contain slices treated without or with PADK for 20 days, and were stained for synaptophysin and the AMPA receptor subunit GluR1. The plotted data represent mean integrated optical densities as determined from 3 to 5 separate groups of slices. Note the different scales used for cathepsin D in the 2 panels, thus depicting more than a 4-fold increase induced by PADK but less than a 2-fold increase by chloroquine.

β-glucuronidase and elastase (Table). While chloroquine-induced lysosomal activation consisted of increases in nearly all enzymes measured, there was an almost 50% decrease in cathepsin S.

As with the lysosomal inhibitor chloroquine, a similar correspondence between synaptic decline and lysosomal activation was found when the slice cultures were treated with Aβ1-42 (Fig. 1b), the β-amyloid peptide shown to be taken up by neurons and causing lysosomal disruption (25–27). The cathepsin D hydrolase was increased by

TABLE
Broad Activation of the Lysosomal System Is Produced by the Lysosomal Disruptor Chloroquine and by the Lysosomal Modulator PADK

	Chloroquine-Induced Change	PADK-Induced Change
Lysosomal System		
Cathepsin B	+104%	+850%
Cathepsin D	+75%	+480%
Cathepsin L	+71%	n.d.
Cathepsin S	−45%	+163%
β-glucuronidase	+135%	n.d.
Elastase	+69%	+119%
Proteasome System		
Proteasome 20S	−2%	−2%

Slices were treated with chloroquine alone for 6 days or followed by PADK modulation (3 μM), then compared to control slices after assessing for enzyme content via immunoblot using selective antibodies. n.d., not determined.

47% ± 5% and synaptophysin was reduced by 54% ± 7% (mean ± SEM, $p < 0.01$) as compared to levels in slices treated for 10 to 17 days with a scrambled version of the 42-residue sequence. Matching previous results with chloroquine treatment (5), the synaptic decline produced by Aβ1-42 was also associated with a 46% decrease in EPSP amplitude measured in the stratum radiatum of CA1a ($p < 0.01$).

Similar to the lysosome responses induced by the broad lysosomal inhibitor chloroquine and Aβ1-42, a specific cathepsin inhibitor also caused hydrolase upregulation. N-Cbz-L-phenylalanyl-L-alanyl-diazomethylketone (PADK) selectively inhibits cathepsins B and L, but was found to cause increases in several lysosomal enzymes (Table). A high concentration of PADK (200 μM) over a 5-day period has been previously shown to induce lysosomal activation (9) as well as the same level of synaptic decline as elicited by chloroquine (5). However, application of much lower concentrations of PADK (1–10 μM) for 20 days or more did not trigger deficits in the postsynaptic AMPA receptor subunit GluR1 (Fig. 2a). The glutamate receptor subunit and the presynaptic marker remained at stable concentrations in the PADK-treated slices (see blots in Fig. 2), while cathepsin levels increased 4- to 5-fold. The synaptic maintenance is in striking contrast to the synaptic decline associated with chloroquine-mediated lysosomal activation (Fig. 2b). The lysosomal response produced by low-level PADK was more pronounced than that expressed by chloroquine-treated slice cultures. In only a few days, appropriate concentrations of PADK elicited modulatory action that generated 2- to 8-fold increases in lysosomal enzymes over the level

of activation found after 6 days of chloroquine treatment (Fig. 3; Table). Moreover, PADK modulation upregulated cathepsin S, which was reduced in concentration by chloroquine. Note that the selective modulation of the lysosomal system by PADK was not influenced by prior exposure of slices to chloroquine.

Having demonstrated the ability to induce positive lysosomal modulation without affecting synaptic maintenance, we addressed whether lysosomal activation represents a compensatory response. We first examined the effects of the lysosomal modulator PADK on the early induction of phosphorylated tau species (12). The induction of tau was 2- to 3-fold resulting from 3 days of chloroquine treatment (Fig. 4a; compare CQN sample to control lane). Interestingly, when chloroquine was infused together with PADK for the same time period, the chloroquine exposure no longer was associated with increased levels of the 55- to 69-kDa tau isoforms (rightmost lane). The same immunoblot sample exhibited a 3- to 4-fold increase in cathepsin immunoreactivity as compared to the nontreated control sample. It is important to note that the tau isoforms as well as actin protein levels were unchanged in slices treated with PADK alone for 3 days and for extended time periods of ≥20 days (first lane). Thus, PADK modulation selectively enhances the cellular content of lysosomal enzymes and promotes the stable maintenance of microtubule-binding proteins and other structural components.

Next, lysosomal function was disrupted prior to PADK treatment in order to examine the modulator's effects on pre-existing protein deposits and associated synaptopathogenesis. Chloroquine was applied to slice cultures for 6 days causing intraneuronal deposition of PHF-immunopositive material, an increase in carboxyl-terminal fragments of APP, as well as a gradual loss of synaptic components as previously reported (5, 12). In spite of removing chloroquine from the media after the 6-day exposure, protein accumulation in pyramidal neurons persisted and appeared unchanged (Fig. 5b; control in Fig. 5a), and levels of the GluR1 subunit continued to decline (Fig. 6a and open triangles in Fig. 6c). In contrast, replacing chloroquine with PADK after the sixth day of treatment led to a marked reduction in APP fragments (Fig. 4b) and evident clearance of the polar deposits of PHF material (Fig. 5c). No polar deposition was produced by a 9-day treatment with PADK alone.

Corresponding with PADK-mediated protein clearance was a marked recovery of the GluR1 postsynaptic marker (see closed triangles in Fig. 6c; $p < 0.00001$, 2-way ANOVA). PADK modulation caused significant recovery of the presynaptic protein synaptophysin as well. Such synaptic repair also was observed a week after the chloroquine exposure, the longest time point tested. Other cathepsin antagonists elicited similar neuroprotective

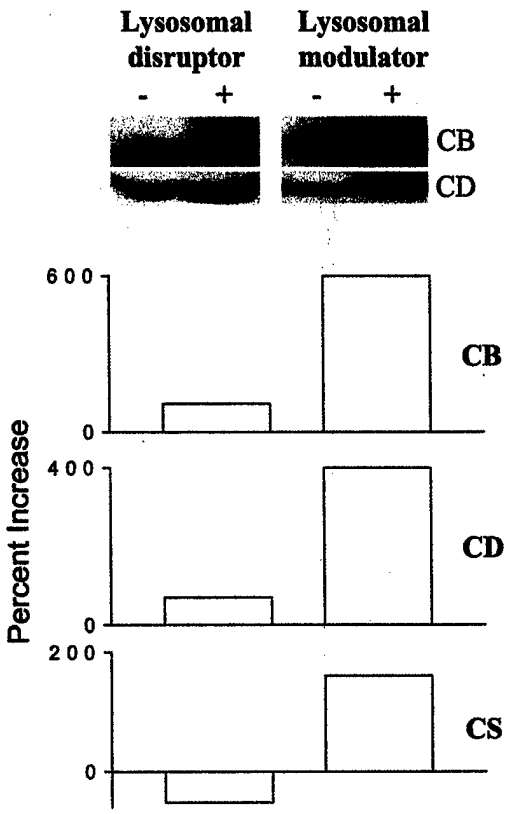


Fig. 3. PADK elicits a larger lysosomal response in hippocampal slices than that induced by the lysosomal disruptor chloroquine. Cultured slices were treated with (+) or without (−) chloroquine (left blots and bars) or the lysosomal modulator PADK (right) for 6 days. Representative immunoblots were probed with anti-cathepsin B and D antibodies, and the bar graphs represent the percent change in integrated optical densities. Similar results were seen in 3 other experiments. Unchanged actin levels were found on all blots tested. Abbreviations: CB, cathepsin B; CD, cathepsin D; CS, cathepsin S.

lysosomal modulation. A specific inhibitor of cathepsin D, diazoacetyl-DL-2-aminohexanoic acid methyl ester (1 μM), and the cathepsin B inhibitor glycyl-phenylalanyl-glycine-aldehyde semicarbazone (30 μM) produced as much as 2-fold increases in cathepsin D and other cathepsins, as well as a corresponding 24% to 42% recovery of synaptic markers following the 6-day exposure to chloroquine ($p < 0.01$).

The PADK-induced synaptic recovery shown in Figure 6c was observed across 17 separate groups of slices. The slice samples exhibiting up to 71% restoration of synaptic markers expressed pronounced upregulation of lysosomal enzymes, including a 6- to 8-fold increase in cathepsin B as compared to chloroquine-treated slices without PADK modulation (Table). Elastase was enhanced by a smaller 40% to 80% during lysosomal modulation. Figure 7 shows that PADK-treated slices also exhibit 2- to 4-fold increases in the active intermediate form of cathepsin D as well as in the cleaved, mature form of the enzyme

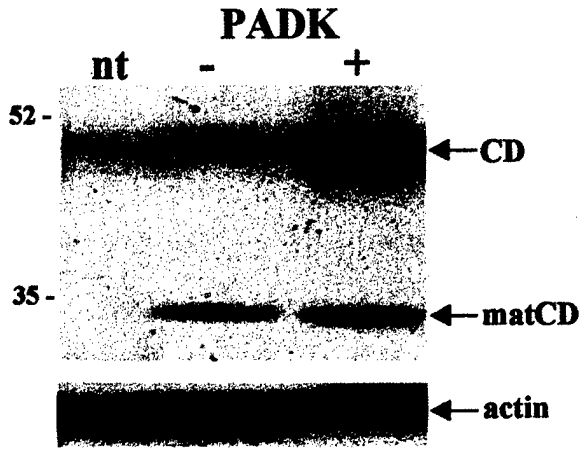


Fig. 7. PADK-mediated lysosomal modulation upregulates the active forms of cathepsin D. Nontreated slices (nt) and slices exposed to chloroquine for 6 days, followed by a 2-day period in the absence (−) or presence (+) of PADK, were analyzed by immunoblot. The antibodies used recognize the active 47-kDa intermediate molecule of cathepsin D (CD) as well as the 34-kDa carboxyl-terminal domain of the mature enzyme (matCD). Actin was assessed on the same blot. The electrophoretic positions of 35- and 52-kDa standards are shown on the left.

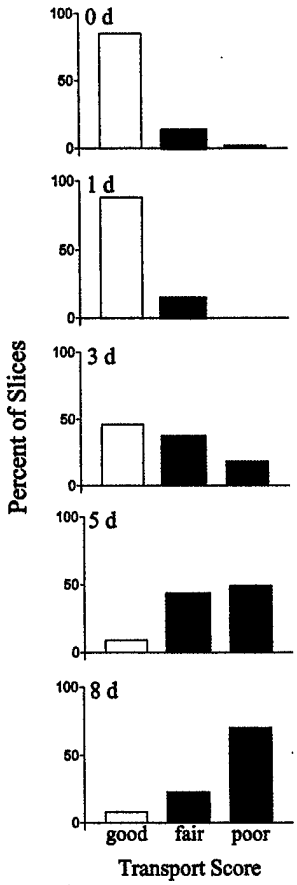


Fig. 8. Lysosomal dysfunction gradually disrupts transport mechanisms. Slices were treated with chloroquine for 0, 1, 3, 5, and 8 days (n = 82, 17, 22, 35, and 68 slices, respectively). HRP was applied to zones of the stratum pyramidale and neurons were allowed to internalize and transport the HRP for 2 h. Apical dendrites were assessed for peroxidase activity and blind scoring categorized the slices as good, fair, or poor, based on the number of neurites exhibiting transport and the extent of such transport.

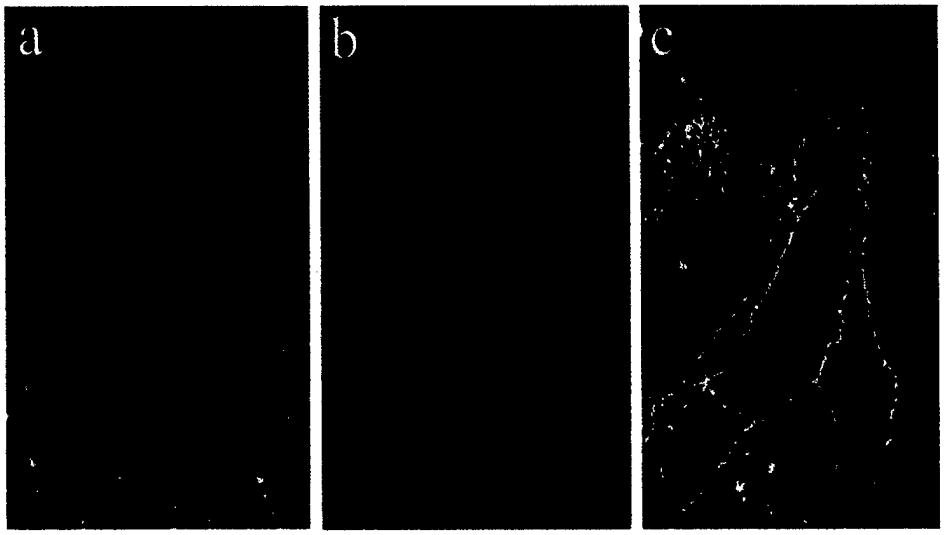
Thus, distinct microtubule changes were elicited in the same slice samples exhibiting pronounced synaptopathogenesis. When PADK was administered after the chloroquine treatment, acetylated tubulin was gradually restored to levels similar to those found in control slices (2 rightmost lanes in Fig. 6a), closely correlating with the recovery of synaptic components.

PADK's positive effect on microtubule integrity was associated with restored transport as part of the modulator's neuroprotective action. After the 2 days of PADK modulation, HRP applied to pyramidal neurons was found to be transported throughout an elaborate array of dendrites (Fig. 9c) in a manner that approximated the HRP distribution observed in control tissue (Fig. 9a). Conversely, slices without PADK treatment exhibited no evidence of transport recovery 2 days after terminating the chloroquine exposure period (Fig. 9b), a result consistent with the continued decline of synaptic markers seen in Figure 6c. Blind scoring of transport capability in 156 slice cultures confirmed the restorative effect on transport by PADK (Fig. 9d).

DISCUSSION

The present study shows that activation of the lysosomal system is triggered during episodes of protein accumulation. Upon potentiation of the lysosomal response, a correspondence between the clearance of protein deposits and synaptic recovery occurred. The current results, thereby, further establish a strong inverse relationship between abnormal protein accumulation and synaptic maintenance. They also indicate that lysosomal activation represents compensatory signaling. Thus, lysosomal dysfunction not only promotes a deleterious cascade leading to synaptic decline; it also activates a compensatory response capable of protecting synapses.

Enzyme upregulation was elicited by experimentally inducing lysosomal disruption with the lysosomotropic agent chloroquine. The changes in lysosomal enzymes are similar to those identified in Alzheimer tissue (3, 15–20), perhaps in response to protein accumulation as in the slice model. Such evidence of a compensatory response induced by lysosomal abnormalities suggests that the brain is not merely the passive recipient of neurological insults. Lysosomal dysfunction is an age-related alteration (51, 52), contributing to protein accumulation events in potentially many ways thereby establishing aging as a primary risk factor for neurodegenerative disorders (1). Evidence exists that indicates lysosomal responses are active in the aging brain and during the early



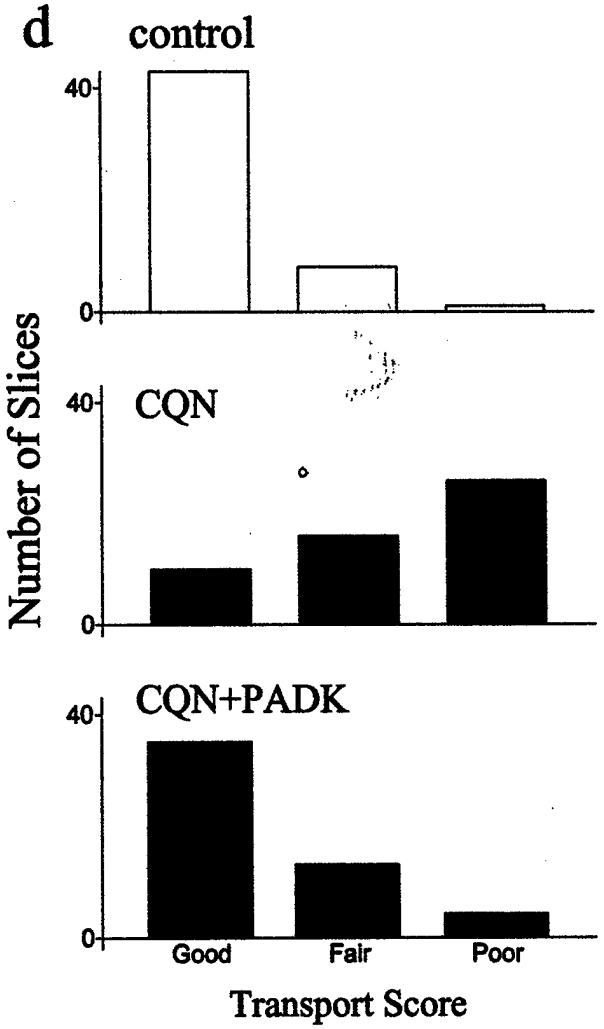


Fig. 9. Lysosomal modulation promotes the recovery of transport capability in hippocampal pyramidal neurons. After 6 days of lysosomal disruption, the chloroquine was removed from the media **(b)** or replaced with PADK **(c)** for a 2-day recovery period. In parallel with nontreated control tissue **(a)**, HRP was applied to several zones of the stratum pyramidale to assess transport as in Figure 8. The cultures were stained for peroxidase activity and their apical dendrites viewed by dark-field microscopy (view-field width: 100 μm). Transport was assessed in the 3 treatment groups (52 slices each) by 2 independent scorers in a blind manner **(d)**. χ^2 analysis: $\chi^2 = 58.9$, $p < 0.0001$ across all 3 treatment groups; $p < 0.0001$ between nontreated controls vs chloroquine-treated slices without lysosomal modulation (CQN); $p < 0.0001$ between the CQN group of slices vs slices that also received lysosomal modulator (CQN+PADK); no significant difference was found between the CQN+PADK group and control cultures.

stages of age-related disorders (15, 16, 18, 20), possibly masking many of the symptoms until they can appear later. The gradual decay of synaptic composition and function in the slice model, as well as the long duration of AD, may be indicative of a prolonged struggle between protein accumulation events and compensatory responses.

Surprisingly, we found that the enzyme regulation events following a lysosomal disturbance are accessible to modulation. The modulating agent PADK allowed the lysosomal activation response to be enhanced in a controlled, nonpathogenic manner. Treatment with PADK greatly upregulated lysosomal enzymes and promoted the stable maintenance of cellular and synaptic components. Increasing cellular levels of lysosomal enzymes is also a potential therapeutic strategy to overcome the abnormal accumulations in neurodegenerative lysosomal storage diseases (53). Upregulation of appropriate proteases may be part of a feedback system that keeps in check any buildup of modified of misfolded protein species. While chloroquine affects many pH-dependent hydrolases, PADK elicits a more pronounced lysosomal response via the mild and selective inhibition of cathepsins B and L. Thus, it may be undigested substrates of cathepsins B and L that are monitored, and their buildup that greatly influences the expression systems controlling lysosomal enzymes.

The compensatory lysosomal response involves the upregulation of at least the aspartic acid protease cathepsin D, the cysteine proteases cathepsins B and L, and possibly β-glucuronidase. Interestingly, β-glucuronidase is among the endosomal-lysosomal components highly expressed in Aβ-resistant neuronal cell lines, thus implicating it in neuronal survival during pathogenic Aβ accumulation (54). While the largest increases were expressed by cathepsins, the cysteine protease cathepsin S was reduced by the chloroquine treatment and positive lysosomal modulation only moderately enhanced its expression level. Such downregulation may be part of compensatory signaling since cathepsin S is capable of generating Aβ variants under certain conditions (55); however, there are indications of increased cathepsin S in AD and Down syndrome (21). Cathepsins B, D, and L exhibited pronounced upregulation following chloroquine exposure and during PADK-mediated lysosomal modulation. This corresponds with the fact that these cathepsins are appropriately positioned for early repair responses. The enzymes are highly expressed in brain regions that are vulnerable to age-related diseases and that contain the highest amounts of APP (16, 56). In addition, cathepsin D has been suggested to reduce Aβ secretion (55) and degrade tau (57–59). Upregulation of cathepsin D occurs before overt cellular damage in neuronal populations targeted by AD as well as in animal models of neurodegeneration (7, 15, 16, 60). It is noteworthy that cathepsin regulation is not restricted to episodes of lysosomal stress or age-related diseases. There are examples of normal biological processes where select proteases are upregulated, including members of the cathepsin family (61, 62). Most interestingly, transcriptional activation of the cathepsin D gene is mediated by 17β-estradiol (62), a hormone shown to accelerate APP trafficking, reduce Aβ generation, and reduce the risk of AD in postmenopausal women (63–66). Together with the current results, cathepsins, particularly the B, D, and L types, appear to be key components of neuroprotective lysosomal activation.

Lysosomal activation occurred in response to pathogenic protein accumulation. Chloroquine-mediated accumulation of proteins and protein fragments is indicative of altered trafficking and/or metabolism in the hippocampal slice model, similar to events associated with many age-related diseases. Such accumulations led to the disruption of microtubule integrity and the loss of microtubule-based transport processes necessary for synaptic maintenance. We recently proposed that these events make up a synaptopathogenic cascade (12). Accordingly, compensatory signaling that affects one element of the cascade would also affect remaining downstream elements. As reported here, positive modulation of the lysosomal response promotes both the clearance of protein deposits and the recovery of synaptic composition. Thus, there is a clear connection between the first and last elements of the pathogenic cascade. As previously shown in the slice model, early protein accumulations were followed by the formation of intracellular deposits (1, 5, 12). PHF-type deposition was composed of phosphorylated tau species that are well-known markers of the aged brain and AD (67–69). Upon terminating chloroquine-induced lysosomal disruption, protein deposits and synaptic decline persisted. The intensity and frequency of PHF-immunopositive deposits remained stable or increased during the postinsult period, and synaptic markers continued to decline. Although insufficient to protect synapses, a strong link between synaptic decline and lysosomal activation was evident, similar to that found in AD neurons containing tau deposits (20, 35). Enhancing the level of lysosomal activation led to clearance of protein accumulations and the return of synaptic maintenance. The clearance of polar tau deposits by lysosomal modulation is consistent with reports suggesting that tau degradation occurs in lysosomal compartments (9, 12, 70, 71). The upregulation of lysosomal enzymes perhaps restores the ability of lysosomes to process modified and misfolded proteins, as evidenced by the reduction of tau species to normal levels.

Microtubule integrity also was rescued by enhancing the lysosomal activation response. The connection between microtubule destabilization and synaptic decline was indicated by the corresponding reduction in tubulin

acetylation, a post-translational modification that provides a marker for microtubule stability (72). Decreases in acetylated tubulin and increases in tubulin fragmentation have been previously shown to correlate with the degree of synaptic decline (12). In addition, AD-related protein deposition events are thought to be involved in the disruption of microtubule integrity (12, 73). In the slice model, persistent intracellular deposits and reduced acetylated tubulin levels occurred together. Interestingly, the opposite trend transpired during PADK-mediated lysosomal modulation, causing tubulin acetylation to be restored to a level similar to that expressed by nontreated control slices.

Stable microtubules are essential for transport mechanisms that support synapses. Thus, restoration of microtubule integrity is not only a consequence of lysosomal modulation but also a key factor in synapse recovery. As shown, microtubule destabilization and somatofugal transport failure were found associated during periods when protein deposits and synaptic decline persisted. Intraneuronal deposits have been linked to the disruption of transport mechanisms, thereby cutting off protein delivery and leading to downregulated expression of affected proteins (5, 12, 20). A previous report indicates that initial transport failure occurs prior to overt protein deposition (12). In the present study, lysosomal modulator applied after several days of lysosomal dysfunction induced both synaptic recovery and restoration of transport. Thus, neurons with severe intracellular deposits and near complete transport failure are capable of expressing synaptic recovery upon activation of appropriate compensatory signaling. Along with recent findings (74), the results also indicate that the deleterious effects of protein deposition on synaptic integrity are not permanent.

The slice model is a valuable tool for studying synaptic pathology because it exhibits gradual degeneration of nerve terminals, synonymous to that which is thought to occur during the initial stages of AD. As shown here and previously (5, 12), lysosomal dysfunction causes a decline in synaptic proteins and their mRNAs, correlating with the degree of intracellular deposition. Such a correlation is similar to that evident in neurons of AD brains (20, 35). Synaptic decline, in fact, is a prominent feature of Alzheimer brains, and the degree of cognitive impairment is more highly correlated with synaptic deterioration than with other pathological features. By clearing protein deposits and re-establishing microtubule-based mechanisms, PADK restored synaptic integrity as evidenced by the marked recovery of pre- and postsynaptic markers. The fact that lysosomal modulation rescues synapse maintenance in the face of acute protein accumulation leads to the idea that the lysosomal system can be exploited to treat protein deposition diseases. The need for early clearance of accumulated protein species is an increasingly recognized theme among a diverse group of protein deposition disorders, thus, implicating lysosomal modulation as a common therapeutic approach.

Lysosomal activation may be among many examples of compensatory systems that are activated in response to disease states or toxin exposure. The presence of compensatory signaling, although at insufficient levels to prevent gradual synaptic deterioration, possibly allows cellular repair to require only modest intervention of the pathogenic cascade. It is important to note that even trace levels of synaptic recovery may lead to progressive cellular recuperation facilitated by the restored neurotransmission and associated signaling events. Particularly in the hippocampus, glutamatergic transmission is vital for synaptic and cellular maintenance (36, 75, 76). Thus, modest levels of recovery might reinstate synaptic signaling to a sufficient degree to activate compensatory pathways, thereby slowing degenerative processes and altering the balance between pathogenesis and repair.

ACKNOWLEDGMENT

The authors wish to thank Ms. Queenie Brown and Ms. Ebru Caba for their excellent technical assistance on this project.

REFERENCES

1. Bahr BA, Bendiske J. The neuropathogenic contributions of lysosomal dysfunction. J Neurochem 2002;83:481–89
2. Benowitz LI, Rodriguez W, Paskevich P, et al. The amyloid precursor protein is concentrated in neuronal lysosomes in normal and Alzheimer disease subjects. Exp Neurol 1989;106:237–50
3. Cataldo AM, Paskevich PA, Kominami E, Nixon RA. Lysosomal hydrolases of different classes are abnormally distributed in brains of patients with Alzheimer disease. Proc Natl Acad Sci U S A 1991; 88:10998–11002
4. Golde TE, Estus S, Younkin LH, Selkoe DJ, Younkin SG. Processing of the amyloid protein precursor to potentially amyloidogenic derivatives. Science 1992;255:728–30
5. Bahr BA, Abai B, Gall CM, et al. Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. Exp Neurol 1994;129:81–94
6. Hajimohammadreza I, Anderson VER, Cavanagh JB, et al. β-Amyloid precursor protein fragments and lysosomal dense bodies are found in rat brain neurons after ventricular infusion of leupeptin. Brain Res 1994;640:25–32
7. Nixon RA, Mathews PM, Cataldo AM. The neuronal endosomal-lysosomal system in Alzheimer's disease. J Alzheimer's Disease 2001;3:97–107
8. Ivy GO. Protease inhibition causes some manifestations of aging and Alzheimer's disease in rodent and primate brain. Ann NY Acad Sci 1992;674:89–102
9. Bahr BA. Long-term hippocampal slices: A model system for investigating synaptic mechanisms and pathologic processes. J Neurosci Res 1995;42:294–305
10. Takauchi S, Miyoshi K. Cytoskeletal changes in rat cortical neurons induced by long-term intraventricular infusion of leupeptin. Acta Neuropathol 1995;89:8–16
11. Bi X, Yong AP, Zhou J, Ribak CE, Lynch G. Rapid induction of intraneuronal neurofibrillary tangles in apolipoprotein E-deficient mice. Proc Natl Acad Sci U S A 2001;98:8832–37

12. Bendiske J, Caba E, Brown QB, Bahr BA. Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. J Neuropathol Exp Neurol 2002;61:640–50
13. Sapp E, Schwarz C, Chase K, et al. Huntingtin localization in brains of normal and Huntington's disease patients. Ann Neurol 1997;42: 604–12
14. DiFiglia M, Sapp E, Chase KO, et al. Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 1997;277:1990–93
15. Cataldo AM, Barnett JL, Berman SA, et al. Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: Evidence for early up-regulation of the endosomal-lysosomal system. Neuron 1995;14:671–80
16. Cataldo AM, Hamilton DJ, Barnett JL, Paskevich PA, Nixon RA. Properties of the endosomal-lysosomal system in the human central nervous system: Disturbance mark most neurons in populations at risk to degenerate in Alzheimer's disease. J Neurosci 1996;16: 186–99
17. Cross AJ, Crow TJ, Dawson JM, et al. Subcellular pathology of human neurodegenerative disorders: Alzheimer-type dementia and Huntington's disease. J Neurochem 1986;47:882–89
18. Haas U, Sparks DL. Cortical cathepsin D activity and immunolocalization in Alzheimer disease, critical coronary artery disease, and aging. Mol Chem Neuropath 1996;29:1–14
19. Ii K, Ito H, Kominami E, Hirano A. Abnormal distribution of cathepsin proteinases and endogenous inhibitors (cystatins) in the hippocampus of patients with Alzheimer's disease, Parkinsonism-dementia complex on Guam, and senile dementia and in the aged. Virchows Arch A, Path Anat Histopath 1993;423:185–94
20. Callahan LM, Vaules WA, Coleman PD. Quantitative decrease in synaptophysin message expression and increase in cathepsin D message expression in Alzheimer disease neurons containing neurofibrillary tangles. J Neuropathol Exp Neurol 1999;58:275–87
21. Lemere CA, Munger JS, Shi GP, et al. The lysosomal cysteine protease, cathepsin S, is increased in Alzheimer's disease and Down syndrome brain. An immunocytochemical study. Am J Path 1995; 146:848–60
22. Kegel KB, Kim M, Sapp E, et al. Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. J Neurosci 2000;20:7268–78
23. Hoffman KB, Bi X, Pham JT, Lynch G. β-Amyloid increases cathepsin D levels in hippocampus. Neurosci Lett 1998;250:75–78
24. Burdick D, Kosmoski J, Knauer MF, Glabe CG. Preferential adsorption, internalization and resistance to degradation of the major isoform of the Alzheimer's amyloid peptide, Aβ 1–42, in differentiated PC12 cells. Brain Res 1997;746:275–84
25. Bahr BA, Hoffman KB, Yang AJ, et al. Amyloid β protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. J Comp Neurol 1998;397:139–47
26. Yang AJ, Chandswangbhuvana D, Margol L, Glabe CG. Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Aβ1-42 pathogenesis. J Neurosci Res 1998;52:691–98
27. Ditaranto K, Tekirian TL, Yang AJ. Lysosomal membrane damage in soluble Aβ-mediated cell death in Alzheimer's disease. Neurobiol Dis 2001;8:19–31
28. Davies CA, Mann DM, Sumpter PQ, Yates PO. A quantitative morphometric analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease. J Neurol Sci 1987;78:151–64
29. Terry RD, Masliah E, Salmon DP, et al. Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. Ann Neurol 1991;30:572–80
30. Honer WG, Dickson DW, Gleeson J, Davies P. Regional synaptic pathology in Alzheimer's disease. Neurobiol Aging 1992;13: 375–82
31. Small DH, Mok SS, Bornstein JC. Alzheimer's disease and Aβ toxicity: From top to bottom. Nat Rev Neurosci 2001;2:595–98
32. Selkoe DJ. Alzheimer's disease is a synaptic failure. Science 2002; 298:789–91
33. Samuel W, Masliah E, Hill LR, Butters N, Terry R. Hippocampal connectivity and Alzheimer's dementia: Effects of synapse loss and tangle frequency in a two-component model. Neurology 1994;44: 2081–88
34. Heinonen O, Soininen H, Sorvari H, et al. Loss of synaptophysin-like immunoreactivity in the hippocampal formation is an early phenomenon in Alzheimer's disease. Neurosci 1995;54:375–84
35. Callahan LM, Vaules WA, Coleman PD. Progressive reduction of synaptophysin message in single neurons in Alzheimer disease. J Neuropathol Exp Neurol 2002;61:384–95
36. Bahr BA, Bendiske J, Brown QB, et al. Survival signaling and selective neuroprotection through glutamatergic transmission. Exp Neurol 2002;174:37–47
37. Garcia-Ladona FJ, Palacios JM, Probst A, Wieser HG, Mengod GA. Excitatory amino acid AMPA receptor mRNA localization in several regions of normal and neurological disease affected human brain. An in situ hybridization histochemistry study. Brain Res Mol Brain Res 1994;21:75–84
38. Thorns V, Mallory M, Hansen L, Masliah E. Alterations in glutamate receptor 2/3 subunits and amyloid precursor protein expression during the course of Alzheimer's disease and Lewy body variant. Acta Neuropathol (Berl) 1997;94:539–48
39. Ikonomovic MD, Mizukami K, Davies P, et al. The loss of GluR2(3) immunoreactivity precedes neurofibrillary tangle formation in the entorhinal cortex and hippocampus of Alzheimer brains. J Neuropathol Exp Neurol 1997;56:1018–27
40. Yasuda RP, Ikonomovic MD, Sheffield R, et al. Reduction of AMPA-selective glutamate receptor subunits in the entorhinal cortex of patients with Alzheimer's disease pathology: A biochemical study. Brain Res 1995;678:161–67
41. Sze CI, Troncoso JC, Kawas C, et al. Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. J Neuropathol Exp Neurol 1997;56: 933–44
42. Masliah E, Mallory M, Alford M, et al. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. Neurology 2001;56:127–29
43. Hsia AY, Masliah E, McConlogue L, et al. Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. Proc Natl Acad Sci USA 1999;96:3228–33
44. Mucke L, Masliah E, Yu GO, et al. High-level neuronal expression of Aβ1-42 in wild-type human amyloid protein precursor transgenic mice: Synaptotoxicity without plaque formation. J Neurosci 2000; 20:4050–58
45. Bahr BA, Hoffman KB, Kessler M, et al. Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (*GR53*) in brain tissue. Neurosci 1996;74:707–21
46. Burdick D, Soreghan B, Kwon M, et al. Assembly and aggregation properties of synthetic Alzheimer's Aβ amyloid peptide analogs. J Biol Chem 1992;267:546–54
47. Bahr BA, Kessler M, Rivera S, et al. Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. Hippocampus 1995;5:425–39
48. Caporaso GL, Gandy SE, Buxbaum JD, Greengard P. Chloroquine inhibits intracellular degradation but not secretion of Alzheimer β/A4 amyloid precursor protein. Proc Natl Acad Sci U S A 1992;89: 2252–56

49. Scornik OA. Effects of inhibitors of protein degradation on the rate of protein synthesis in Chinese hamster ovary cells. J Cell Physiol 1984;121:257–62
50. Stamer K, Vogel R, Thies E, Mandelkow E, Mandelkow EM. Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress. J Cell Biol 2002;156:1051–63
51. Brunk U, Brun A. The effect of aging on lysosomal permeability in nerve cells of the central nervous system. An enzyme histochemical study in rat. Histochemie 1972;30:315–24
52. Nakamura Y, Takeda M, Suzuki K, et al. Lysosome instability in aged rat brain. Neurosci Lett 1998;97:215–20
53. Dobrenis K, Joseph A, Rattazzi MC. Neuronal lysosomal enzyme replacement using fragment C of tetanus toxin. Proc Natl Acad Sci U S A 1992;6:2297–2301
54. Li Y, Xu C, Schubert D. The up-regulation of endosomal-lysosomal components in amyloid β-resistant cells. J Neurochem 1999;73: 1477–82
55. Munger JS, Haass C, Lemere CA, et al. Lysosomal processing of the amyloid precursor protein to Aβ peptides: A distinct role for cathepsin S. Biochem J 1995;311:299–305
56. Petanceska S, Burke S, Watson SJ, Devi L. Differential distribution of messenger RNAs for cathepsins B, L and S in adult rat brain: An in situ hybridization study. Neurosci 1994;59:729–38
57. Kenessey A, Nacharaju P, Ko LW, Yen SH. Degradation of tau by lysosomal enzyme cathepsin D: Implication for Alzheimer neurofibrillary degeneration. J Neurochem 1997;69:2026–38
58. Ikeda K, Akiyama H, Arai T, et al. Alz-50/Gallyas-positive lysosome-like intraneuronal granules in Alzheimer's disease and control brains. Neurosci Lett 1998;258:113–16
59. Bi X, Haque TS, Zhou J, et al. Novel cathepsin D inhibitors block the formation of hyperphosphorylated tau fragments in hippocampus. J Neurochem 2000;74:1469–77
60. Adamec E, Mohan PS, Cataldo AM, Vonsattel JP, Nixon RA. Up-regulation of the lysosomal system in experimental models of neuronal injury: Implications for Alzheimer's disease. Neurosci 2000; 100:663–75
61. Robker RL, Russell DL, Espey LL, et al. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. Proc Natl Acad Sci U S A 2000;97:4689–94
62. Wang F, Samudio I, Safe S. Transcriptional activation of cathepsin D gene expression by 17β-estradiol: Mechanism of aryl hydrocarbon receptor-mediated inhibition. Mol Cell Endocrinol 2001;172: 91–103
63. Greenfield JP, Leung LW, Cai D, et al. Estrogen lowers Alzheimer β-amyloid generation by stimulating trans-Golgi network vesicle biogenesis. J Biol Chem 2002;277:12128–36
64. Petanceska SS, Nagy V, Frail D, Gandy S. Ovariectomy and 17β-estradiol modulate the levels of Alzheimer's amyloid β peptides in brain. Neurology 2000;54:2212–17
65. Zheng H, Xu H, Wang H, et al. Modulation of Aβ peptides by estrogen in mouse models. J Neurochem 2002;80:191–96
66. Turner RS. Alzheimer's disease in man and transgenic mice: Females at higher risk. Am J Pathol 2001;158:797–801
67. Bahr BA, Vicente JS. Age-related phosphorylation and fragmentation events influence the distribution profiles of distinct tau isoforms in mouse brain. J Neuropathol Exp Neurol 1998;57:111–21
68. Delacourte A, Flament S, Dibe EM, et al. Pathological proteins Tau 64 and 69 are specifically expressed in the somatodendritic domain of the degenerating cortical neurons during Alzheimer's disease: Demonstration with a panel of antibodies against tau proteins. Acta Neuropathol (Berl) 1990;80:111–17
69. Goedert M, Spillantini MG, Cairns NJ, Crowther RA. Tau proteins of Alzheimer paired helical filaments: Abnormal phosphorylation of all six brain isoforms. Neuron 1992;8:159–68
70. Ikeda K, Akiyama H, Arai T, et al. Neurons containing Alz-50-immunoreactive granules around the cerebral infarction: Evidence for the lysosomal degradation of altered tau in human brain? Neurosci Lett 2000;284:187–89
71. Oyama F, Murakami N, Ihara Y. Chloroquine myopathy suggests that tau is degraded in lysosomes: Implication for the formation of paired helical filaments in Alzheimer's disease. Neurosci Res 1998; 31:1–8
72. Black MM, Baas PW, Humphries S. Dynamics of α-tubulin deacetylation in intact neurons. J Neurosci 1989;9:358–68
73. Hempen B, Brion JP. Reduction of acetylated α-tubulin immunoreactivity in neurofibrillary tangle-bearing neurons in Alzheimer's disease. J Neuropathol Exp Neurol 1996;55:964–72
74. Kotilinek LA, Bacskai B, Westerman M, et al. Reversible memory loss in a mouse transgenic model of Alzheimer's disease. J Neurosci 2002;22:6331–35
75. Bambrick LL, Yarowsky PJ, Krueger BK. Glutamate as a hippocampal neuron survival factor: An inherited defect in the trisomy 16 mouse. Proc Natl Acad Sci U S A 1995;92:9692–96
76. McKinney RA, Capogna M, Durr R, Gahwiler BH, Thompson SM. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat Neurosci 1999;2:44–49

Received September 4, 2002
Revision received December 16, 2002
Accepted January 9, 2003

European Journal of Neuroscience, Vol. 16, pp. 1463–1471, 2002

Caspase-mediated death of newly formed neurons in the adult rat dentate gyrus following status epilepticus

Christine T. Ekdahl,[1] Paul Mohapel,[1] Ekkehard Weber,[2] Ben Bahr,[3] Klas Blomgren[4,5] and Olle Lindvall[1]
[1]Section of Restorative Neurology, Wallenberg Neuroscience Center, BMC A11, 221 84 Lund, Sweden
[2]Institute of Physiological Chemistry, Martin Luther University Halle-Wittenberg, Hollystr. 1, D-06097 Halle, Germany
[3]Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut 06269-2092, USA
[4]Department of Physiology, Box 432, SE-405 30, Göteborg, Sweden
[5]Department of Paediatrics, The Queen Silvia Children's Hospital, SE-416 85, Göteborg, Sweden

Abstract

A large proportion of cells that proliferate in the adult dentate gyrus under normal conditions or in response to brain insults exhibit only short-term survival. Here, we sought to determine which cell death pathways are involved in the degeneration of newly formed neurons in the rat dentate gyrus following 2 h of electrically induced status epilepticus. We investigated the role of three families of cysteine proteases, caspases, calpains, and cathepsins, which can all participate in apoptotic cell death. Status epilepticus increased the number of bromodeoxyuridine (BrdU)-positive proliferated cells in the subgranular zone of the dentate gyrus. At the time of maximum cell proliferation, immunohistochemical analyses revealed protein expression of active caspase-cleaved poly (ADP-ribose) polymerase (PARP) in ≈66% of the BrdU-positive cells, while none of them expressed cathepsin B or the 150-kDa calpain-produced fodrin breakdown product. To evaluate the importance of cysteine proteases in regulating survival of the newly formed neurons, we administered intracerebroventricular infusions of a caspase inhibitor cocktail (zVAD-fmk, zDEVD-fmk and zLEHD-fmk) over a 2-week period, sufficient to allow for neuronal differentiation, starting 1 week after the epileptic insult. Increased numbers of cells double-labelled with BrdU and neuron-specific nuclear protein (NeuN) marker were detected in the subgranular zone and granule cell layer of the caspase inhibitor-treated rats. Our data indicate that caspase-mediated cell death pathways are active in progenitor cell progeny generated by status epilepticus and compromise survival during neuronal differentiation.

Keywords: apoptosis, epilepsy, neurogenesis, PARP, proliferation

Introduction

The generation of new neurons in the adult mammalian brain is often referred to as a single process called neurogenesis. However, neurogenesis comprises at least four distinct steps: proliferation, survival, migration, and differentiation (Gage *et al.*, 1998), and each step has its own machinery of initiators, executors, and brakes. Many factors influence the number of new neurons formed in the two neurogenic areas of the adult rodent brain, the subventricular zone in the wall of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG) (Scharff, 2000; Duman *et al.* 2001). Brain insults such as epileptic seizures, stroke, and traumatic brain injury (Bengzon *et al.*, 1997; Parent *et al.*, 1997; Gray & Sundstrom, 1998; Liu *et al.*, 1998; Covolan *et al.* 2000a; Nakagawa *et al.* 2000; Arvidsson *et al.* 2001) stimulate the initial phase of neurogenesis, i.e. cell proliferation. Status epilepticus (SE) induces a several-fold increase in the cell proliferation rate in the adult rat DG, starting a couple of days after the insult and remaining at peak levels for a 2-week period, before returning to control levels after 4 weeks. Of the surviving newborn cells, more than 90% express markers of mature neurons at 5 weeks after the seizures, and many of these cells have migrated into the granule cell layer (GCL) (Parent *et al.*, 1997; Ekdahl *et al.* 2001).

It is not clear how the survival of the proliferated cells is regulated either in the normal or the injured brain (Cameron & McKay, 2001; Ekdahl *et al.* 2001). Apoptotic cell death, which is observed in the SGZ following both mild seizures (Bengzon *et al.*, 1997) and SE (Sloviter *et al.*, 1996; Covolan *et al.* 2000b), was recently suggested to mediate the degeneration of the newly formed cells (Covolan *et al.* 2000a; Ekdahl *et al.* 2001). In agreement with this, administration of the cell-cycle inhibitor cyclohexamide (Covolan *et al.* 2000a) or cysteinyl aspartate-specific proteinase (caspase) inhibitors (Ekdahl *et al.* 2001) increased the number of newborn progenitor cell progeny in the DG following SE. This result of caspase inhibitor administration raised the possibility that it might be the caspase-mediated apoptotic pathway that compromises the survival of progenitor cell progeny in the SGZ (Ekdahl *et al.* 2001). However, the specificity of the caspase inhibitors used has recently been questioned, as they may not only affect caspase activity but also other cysteine proteases such as calpains and cathepsins (Wolf *et al.*, 1999; Gray *et al.* 2001). It should also be noted, that caspase inhibitor infusions only during and up to 1 week after the epileptic insult resulted in a transient increase in progenitor cell progeny, whereas no effect on the number of new

Correspondence: Dr C. T. Ekdahl, as above.
E-mail: christine.ekdahl@neurol.lu.se

Received 7 May 2002, revised 26 July 2002, accepted 6 August 2002

doi:10.1046/j.1460-9568.2002.02202.x

neurons was observed (Ekdahl *et al.* 2001). It is therfore unknown whether treatment can promote survival of seizure-generated cells until they have differentiated into neurons.

We set out to explore whether three different cysteine protease families, the caspases, calpains, and cathepsins, are active in newly formed progenitor cell progeny and involved in the regulation of their survival following an epileptic insult. After kainic acid-induced SE, activation of caspases, calpains, and cathepsins has been detected in vulnerable pyramidal and hilar neurons (Hetman *et al.*, 1995; Bi *et al.*, 1996; Gillardon *et al.*, 1997; Tominaga *et al.*, 1998; Becker *et al.*, 1999; Faherty *et al.*, 1999; Henshall *et al.* 2000; Kondratyev & Gale, 2000). The caspases play a major role in apoptotic cell death and caspase 3 is one of the key executors (Cohen, 1997). Increased activation of calpains often results in necrosis, but can also induce apoptosis (Johnson, 2000; Kidd *et al.* 2000; Wang, 2000). The cathepsins, a lysosomal cysteine protease family, have been implicated in both necrosis and apoptosis (Kidd *et al.* 2000; Foghsgaard *et al.* 2001). We used immunohistochemical methods to analyse protein expression of activated caspase-cleaved poly (ADP-ribose) polymerase (cPARP), cathepsin B, and a breakdown product of fodrin, produced by calpain cleavage (fodrin breakdown product of 150 kDa, FBDP), in newly formed cells in the SGZ following 2 h of electrically induced SE. We then examined whether the number of surviving new cells could be increased by infusions of caspase inhibitors into the lateral ventricle until neuronal differentiation had occurred. The daily infusions were not started until 1 week after SE, at the time of maximum seizure-induced proliferation, and were then continued for 2 weeks.

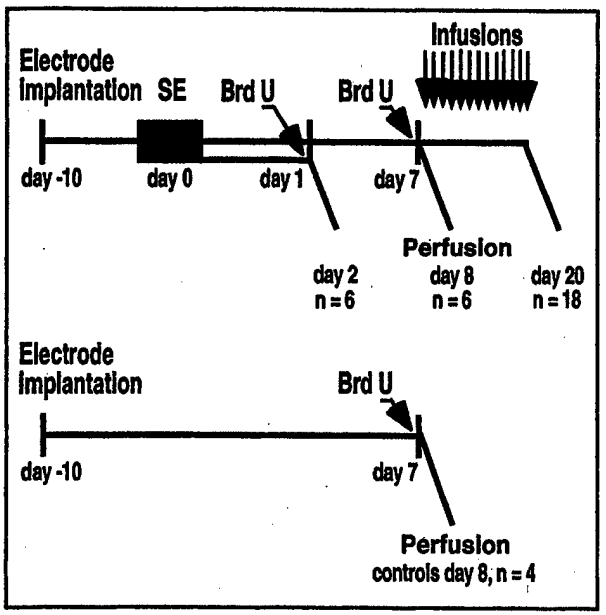


FIG. 1. Experimental design and group assignment. All rats ($n = 34$) were implanted with one or two electrodes into the CA1/CA3 region of the ventral hippocampus. One group of rats ($n = 18$) was also implanted with an intracerebroventricular cannula. Ten days later, all rats except four nonstimulated controls were subjected to electrically induced status epilepticus. Six animals received BrdU injections the day after the insult and were perfused 24 h later. The other rats were given BrdU injections day 7, and were either perfused at day 8 ($n = 10$) or received daily infusions of caspase inhibitors or vehicle over 2 weeks prior to perfusion ($n = 18$).

Materials and methods

Animals and surgery

Thirty-four adult male Sprague–Dawley rats (B&K Universal, Stockholm, Sweden), weighing 270–340 g at the time of surgery, were used. Rats were housed separately under 12 h light : 12 h dark conditions with *ad libitum* access to water and food. Experimental procedures followed guidelines set by the Malmoe–Lund Ethical Committee for the use and care of laboratory animals. All rats were anaesthetized with sodium pentobarbital (65 mg/kg i.p.) and implanted with a twisted insulated stainless-steel stimulating/recording electrode (Plastics One, Roanoke, VA, USA) unilaterally (left side; $n = 18$) or bilaterally ($n = 16$, including four nonstimulated controls) into the CA1–CA3 region of the ventral hippocampus [coordinates, 4.8 mm caudal and 5.2 mm lateral to bregma, 6.3 mm ventral from dura, toothbar at ≈3.3 mm (Paxinos & Watson, 1997)]. Rats with a unilateral electrode also had a guiding cannula (Plastics One) implanted into the right lateral ventricle (coordinates, 0.8 mm caudal and 1.3 mm lateral to bregma, 3.5 mm ventral from dura, toothbar at –3.3 mm).

Induction of status epilepticus

Ten days following surgery, all rats except the nonstimulated controls were subjected to electrically induced, self-sustained SE (eSE), as originally described by Lothman *et al.* (1989). In brief, the rats received 1 h of suprathreshold stimulation consisting of 10 s trains of 1-ms biphasic square wave pulses, at a frequency of 50 Hz. The stimulation was interrupted every 10 min for 1 min to allow for electroencephalogram (EEG) recording and measurement of afterdischarges (MacLab; AD Systems, Hastings, UK). After cessation of stimulation, all rats exhibited self-sustained continuous ictal EEG activity, which was associated with varying severity of motor behavioural seizures, categorized into distinct eSE profiles (P. Mohapel, C. T. Ekdahl and O. Lindvall, unpublished observation). The milder profiles consisted of hyperactive motor behaviour, or partial seizures, scored as grade 1 and 2 in the classical kindling motor behaviour scale. The more severe eSE profile corresponded to clonic seizures rated as grade 3–5 (Racine, 1972). Both behavioural convulsions and ictal EEG activity were arrested in all rats with pentobarbital (65 mg/kg i.p.) at 2 h after stimulation offset. With rats that had received intracerebral infusions ($n = 18$), EEG recordings were sampled for 2–4 min every other morning from day 7 and onwards to measure hippocampal EEG frequency.

Bromodeoxyuridine (BrdU) administration

Seven days after eSE, 28 rats (including four nonstimulated controls) were given a series of four injections (every 2 h during a 6-h period) of the thymidine analogue BrdU (50 mg/kg, i.p.) dissolved in potassium phosphate-buffered saline (KPBS), in order to label mitotic cells (Dolbeare, 1995). In addition, BrdU was administered to six rats without an implanted cannula at 1 day after eSE. All rats without cannula implantation ($n = 16$) were perfused (see details below) the day after BrdU injections (day 2, $n = 6$; day 8, $n = 6$; nonstimulated controls, $n = 4$), while those with cannula implantation received intracerebroventricular infusions during an additional 2 weeks before perfusion ($n = 18$) (Fig. 1). This group assignment intended to cover both the early gliosis phase (day 2), the neuronal proliferation phase (day 8), and the neuronal differentiation phase (day 20) following SE.

Intracerebroventricular administration of caspase inhibitors

The caspase inhibitor cocktail comprised equal portions (2 μg) of three different caspase inhibitors: zVAD-fmk (a multicaspase inhibitor), zDEVD-fmk (a caspase 3 inhibitor), and zLEHD-fmk (a caspase 9 inhibitor) (Enzyme Systems, Livermore, CA, USA). All inhibitors were dissolved in 14% dimethylsulfoxide (DMSO) in KPBS at a concentration of 1 μg/μL. The vehicle solution consisted of 14% DMSO. With any given infusion, rats received 6 μL of either caspase inhibitor cocktail ($n = 9$) or vehicle solution ($n = 9$), which was delivered over 10 min using a 10-μL Hamilton syringe attached to a microinjection pump (CMA, Stockholm, Sweden). The rats were given daily infusions over 13 days, starting directly after the BrdU injections on day 7.

Tissue fixation and sectioning

Rats received an overdose of anaesthesia (sodium pentobarbital; 200 mg/kg i.p.) and were transcardially perfused with 50 mL of saline followed by 250 mL of ice-cold formaldehyde solution [4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4]. Brains were removed, postfixed overnight in the same medium, and then placed in 20% sucrose/0.1 M phosphate buffer for at least 24 h. Coronal sections (30 μm) through the dorsal hippocampus were cut on a freezing microtome and stored in cryoprotective solution.

Caspase-cleaved poly (ADP-ribose) polymerase (cPARP), calpain-specific fodrin breakdown product 150 kDa (FBDP), and cathepsin B immunohistochemistry

Free-floating sections were mounted in KPBS onto microscope slides and dried before pre-incubation in 4% goat serum for 1.5 h. Subsequently, the sections were incubated overnight with rabbit anti-(rat cPARP) antibody (1 : 50; Cell Signalling Technology, Beverly, MA, USA; Au-Yeung *et al.* 2001; Erhardt *et al.* 2001; Han *et al.* 2001) or rabbit anti-FBDP antibody (1 : 50; Bahr *et al.*, 1995; Bednarski *et al.*, 1995; Bahr *et al.* 2002) in 3% bovine serum albumin (only for the cPARP staining) and 0.25% Triton X-100 at 4 °C. Biotinylated goat anti-(rabbit IgG) antibody (1 : 50; Vector Laboratories, Burlingame, CA, USA) was applied for 1.5 h, followed by quenching for 12 min in 3% hydrogen peroxide before incubation with avidin–biotin–peroxidase complex (Elite ABC kit, Vector Laboratories) for 1.5 h and treatment with diaminobenzidine (0.5 mg/mL) and hydrogen peroxide. For the cathepsin B staining, free-floating sections were quenched for 20 min in 3% hydrogen peroxide and 10% methanol before pre-incubation with 5% goat serum in 0.25% Triton X-100 for 1 h, followed by incubation at 4 °C overnight with rabbit anti-(rat cathepsin B) antibody (1 : 5000; prepared from rat liver lysosomes, electrophoretically homogeneous, and no cross-reactivities with other related lysosomal cysteine proteinases, i.e. cathepsin L, S, and H; Kirschke *et al.*, 1997; Fiebiger *et al.* 2001). Sections were then incubated for 2 h with biotinylated goat anti-(rabbit IgG) antibody (1 : 200) before incubation with avidin–biotin–peroxidase complex for 1.5 h and treatment with diaminobenzidine (0.5 mg/mL) and hydrogen peroxide. Rinsing in KPBS was applied between each step for all three staining procedures.

TUNEL staining

In situ detection of single- and double-stranded DNA breaks was performed by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling (TUNEL). Free-floating sections were mounted in KPBS onto glass microscope slides and dried. The sections were pretreated with 4% PFA for 20 min, methanol for 30 min, proteinase K (10 μg/mL KPBS) for 6 min, 4% PFA for 5 min, and ice-cold 0.1% Triton X-100 in 0.1% sodium citrate for 2 min with KPBS rinses between each step. Each section was then incubated in the dark at 37 °C for 60 min in terminal deoxynucleotidyl transferase (TdT) buffer containing 17 μL TdT enzyme solution and 150 μL TUNEL label solution with fluoroscein-conjugated dUTP (Boehringer Mannheim, Germany). Sections were counterstained with Hoechst 33342 (10 μg/mL, Molecular Probes, Leiden, the Netherlands) for 10 min in the dark. Slides were cover-slipped with glycerol-based mounting medium. In the TUNEL/cPARP double staining, the TUNEL staining protocol, with omitted proteinase K and sodium citrate treatment, was directly followed by the cPARP staining, which was visualized with Cy3-conjugated donkey anti-(rabbit IgG) antibody (1 : 200; Jackson ImmunoResearch, West Grove, PA, USA).

Double-label immunohistochemistry

For BrdU/neuron-specific nuclear protein (NeuN) double-label immunofluorescence, free-floating sections were first denatured in 1 M HCl for 30 min at 65 °C. The sections were then rinsed in KPBS to neutral pH before pre-incubation with 2% goat and donkey serum in 0.25% Triton X-100 for 1 h. The sections were incubated overnight with rat anti-BrdU antibody (1 : 100; Sigma, Stockholm, Sweden) and mouse anti-NeuN antibody (1 : 100; Chemicon, Temecula, CA, USA) at 4 °C followed by rinsing and incubation in the dark for 2 h with Cy3-conjugated donkey anti-(rat IgG) antibody (1 : 400; Jackson ImmunoResearch) and biotinylated horse anti-(mouse IgG) antibody (1 : 200; Vector Laboratories). After rinsing, sections were incubated in Streptavidin Alexa Fluor 488 (1 : 200; Molecular Probes) in the dark for 2 h, mounted on glass slides, and finally cover-slipped with glycerol-based mounting medium. The staining protocol was similar for BrdU/glial fibrillary acidic protein (GFAP) double labelling where a mouse anti-GFAP primary antibody (1 : 1000; Sigma) was used for labelling of glial cells. In the BrdU/cPARP and cathepsin B double stainings, the BrdU staining protocol was directly followed by either the cPARP or cathepsin B staining, which were visualized with biotinylated goat anti-(rabbit IgG) antibody (1 : 200) and Streptavidin Alexa Fluor 488.

Cell quantification

All analyses were performed by an observer blind to the treatment conditions. Immunostaining was examined with an Olympus AX-70 light microscope. The number of positive cells was counted in the GCL and within two cell diameters below this region in the SGZ (referred to as GCL/SGZ) as well as in the dentate hilus. FBDP and TUNEL/Hoechst staining were also assessed in the CA1 and CA3 regions. In order to reduce counting bias, only central cell profiles (Coggeshall & Lekan, 1996) exceeding 3 μm were included. Furthermore, the TUNEL-positive cells had to exhibit pycnotic morphology and condensed nuclei with the Hoechst stain in order to be included in the analyses. With each staining, the number of labelled cells in three coronal sections from each rat (5–7 sections for the BrdU counts), located between 3.3 and 4.3 mm posterior to bregma (encompassing the dorsal hippocampal region), was counted and expressed as average number of cells per section. For cPARP, cathepsin B, FBDP, and BrdU staining, the counts are reported as mean number of cells per section in one hemisphere, while the TUNEL/Hoechst staining is reported as mean number of cells per section in both hemispheres due to the low number of labelled cells. Co-localization of BrdU-positive cells with either NeuN, GFAP,

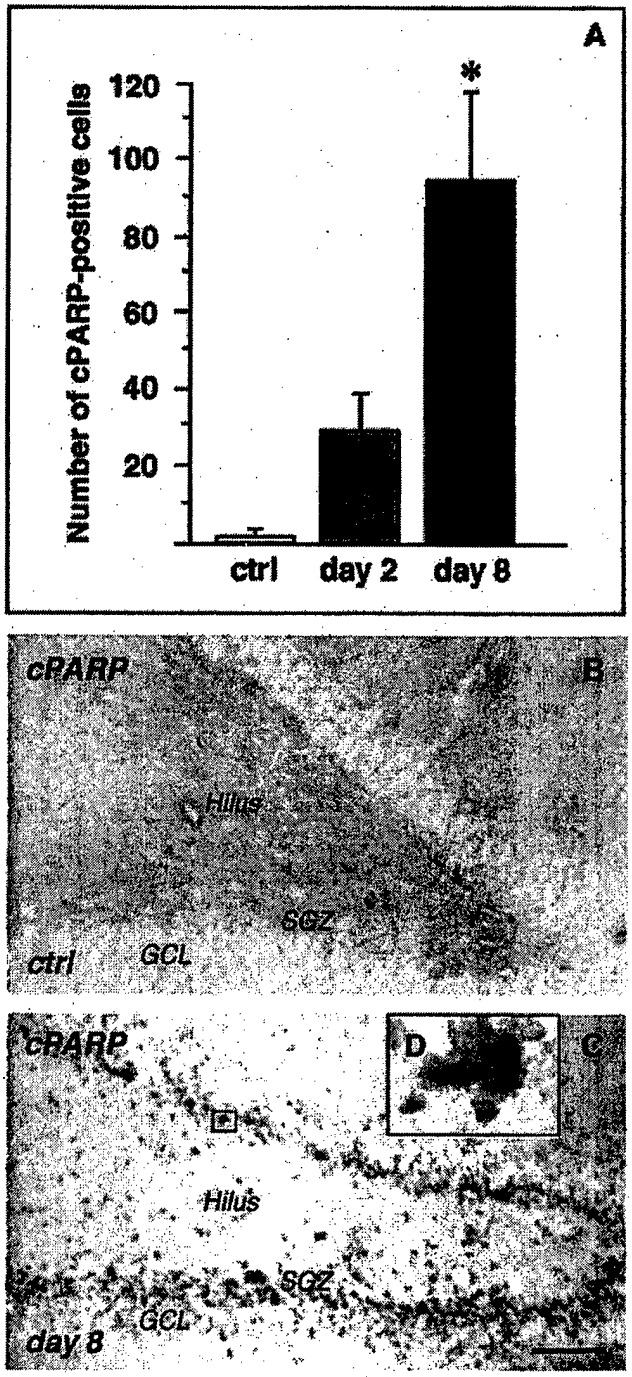


FIG. 2. Cleaved PARP (cPARP) is expresed in the subgranular zone following status epilepticus. (A) The number of cPARP-positive cells per section in the SGZ of one hemisphere at 2 and 8 days after the insult and in nonstimulated controls. (B) The absence of cPARP expression in the SGZ of a nonstimulated control rat. (C) cPARP expression in the SGZ at 8 days. The inset (D) is a higher magnification of a cluster of cPARP-positive cells in the SGZ marked by the square in C. Means ± SEM. $*P < 0.05$, one-way ANOVA followed by a posthoc Bonferroni test. GCL, granule cell layer; SGZ, subgranular zone; Hilus, dentate hilus. Scale bar, 100 μm (B and C), and 15 μm (D).

cPARP, or cathepsin B was assessed using a confocal scanning light microscope (Bio-Rad MRC1024UV, UK) with Kr/Ar 488 and 568 excitation filter. A double-stained cell was defined as having the strongest intensity of both stainings within the same or directly neighbouring 1-μm thick optical section through the cell in a consecutive Z-series of at least four sections, with an overlap of the stainings in at least three sections. Fifty BrdU-positive cells were analysed for either NeuN and GFAP, or cPARP double-labelling in the SGZ/GCL from each rat, and 250 BrdU-positive cells were examined for cathepsin B expression.

Statistical analyses

Seizure parameters and number of BrdU- and TUNEL/Hoechst-positive cells were analysed with nonparametric Mann–Whitney *U*-test. Other comparisons were performed using one-way analysis of variance (ANOVA) followed by posthoc Bonferroni test. Data are

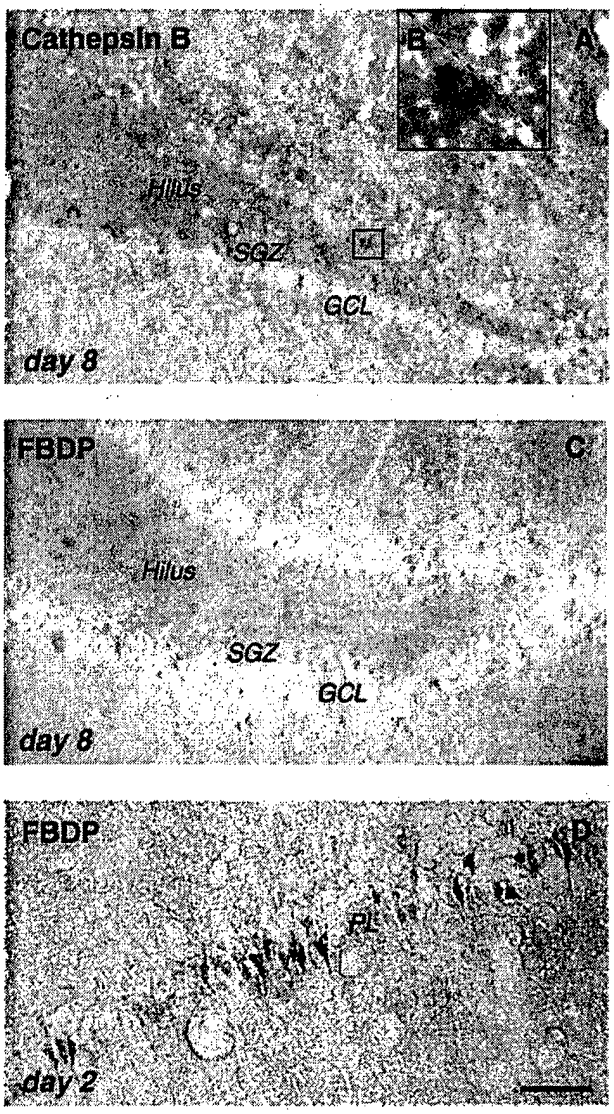


FIG. 3. Cathepsin B but not calpain-cleaved fodrin breakdown product 150 kDaA (FBDP) is expressed in the subgranular zone following status epilepticus. (A and B) Cathepsin B expression in large multipolar cells located in the SGZ at 8 days. The inset (B) is a higher magnification of the cell marked by the square. The dotted line depicts the border between SGZ and GCL. (C) The absence of FBDP expression in the SGZ at 8 days. (D) The expression of FBDP in the CA1 pyramidal layer at 2 days after the insult. GCL, granule cell layer; SGZ, subgranular zone; Hilus, dentate hilus; PL, pyramidal layer. Scale bar, 100 μm (A and C); 16.7 μm (B), and 28.6 μm (D).

presented as means ± SEM, and differences are considered significant at $P < 0.05$. All statistical analyses were conducted with STATVIEW software, version 5.0 (Abacus Concepts, Berkeley, CA).

Results

Cleaved PARP and cathepsin B are expressed in the subgranular zone following status epilepticus

All stimulated rats used in the first experiment developed mild eSE profiles, and were perfused either 2 ($n = 6$) or 8 days ($n = 6$) following the insult. In addition, four rats were nonstimulated controls. The mild eSE profiles comprised either hyperactive and excessive running behaviour with facial twitches, or continuous ongoing partial seizures. None of the rats exhibited the more severe clonic eSE profile.

At just 2 days after the eSE, there seemed to be increased numbers of cPARP-positive cells in the SGZ/GCL (29.1 ± 10.1 cells per section in one hemisphere compared to 2.0 ± 0.8 in controls), but due to large variation between rats, this difference did not reach statistical significance unless the data were subjected to logarithmic transformation. However, at 8 days, the number of cPARP-positive cells in the SGZ/GCL had increased further to 94.4 ± 23.3 cells (Fig. 2A). The cPARP-immunoreactive cells were often located in clusters. They exhibited no clear morphological characteristics of either apoptosis or necrosis, but rather had small and irregularly shaped cell bodies (Fig. 2D). No cPARP-labelled cells could be detected in other parts of the DG, or in the CA1 or CA3 regions, at 2 or 8 days after eSE.

Cathepsin B-stained cells of varying intensities were scattered throughout the CA1 and CA3 regions at 2 and 8 days after eSE. Variation in labelling intensity patterns could reflect the ability of the antibody to detect both the proform and the mature active enzyme. However, the levels of the proform were low, and thus most of the detected antigen was probably active cathepsin B (E. Weber, unpublished observation). We only included strongly stained cells in the analysis. Cells were detected exclusively in the hilus and SGZ of the DG. Both at 2 and 8 days after eSE, the mean number of strongly stained cathepsin B-positive cells increased slightly in the dentate hilus (day 2, 2.6 ± 1.0; day 8, 3.5 ± 1.8 cells per section in one hemisphere) and SGZ (day 2, 4.3 ± 1.3; day 8, 9.6 ± 5.3), but the difference compared to nonstimulated controls (dentate hilus, 0.8 ± 0.1; SGZ, 0.3 ± 0.2) did not reach statistical significance, probably due to the large variations within each group. In the DG, all immunoreactive cells were strongly labelled with the morphological characteristics of large multipolar interneurons (Fig. 3B).

No FBDP staining was detected in the DG using immunohistochemistry (Fig. 3C). A substantial number of FBDP-labelled cells were observed in the pyramidal layer of the CA1 region (70.0 ± 23.2 cells per section in one hemisphere) (Fig. 3D) and some positive cells were also observed in the CA3 region (1.2 ± 0.6) at 2 days after eSE. At 8 days, FBDP staining was not observed in any hippocampal subregion. The morphology of the FBDP-labelled cells varied from big and swollen round cells with necrotic features to cells exhibiting pyknotic nuclei and apoptotic bodies typical of apoptosis. However, most of the cells showed intermediate forms with less distinct morphological features.

Only cleaved PARP is colocalized with BrdU in the subgranular zone following status epilepticus

In agreement with previous studies (Parent *et al.*, 1997; Gray & Sundstrom, 1998; Ekdahl *et al.* 2001), we observed a several-fold increase of the number of BrdU-labelled proliferated cells in the SGZ/GCL at 8 days following SE (data not shown). Confocal microscopy showed that 65.8 ± 7.2% of the BrdU-positive cells also expressed cPARP (Fig. 4). About one-third of the cPARP-positive cells were double-labelled with BrdU, and thus, there was also a substantial number of cells single stained for cPARP that surrounded the double-labelled cells in the SGZ (Fig. 4D–F). Less then 1% of the cPARP-expressing cells were double-labelled with TUNEL (data not shown). At 2 days, only 26.3 ± 6.2% of the BrdU-positive cells located in the SGZ were double-labelled with cPARP, which was significantly less than at 8 days. None of the BrdU-positive cells in

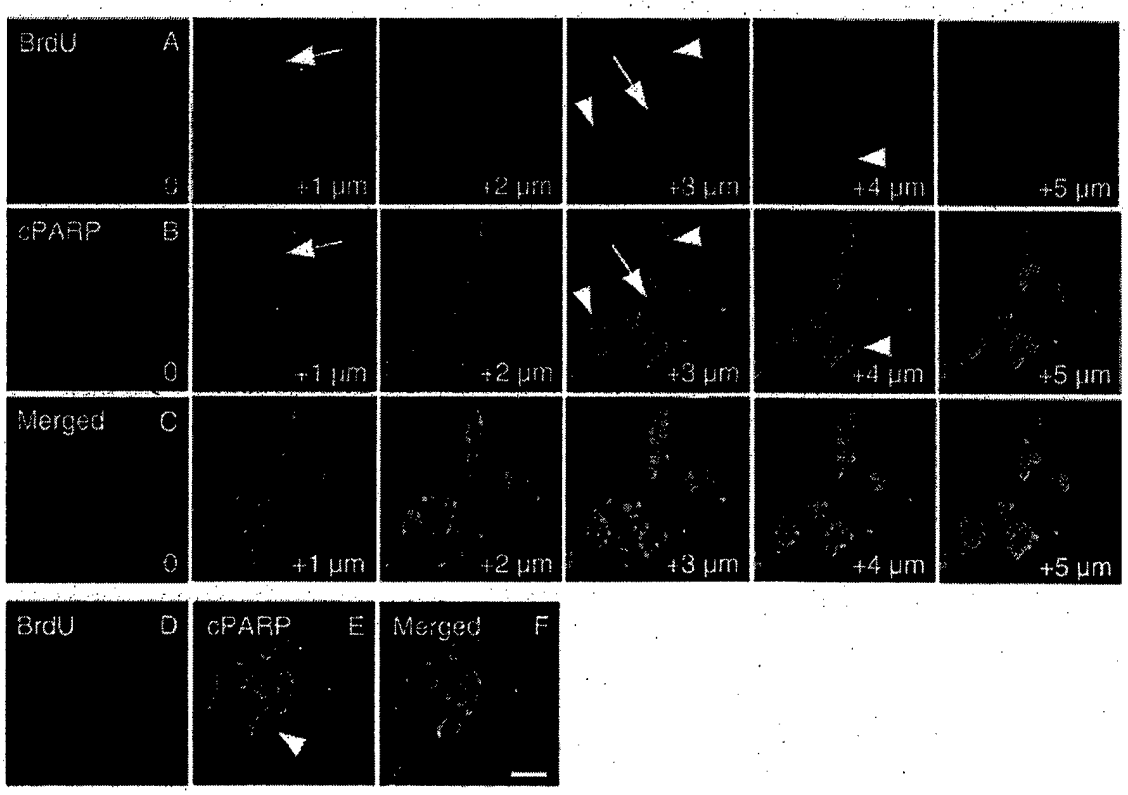


FIG. 4. BrdU-positive cells express cPARP in the subgranular zone at 8 days following status epilepticus. Confocal images illustrating labelling for BrdU (red) and cPARP (green): consecutive optical sections of a *z*-series showing BrdU (A) or cPARP (B) immunoreactivity seperately or as a merged image (C). Arrowheads in A and B mark three BrdU/cPARP double-labelled cells in a cluster of BrdU-positive cells located in the SGZ. Arrows in A and B point at two BrdU-positive cell not expressing cPARP. (D-F) Images from the same confocal plane showing several single stained cPARP-positive cells surrounding a BrdU/cPARP double-labelled cell (arrowhead in E) located in the SGZ. Scale bar, 5 µm.

nonstimulated controls expressed cPARP. Cathepsin B was never co-expressed with BrdU-positive DG cells at any time-point.

Caspase inhibitors increase survival of new neurons in the dentate gyrus following status epilepticus

In a separate experiment, we determined whether caspase inhibitor infusions into the lateral ventricle could increase the survival of new neurons generated following eSE. All 18 cannula-implanted rats developed eSE. Only three rats developed a more severe generalized eSE profile. Because the partial and generalized eSE profiles lead to different survival ratio over time for the newborn cells (P. Mohapel, C. T. Ekdahl and O. Lindvall, unpublished observations), these groups could not be collapsed and the three rats with generalized eSE profiles were therefore omitted from the analyses. The remaining rats with mild partial eSE profiles were randomly divided into two groups, which were given either caspase inhibitors (n = 7) or vehicle (n = 8). There were no significant differences between treatment groups, with respect to time, exhibiting clonic (6.3 ± 1.0 *vs* 7.3 ± 3.2 min) or partial convulsive behaviour (42.0 ± 2.2 *vs* 45.0 ± 2.5 min), the mean duration of after-discharges (40.8 ± 4.3 *vs* 49.0 ± 3.8 s), or the mean onset to continuous behavioural convulsions (36 ± 5 *vs* 26 ± 11 min) over 1-h stimulation period. In addition, no differences in the mean frequency of discharges were detected during the 2 weeks of infusions between vehicle- and caspase inhibitor-treated animals (0.15 ± 0.03 *vs* 0.21 ± 0.03 Hz). Spontaneous seizures were occasionally observed at all times during the day in both groups.

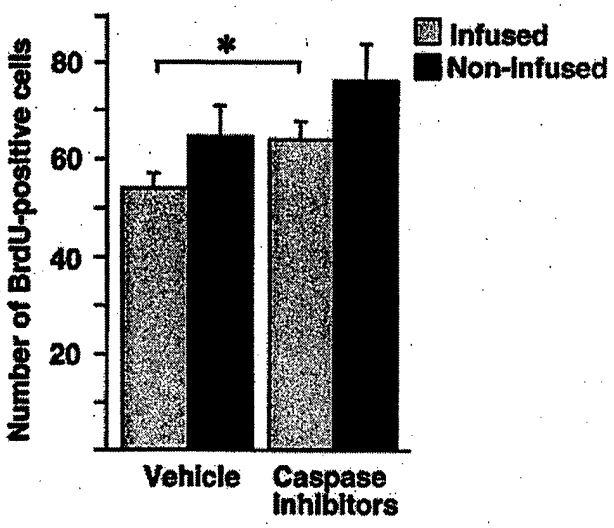


FIG. 5. Caspase inhibitors increase the number of BrdU-positive cells in the subgranular zone and granule cell layer at 3 weeks following status epilepticus. Means ± SEM per section in the SGZ and GCL on the infused- and noninfused side of the brain. *P < 0.05, Mann–Whitney U-test.

An increased number of BrdU-positive cells was observed at 3 weeks after eSE in caspase inhibitor- as compared to vehicle-treated rats. This difference was statistically significant in the SGZ/

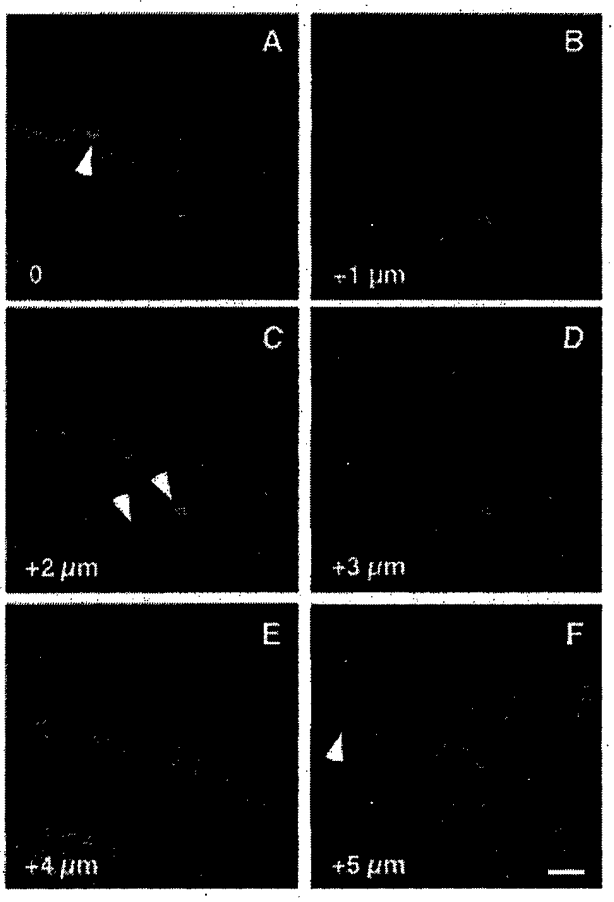


FIG. 6. The vast majority of BrdU-positive cells surviving at 3 weeks following status epilepticus express the neuronal marker NeuN. Confocal images illustrating labelling for NeuN (green) and BrdU (red), (A–F), consecutive optical sections of a *z*-series showing merged images with double labelled cells (arrowheads; yellow) located in the SGZ and GCL. Scale bar, 20 μm.

GCL in the infused (63.7 ± 4.0 *vs* 53.6 ± 3.3 cells per section) but not in the non-infused hemisphere (76.5 ± 7.4 *vs* 64.7 ± 6.2) (Fig. 5). The increase in number of cells was relatively higher in the ventral blade of the dentate gyrus (30.8% increase), as compared to the dorsal blade (11.1% increase) on the infused side of the brain. There were no differences between the two groups in the number of BrdU-labelled cells in the dentate hilus (infused side, 6.0 ± 1.2 *vs* 5.9 ± 1.0). In both treatment groups, the majority of the BrdU-positive cells were located in the SGZ or within two cell diameters into the GCL. Confocal microscopy confirmed that virtually all BrdU-positive cells were also NeuN-positive (Fig. 6), with no differences between caspase inhibitor- and vehicle-treated groups (89.1% ± 1.5 *vs* 88.4% ± 2.3). Less than 1% of the BrdU-positive cells were double-labelled with the GFAP antibody. No BrdU/cPARP staining was performed on the infused rats, as the cell death at this late timepoint was not the primary target with the caspase inhibitor administration.

TUNEL/Hoechst staining showed low numbers of positive cells in the GCL/SGZ at 3 weeks after eSE, similar to that observed in rats perfused 5 weeks after pilocarpine-induced SE (Ekdahl *et al.* 2001). There were no differences between caspase inhibitor- and vehicle-treated groups (0.4 ± 0.1 *vs* 0.4 ± 0.3 cells per section). Furthermore, in the dentate hilus, CA1 and CA3, there were low numbers with no differences between the groups (data not shown).

Discussion

Status epilepticus and other brain insults lead to dramatic increases in cell proliferation in the adult DG, but many of the new cells only survive for a limited time (Ekdahl *et al.* 2001). This study provides the first direct evidence for caspase-mediated death of newly proliferated cells in the adult DG following status epilepticus. At the time of maximum seizure-induced cell proliferation, 66% of the newly generated progenitor cell progeny in the SGZ/GCL coexpressed active caspase-cleaved PARP. Moreover, administration of caspase inhibitors during a 2-week period following SE increased the number of surviving newly formed neurons. Taken together, these data support an important regulating role for caspase activation in survival of new neurons generated by SE, whereas we obtained no evidence of an involvement of two other cysteine protease families, the cathepsins or the calpains.

The caspases play a key role in many apoptotic processes and have the capacity to cleave a wide range of endogenous substrates (Cohen, 1997; Chan & Mattson, 1999; Wang, 2000). The PARP protein is one of several substrates for active caspases and has been implicated in DNA repair, proliferation, stress responses and necrosis. Upon cleavage by caspases, the PARP protein becomes inactivated as apoptotic cell death is initiated (Herceg & Wang, 2001). Based on these data, our findings of cPARP in newly proliferated cells in the DG would suggest apoptosis. Few cPARP/TUNEL double-labelled cells could indicate that PARP degradation preceded DNA cleavage (Soldani *et al.* 2001). Also, our observation that administration of caspase inhibitors increased the survival of proliferated neurons strongly argues for the involvement of caspases in the death of these newly generated cells. Such a role for the caspases is clearly supported by recent *in vitro* studies on apoptotic death of newly proliferated cells. Caspase 3 activation was found in telencephalic neuronal progenitor cells after both arabinoside- and staurosporine-induced apoptosis, and the cell death was prevented by administration of a caspase inhibitor (D'Sa-Eipper & Roth, 2000). Furthermore, both activation of p38 MAP kinase and PARP and caspase cleavage have been detected in the clonal neural progenitor cell line C17.2 after nitric-oxide-induced apoptosis (Cheng *et al.* 2001). So far, there is no clear experimental evidence, either *in vitro* or *in vivo*, indicating that the expression of caspase-cleaved PARP would not be followed by the death of the cell. However, the high frequency of BrdU-labelled cells containing caspase-cleaved PARP as compared to the low number of cPARP/TUNEL double-labelled cells at 8 days after eSE raises the question whether cPARP expression in newborn cells is necessarily a sign of ensuing cell death. In a recent study in rats, expression of active caspase 3 was detected in some BrdU-positive cells, as well as in TUJ1-positive cells (marker for immature postmitotic neurons) in the subventricular zone, rostral migratory stream and olfactory bulb during the first two postnatal weeks. As these cells were not double-labelled with TUNEL, it was suggested that caspase 3 activation could play a role in other cellular processes, besides cell death (Yan *et al.* 2001). In addition, although the relative modest degree of neuroprotection exerted by our inhibitor cocktail following 2 weeks of infusion, as compared to the high percentage of BrdU-positive cells expressing cPARP, could be explained by incomplete inhibition of caspase activity, this observation may also indicate that the role of the caspase activation extends beyond that of the cell death machinery (Chan & Mattson, 1999).

Therefore, cleavage of PARP in a cell may not always lead to its death.

Neither calpain-induced FBDP nor cathepsin B was expressed in the progenitor cell progeny after eSE, which indicates that their improved survival after the infusions was most likely mediated through inhibition of caspases. However, considering the non-specific nature of the caspase inhibitors, we cannot exclude the possibility that other cysteine protease families, in addition to the caspases, calpains, and the cathepsin B, are involved. The lack of calpain and cathepsin B activation was unexpected considering the close relationship between all three pathways (Yamashima, 2000). Caspases are able to induce calpain activation (Wang *et al.*, 1998), and calpains can both activate (McGinnis *et al.*, 1999; Gao & Dou, 2000; Nakagawa & Yuan, 2000; Blomgren *et al.* 2001) and inhibit caspases (Chua *et al.* 2000). Caspases and calpains are also co-expressed in vulnerable axonal foci after traumatic axonal injury (Buki *et al.* 2000). Calpains have been suggested to activate the lysosomal cathepsins (Yamashima, 2000), and cathepsin B either directly cleaves caspases or activates them via Bid cleavage (Ishisaka *et al.*, 1998; Vancompernolle *et al.*, 1998; Stoka *et al.* 2001). In addition, transient calpain-cleaved FBDP expression in mature pyramidal and hilar neurons has been detected within the first hours and days following SE, preceding neuronal pathology (Bi *et al.*, 1996). These observations together with our data indicate that SE-induced cell death mechanisms may differ between mature pyramidal/hilar neurons and both newly formed and fully developed granule cells.

The cells generated by SE are born into a pathological environment due to both the initial insult and the ensuing recurring spontaneous seizures. It is conceivable that this pathological environment triggers a continual cycle of proliferation and apoptosis. The relationship between the proliferation and survival of newly generated cells following eSE is presently unclear. In the normal adult rat DG, about 50% of the newborn cells have been estimated to survive until they express a mature phenotype (Cameron *et al.*, 1993; Cameron & McKay, 2001). After chemically induced status epilepticus, the number of proliferated cells is increased (Parent *et al.*, 1997; Gray & Sundstrom, 1998; Ekdahl *et al.* 2001). However, the cell death machinery is also more active and thereby the overall survival rate is still relatively low (Ekdahl *et al.* 2001). Here, we observed that caspase inhibitor infusions resulted in an increase of newly born neurons in the DG at 3 weeks following status epilepticus. At this time-point, by the end of the 2-week infusion period, the apoptotic cell death had tapered off, as reflected by the low number of TUNEL/Hoechst-positive cells. Our findings indicate that the cell death machinery in newly proliferated cells can be suppressed, even in the pathological environment of the epileptic brain, leading to prolonged survival of the new neurons.

Whether the new neurons rescued by caspase inhibitors are functionally integrated and active is presently unknown. It is yet to be determined whether increased number of newly generated neurons following SE could contribute adversely by elevating seizure susceptibility, or act beneficially by having a reparative function in replacing neurons lost due to the epileptic insult. Also, the present observation that caspases can mediate death of new neurons generated by epileptic seizures could have broader implications to other brain insults, such as stroke where a major loss of newly generated striatal neurons seems to occur (Arvidsson *et al.* 2002). Thus, inhibition of caspase-mediated cell death pathways might be a useful tool to promote neuronal replacement from endogenous precursor cells and thereby increase functional recovery following brain insults.

Acknowledgements

Supported by grants from the Swedish Research Council, the Lundbeck, Söderberg, Kock, Åhlén, and Wilhelm and Martina Lundgren Foundations and the Royal Physiographic Society. We thank Z. Kokaia for helpful discussions. P.M. was supported by a fellowship from Natural Sciences and Engineering Research Council of Canada and by the Thorsten and Elsa Segerfalk Foundation.

Abbreviations

BrdU, bromodeoxyuridine; Casp, caspase inhibitor-treated; capsases, cysteinyl aspartate-specific proteinases cPARP; caspase-cleaved, poly (ADP-ribose) polymerase (PARP); DG, dentate gyrus; DMSO, dimethylsulfoxide; FBDP, calpain-specific fodrin breakdown product 150 kDa; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; NeuN, neuron-specific nuclear protein; SE, status epilepticus; SGZ, subgranular zone; TUNEL, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling; Veh, vehicle-treated.

References

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z. & Lindvall, O. (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nature Med.*, **8**, 963–970.

Arvidsson, A., Kokaia, Z. & Lindvall, O. (2001) *N*-Methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. *Eur. J. Neurosci.*, **14**, 10–18.

Au-Yeung, K.K., Zhu, D.Y.O., K. & Siow, Y.L. (2001) Inhibition of stress-activated protein kinase in the ischemic/reperfused heart: role of magnesium tanshinoate B in preventing apoptosis. *Biochem. Pharmacol.*, **62**, 483–493.

Bahr, B.A., Bendiske, J., Brown, Q.B., Munirathinam, S., Caba, E., Rudin, M., Urwyler, S., Sauter, A. & Rogers, G. (2002) Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp. Neurol.*, **174**, 37–47.

Bahr, B.A., Tiriveedhi, S., Park, G.Y. & Lynch, G. (1995) Induction of calpain-mediated spectrin fragments by pathogenic treatments in long-term hippocampal slices. *J. Pharmacol. Exp. Ther.*, **273**, 902–908.

Becker, A.J., Gillardon, F., Blumcke, I., Langendorfer, D., Beck, H. & Wiestler, O.D. (1999) Differential regulation of apoptosis-related genes in resistant and vulnerable subfields of the rat epileptic hippocampus. *Brain Res. Mol. Brain Res.*, **67**, 172–176.

Bednarski, E., Vanderklish, P., Gall, C., Saido, T.C., Bahr, B.A. & Lynch, G. (1995) Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices. *Brain Res.*, **694**, 147–157.

Bengzon, J., Kokaia, Z., Elmer, E., Nanobashvili, A., Kokaia, M. & Lindvall, O. (1997) Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. *Proc. Natl Acad. Sci. USA*, **94**, 10432–10437.

Bi, X., Chang, V., Siman, R., Tocco, G. & Baudry, M. (1996) Regional distribution and time-course of calpain activation following kainate-induced seizure activity in adult rat brain. *Brain Res.*, **726**, 98–108.

Blomgren, K., Zhu, C., Wang, X., Karlsson, J.O., Leverin, A.L., Bahr, B.A., Mallard, C. & Hagberg, H. (2001) Synergistic activation of caspase-3 by *m*-calpain after neonatal hypoxia- ischemia: a mechanism of 'pathological apoptosis'? *J. Biol. Chem.*, **276**, 10191–10198.

Buki, A., Okonkwo, D.O., Wang, K.K. & Povlishock, J.T. (2000) Cytochrome c release and caspase activation in traumatic axonal injury. *J. Neurosci.*, **20**, 2825–2834.

Cameron, H.A. & McKay, R.D. (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J. Comp. Neurol.*, **435**, 406–417.

Cameron, H.A., Woolley, C.S., McEwen, B.S. & Gould, E. (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience*, **56**, 337–344.

Chan, S.L. & Mattson, M.P. (1999) Caspase and calpain substrates: roles in synaptic plasticity and cell death. *J. Neurosci. Res.*, **58**, 167–190.

Cheng, A., Chan, S.L., Milhavet, O., Wang, S. & Mattson, M.P. (2001) p38 MAP Kinase Mediates Nitric Oxide-induced Apoptosis of Neural Progenitor Cells. *J. Biol. Chem.*, **276**, 43320–43327.

Chua, B.T., Guo, K. & Li, P. (2000) Direct cleavage by the calcium-activated

protease calpain can lead to inactivation of caspases. *J. Biol. Chem.*, **275**, 5131–5135.
Coggeshall, R.E. & Lekan, H.A. (1996) Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J. Comp. Neurol.*, **364**, 6–15. [Erratum appears in *J. Comp. Neurol.*, **369**, 162].
Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.*, **326**, 1–16.
Covolan, L., Ribeiro, L.T., Longo, B.M. & Mello, L.E. (2000a) Cell damage and neurogenesis in the dentate granule cell layer of adult rats after pilocarpine- or kainate-induced status epilepticus. *Hippocampus*, **10**, 169–180.
Covolan, L., Smith, R.L. & Mello, L.E.A.M. (2000b) Ultrastructural identification of dentate granule cell death from pilocarpine-induced seizures. *Epilepsy Res.*, **41**, 9–21.
D'Sa-Eipper, C. & Roth, K.A. (2000) Caspase regulation of neuronal progenitor cell apoptosis. *Dev. Neurosci.*, **22**, 116–124.
Dolbeare, F. (1995) Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: historical perspectives, histochemical methods and cell kinetics. *Histochem. J.*, **27**, 339–369.
Duman, R.S., Malberg, J. & Nakagawa, S. (2001) Regulation of adult neurogenesis by psychotropic drugs and stress. *J. Pharmacol. Exp. Ther..*, **299**, 401–407.
Ekdahl, C.T., Mohapel, P., ElmÈr, E. & Lindvall, O. (2001) Caspase inhibitors increase short-term survival of progenitor cell progeny in the adult rat dentate gyrus following status epilepticus. *Eur. J. Neurosci.*, **14**, 937–945.
Erhardt, J.A., Ohlstein, E.H., Toomey, J.R., Gabriel, M.A., Willette, R.N., Yue, T.L., Barone, F.C. & Parsons, A.A. (2001) Activation of caspase-3/caspase-3-like activity in rat cardiomyocytes by an RGD peptide, but not the GPIIb/IIIa antagonist lotrafiban. *Thromb. Res.*, **103**, 143–148.
Faherty, C.J., Xanthoudakis, S. & Smeyne, R.J. (1999) Caspase-3-dependent neuronal death in the hippocampus following kainic acid treatment. *Brain Res. Mol. Brain Res.*, **70**, 159–163.
Fiebiger, E., Meraner, P., Weber, E., Fang, I.F., Stingl, G., Ploegh, H. & Maurer, D. (2001) Cytokines regulate proteolysis in major histocompatibility complex class II-dependent antigen presentation by dendritic cells. *J. Exp. Med.*, **193**, 881–892.
Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M. & Jaattela, M. (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J. Cell. Biol.*, **153**, 999–1010.
Gage, F.H., Kempermann, G., Palmer, T.D., Peterson, D.A. & Ray, J. (1998) Multipotent progenitor cells in the adult dentate gyrus. *J. Neurobiol.*, **36**, 249–266.
Gao, G. & Dou, Q.P. (2000) N-Terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J. Cell. Biochem.*, **80**, 53–72.
Gillardon, F., Bottiger, B., Schmitz, B., Zimmermann, M. & Hossmann, K.A. (1997) Activation of CPP-32 protease in hippocampal neurons following ischemia and epilepsy. *Brain Res. Mol. Brain Res.*, **50**, 16–22.
Gray, J., Haran, M.M., Schneider, K., Vesce, S., Ray, A.M., Owen, D., White, I.R., Cutler, P. & Davis, J.B. (2001) Evidence that inhibition of cathepsin-B contributes to the neuroprotective properties of caspase inhibitor Ac-YVAD-cmk. *J. Biol. Chem.*, **26**, 26.
Gray, W.P. & Sundstrom, L.E. (1998) Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. *Brain Res.*, **790**, 52–59.
Han, H., Wang, H., Long, H., Nattel, S. & Wang, Z. (2001) Oxidative preconditioning and apoptosis in L-cells. Roles of protein kinase B and mitogen-activated protein kinases. *J. Biol. Chem.*, **276**, 26357–26364.
Henshall, D.C., Chen, J. & Simon, R.P. (2000) Involvement of caspase-3-like protease in the mechanism of cell death following focally evoked limbic seizures. *J. Neurochem.*, **74**, 1215–1223.
Herceg, Z. & Wang, Z.Q. (2001) Functions of poly (ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat. Res.*, **477**, 97–110.
Hetman, M., Filipkowski, R.K., Domagala, W. & Kaczmarek, L. (1995) Elevated cathepsin D expression in kainate-evoked rat brain neurodegeneration. *Exp. Neurol.*, **136**, 53–63.
Ishisaka, R., Utsumi, T., Yabuki, M., Kanno, T., Furuno, T., Inoue, M. & Utsumi, K. (1998) Activation of caspase-3-like protease by digitonin-treated lysosomes. *FEBS Lett.*, **435**, 233–236.
Johnson, D.E. (2000) Noncaspase proteases in apoptosis. *Leukemia*, **14**, 1695–1703.
Kidd, V.J., Lahti, J.M. & Teitz, T. (2000) Proteolytic regulation of apoptosis. *Semin. Cell Dev. Biol.*, **11**, 191–201.
Kirschke, H., Clausen, T., Gohring, B., Gunther, D., Heucke, E., Laube, F., Lowe, E., Neef, H., Papesch, H., Peinze, S., Plehn, G., Rebmann, U., Rinne, A., Rudrich, R. & Weber, E. (1997) Concentrations of lysosomal cysteine proteases are decreased in renal cell carcinoma compared with normal kidney. *J. Cancer Res. Clin. Oncol.*, **123**, 402–406.
Kondratyev, A. & Gale, K. (2000) Intracerebral injection of caspase-3 inhibitor prevents neuronal apoptosis after kainic acid-evoked status epilepticus. *Brain Res. Mol. Brain Res.*, **75**, 216–224.
Liu, J., Solway, K., Messing, R.O. & Sharp, F.R. (1998) Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J. Neurosci.*, **18**, 7768–7778.
Lothman, E.W., Bertram, E.H., Bekenstein, J.W. & Perlin, J.B. (1989) Self-sustaining limbic status epilepticus induced by 'continuous' hippocampal stimulation: electrographic and behavioral characteristics. *Epilepsy. Res.*, **3**, 107–119.
McGinnis, K.M., Gnegy, M.E., Park, Y.H., Mukerjee, N. & Wang, K.K. (1999) Procaspase-3 and poly (ADP) ribose polymerase (PARP) are calpain substrates. *Biochem. Biophys. Res. Commun.*, **263**, 94–99.
Nakagawa, E., Aimi, Y., Yasuhara, O., Tooyama, I., Shimada, M., McGeer, P.L. & Kimura, H. (2000) Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy. *Epilepsia*, **41**, 10–18.
Nakagawa, T. & Yuan, J. (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.*, **150**, 887–894.
Parent, J.M., Yu, T.W., Leibowitz, R.T., Geschwind, D.H., Sloviter, R.S. & Lowenstein, D.H. (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J. Neurosci.*, **17**, 3727–3738.
Paxinos, G. & Watson, C. (1997). *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Inc., San Diego.
Racine, R.J. (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.*, **32**, 281–294.
Scharff, C. (2000) Chasing fate and function of new neurons in adult brains. *Curr. Opin. Neurobiol.*, **10**, 774–783.
Sloviter, R.S., Dean, E., Sollas, A.L. & Goodman, J.H. (1996) Apoptosis and necrosis induced in different hippocampal neuron populations by repetitive perforant path stimulation in the rat. *J. Comp. Neurol.*, **366**, 516–533.
Soldani, C., Bottone, M.G., Pellicciari, C. & Scovassi, A.I. (2001) Two-color fluorescence detection of Poly (ADP-Ribose) Polymerase-1 (PARP-1) cleavage and DNA strand breaks in etoposide-induced apoptotic cells. *Eur. J. Histochem.*, **45**, 389–392.
Stoka, V., Turk, B., Schendel, S.L., Kim, T.H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M., Bromme, D., Krajewski, S., Reed, J.C., Yin, X.M., Turk, V. & Salvesen, G.S. (2001) Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J. Biol. Chem.*, **276**, 3149–3157.
Tominaga, K., Nakanishi, H., Yasuda, Y. & Yamamoto, K. (1998) Excitotoxin-induced neuronal death is associated with response of a unique intracellular aspartic proteinase, cathepsin. *Eur. J. Neurochem.*, **71**, 2574–2584.
Vancompernolle, K., Van Herreweghe, F., Pynaert, G., Van de Craen, M., De Vos, K., Totty, N., Sterling, A., Fiers, W., Vandenabeele, P. & Grooten, J. (1998) Atractyloside-induced release of cathepsin B, a protease with caspase- processing activity. *FEBS Lett.*, **438**, 150–158.
Wang, K.K. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci.*, **23**, 20–26.
Wang, K.K., Posmantur, R., Nadimpalli, R., Nath, R., Mohan, P., Nixon, R.A., Talanian, R.V., Keegan, M., Herzog, L. & Allen, H. (1998) Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Arch. Biochem. Biophys.*, **356**, 187–196.
Wolf, B.B., Goldstein, J.C., Stennicke, H.R., Beere, H., Amarante-Mendes, G.P., Salvesen, G.S. & Green, D.R. (1999) Calpain functions in a caspase-independent manner to promote apoptosis- like events during platelet activation. *Blood*, **94**, 1683–1692.
Yamashima, T. (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog. Neurobiol.*, **62**, 273–295.
Yan, X.X., Najbauer, J., Woo, C.C., Dashtipour, K., Ribak, C.E. & Leon, M. (2001) Expression of active caspase-3 in mitotic and postmitotic cells of the rat forebrain. *J. Comp. Neurol.*, **433**, 4–22.

Toxicology and Applied Pharmacology **185,** 111–118 (2002)
doi:10.1006/taap.2002.9534

8

Positive Modulation of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid-Type Glutamate Receptors Elicits Neuroprotection after Trimethyltin Exposure in Hippocampus

Subramani Munirathinam,*,† Gary Rogers,‡ and Ben A. Bahr*,†

Department of Pharmaceutical Sciences and the Neurosciences Program, †Center for Drug Discovery, University of Connecticut, Storrs, Connecticut 06269-2092; and ‡Cortex Pharmaceuticals, Irvine, California 92618

Received April 18, 2002; accepted September 23, 2002

Positive Modulation of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid-Type Glutamate Receptors Elicits Neuroprotection after Trimethyltin Exposure in Hippocampus. Munirathinam, S., Rogers, G., and Bahr, B. A. (2002). *Toxicol. Appl. Pharmacol.* 185, 111–118.

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamatergic receptors have been linked to survival signaling, especially when the receptors are allosterically modulated by members of the Ampakine family. While increased glutamatergic communication through AMPA receptors has been shown to protect against toxic conditions that target hippocampal subfield CA1, protection in other subfields has not been shown. Accordingly, positive modulation of AMPA receptors by Ampakine compounds CX727 and CX516 was tested for effects on trimethyltin (TMT) neurotoxicity in rat hippocampal slice cultures. TMT was applied for 4 h followed by a rapid washout and antagonistic quenching of AMPA and *N*-methyl-D-aspartate (NMDA) receptors. After a 24-h period, the TMT-exposed slices exhibited increased levels of calpain-mediated spectrin breakdown as well as synaptic deterioration. TMT selectively targeted CA3 pyramidal neurons and dentate gyrus (DG) granule cells as evidenced by degeneration and neuronal loss. The cytoskeletal and synaptic damage was reduced when Ampakine modulation was initiated during the postinsult period. Furthermore, the extent of protection was comparable to that produced by the NMDA receptor antagonist AP5. The above results were substantiated by histological experiments, revealing that Ampakine treatment prevented TMT-induced cell loss in CA3 and DG. These results indicate that AMPA receptor signals are part of cellular repair responses following exposure to an environmental toxin. © 2002 Elsevier Science (USA)

Key Words: **Ampakines; BDP; calpain; CX516; excitotoxicity; hippocampal slice culture; spectrin breakdown product.**

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors are known to participate in higher cognitive functions in the mammalian brain. Positive modulation of AMPA receptors by the Ampakine class of compounds improves channel function by making the receptors more responsive to endogenous ligand and specific agonists (Tang *et al.*, 1991; Arai *et al.*, 1996; Suppiramaniam *et al.*, 2001). Positive modulators in the Ampakine family of benzamide derivatives act selectively on AMPA receptors. The resultant increase in glutamatergic transmission has been shown to be associated with enhanced synaptic plasticity and memory retention (Staubli *et al.*, 1992, 1994; Granger *et al.*, 1996; Hampson *et al.*, 1998; Lebrun *et al.*, 2000).

Excessive glutamatergic activity through AMPA receptors is known to facilitate excitotoxic damage, and negative modulation of the receptor activity through antagonists is well known to be protective (Buchan *et al.*, 1991; Nellgard and Wieloch, 1992; Sheardown *et al.*, 1993). Interestingly, low-level stimulation of AMPA receptors by endogenous glutamate has been shown to enhance neuronal survival and promote synaptic maintenance (Bambrick *et al.*, 1995; McKinney *et al.*, 1999). This is likely related to the fact that, besides having ionotropic properties, AMPA receptors are linked to the neuroprotective mitogen-activated protein kinase (MAPK) pathway (Wang and Durkin, 1995; Hayashi *et al.*, 1999; Bahr *et al.*, 2002; Limatola *et al.*, 2002). This signaling pathway can be positively modulated (Bahr *et al.*, 2002), thereby implicating it in Ampakine induction of neurotrophic factor expression (Lauterborn *et al.*, 2000) and neuroprotection against stroke-type excitotoxicity (Bahr *et al.*, 2002). Thus, AMPA receptors are part of a cellular mechanism(s) that is necessary for both information processing and compensatory signals in response to injury.

In the present study, we tested whether enhanced glutamatergic signaling elicits protection across different hippocampal subfields. Thus, AMPA receptor modulation was tested for neuroprotection against trimethyltin (TMT) exposure. TMT is an organotin molecule that has been used to model selective brain damage (Dyer *et al.*, 1982; Chang, 1996; Ishida *et al.*, 1997; Noraberg *et al.*, 1998; Philbert *et al.*, 2000). The potent neurotoxin targets vulnerable neurons and is known to cause amnesia and selective damage in the hippocampus, possibly through excitotoxic mechanisms (Brodie *et al.*, 1990; Feldman *et al.*, 1993; Bahr *et al.*, 1995b; Dawson *et al.*, 1995). TMT exposure causes seizures in animal models and humans (Feld-

0041-008X/02 $35.00

man *et al.*, 1993; Ishida *et al.*, 1997) as well as severe deficits in learning and memory (Kreyberg *et al.*, 1992; Ishida *et al.*, 1997; Ishikawa *et al.*, 1997). At the molecular level, TMT exposure leads to distinct changes in growth factor and activity-dependent gene expression (Andersson *et al.*, 1997), evidence of cytoskeletal damage (Bahr *et al.*, 1995b, 2002), and selective loss of adhesion molecules (Dey *et al.*, 1994) and synaptic markers (Harry *et al.*, 1985; Brock and O'Callaghan, 1987; Bahr *et al.*, 2002). The hippocampal slice model was used since the hippocampus is a brain area that has been extensively utilized to study TMT toxicity. It is advantageous to use the slice model because of its features characteristic of the adult brain, including the circuitry, integrity, and organization of neuronal subfields (Bahr *et al.*, 1995a). Neurotoxicity measures of particular interest were the loss of synaptic proteins and the activation of the calcium-dependent protease calpain. The decline in expression of synaptic markers represents early neurodegeneration and is useful for identifying potential compensatory pathways (Harry *et al.*, 1985; Brock and O'Callaghan, 1987; Bahr *et al.*, 1994, 1998, 2002; Bendiske *et al.*, 2002; Bendiske and Bahr, 2002). Many types of toxic insults are known to promote calpain-mediated degradation of the cytoskeleton, a sensitive and very early marker of pathology (see Vanderklish and Bahr, 2000). Experiments were conducted to study the neuroprotective effect of two positive modulators of AMPA receptors, Ampakine CX516 [1-(quinoxalin-6-ylcarbonyl)piperidine] and the related compound CX727. CX727 is a metabolically stable analog of the Ampakine CX516.

METHODS

Hippocampal slice preparation. Sprague–Dawley rat litters (Charles River Laboratories, Wilmington, MA) were housed in accordance with guidelines from the National Institutes of Health. Animals 11–12 days postnatal were quickly treated with isoflurane anesthesia then decapitated to prepare organotypic hippocampal slices. The brains were rapidly removed and cooled in ice-cold buffer containing 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 4 mM $MgSO_4$, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM D-glucose, 2 mM ascorbic acid, and 75 μM adenosine (pH 7.2). Hippocampi were collected and 400-μm transverse slices were prepared starting from the septal to the temporal end. Groups of seven to eight slices were distributed onto Biopore insert membranes (Millicell-CM, Millipore Co., Bedford, MA), which remained in contact with the culture media. The media included 50% basal medium Eagle, 25% Earle's salt solution, 25% and horse serum, and was supplemented to the following final concentrations: 136 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$, 4 mM $NaHCO_3$, 3 mM glutamine, 40 mM glucose, 0.4 mM ascorbic acid, 20 mM Hepes buffer (pH 7.3 at 23°C), 1 mg/L insulin (24 IU/mg), 5 units/ml penicillin, and 5 mg/L streptomycin. The slices were maintained for 15–20 days at 37°C in a humid incubator supplied with 5% CO_2, with the media being changed every 2–3 days.

Treatment schedule. Freshly prepared medium was changed 1 day before the actual experiment. Slices were treated with 100 μM TMT (Aldrich Chemical Co., Milwaukee, WI) in serum-free media for 1–4 h. The slices were then washed twice (5 min each) with media containing 20 μM MK801 and 40 μM CNQX (TOCRIS, Ballwin, MO) in order to establish a controlled, acute toxic exposure. Glutamate receptor antagonists inhibit the progressive cellular changes induced by TMT as previously shown (Bahr *et al.*, 1995b). Groups of TMT-treated slices were allowed to recover in media containing serum for 24 h in the presence of Ampakine compounds (0–100 μM). Ampakine compounds were used at the respective concentrations that produced mild-to-maximal increases in electrophysiological response size measured in hippocampal slices (e.g., see Arai *et al.*, 1996). A separate group of slices were coexposed with TMT and the *N*-methyl-D-aspartate (NMDA) receptor antagonist AP5 (TOCRIS) for 4 h. All treatment groups were harvested after the 24-h recovery period.

Immunoblot analyses. Immunoblot technique was carried out as described (Bahr *et al.*, 1995b, 2002). Slice cultures prepared from 25 brains were harvested in ice-cold homogenization buffer containing 0.32 M sucrose, 5 mM Hepes buffer (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3, 10 μg/L antipain, and 2 μg/L each of leupeptin, aprotinin, and pepstatin. In groups of six to eight, the slices were gently harvested using a soft brush. Slice samples were then subjected to one freeze–thaw cycle and homogenized by sonication in 50 μl of hyposomotic lysis buffer containing 8 mM Hepes buffer (pH 7.4), 1 mM EDTA, 0.3 mM EGTA, and protease inhibitors listed above. Protein concentration was determined with BSA as the standard. Equal aliquots of the slice samples (85 μg protein) were prepared in 2.5% (wt/vol) SDS in the presence of 3% (vol/vol) β-mercaptoethanol and incubated at 100°C for 5 min. The denatured samples were then subjected to electrophoresis on polyacrylamide gradient gels (4–16% wt/vol) and transferred to nitrocellulose (0.2-μm pore size) for 12 h. Immunodetection of transferred proteins used the following antibodies diluted in 1.5% nonfat dry milk: anti-GluR1 prepared as described (Bahr *et al.*, 1996), anti-synaptophysin (Boehringer Mannheim, Indianapolis, IN), and anti-spectrin BDP_N (Bahr *et al.*, 1995b). The nitrocellulose membrane was incubated with primary antibodies at 4°C overnight with gentle agitation. Secondary antibody incubation utilized anti-IgG-alkaline phosphatase conjugates. For color development, the 5-bromo-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate system was used. Color development was stopped prior to maximal intensity to avoid saturation, and single blots were used for comparative studies between different antigens. Integrated densities of the labeled antigens were quantitatively compared within single immunoblots using software from Bioquant Image Analysis System (R&M Biometrics, Nashville, TN).

Tissue staining by Nissl. Cultured hippocampal slices prepared from 10 brains were fixed with 4% paraformaldehyde for 2 h, and then fixative was removed and replaced with 20% sucrose for 1 h. Sections of 20-μm thickness were cut and stained with cresyl violet.

Statistics. Data were expressed as mean ± SEM and statistical significance was determined by one-way analyses of variance followed by the Tukey–Kramer multiple range tests. Differences were considered significant at $p < 0.05$.

RESULTS

TMT Toxicity in Hippocampal Slice Cultures

The toxic action of TMT was assessed in long-term hippocampal slice cultures. Time-dependent changes were found in markers of cytoskeletal and synaptic components after exposure to 100 μM TMT. TMT was used at a sufficient level to elicit acute toxicity in a rapid manner throughout the multiple cell layers of the organotypic cultures (see Bahr *et al.*, 1995a). Cleavage of the cytoskeletal protein spectrin via the protease calpain increased over time of TMT exposure (Fig. 1A). The amino-terminal spectrin breakdown product (BDP_N) of 145 kDa was labeled in immunoblot samples with antibodies directed against the calpain recognition site. The TMT insult of 1 h caused calpain activation, and the BDP response was still evident 5 h postinsult (Fig. 1A, solid line). This BDP signal dissipated by 24 h postinsult (Fig. 1A, dotted line), concluding

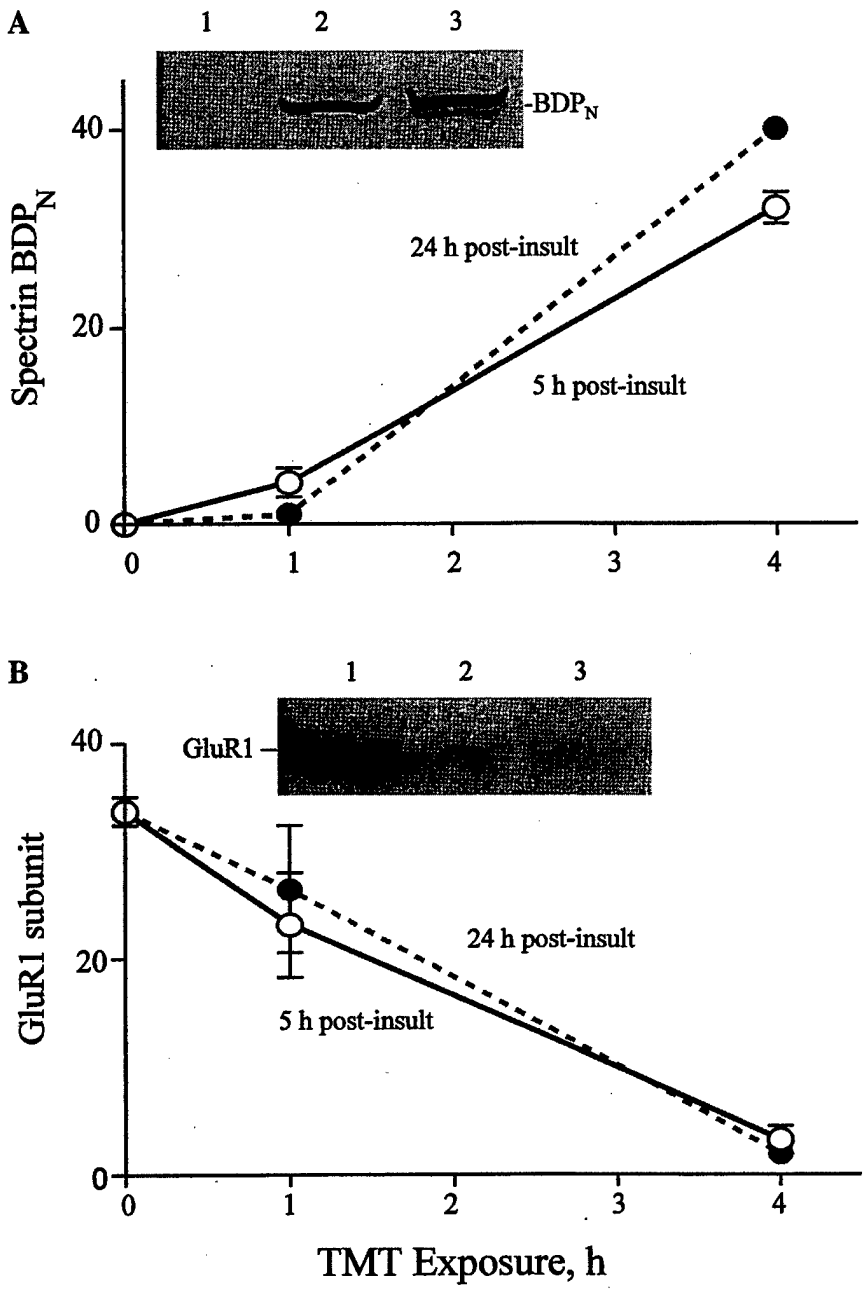


FIG. 1. Markers of cytoskeletal damage and synaptic decay illustrate the time course of TMT toxicity. Cultured hippocampal slices were treated with 100 μM TMT for 1 or 4 h and then rapidly washed and quenched with CNQX and MK-801. The slices were maintained in culture during the 5-h (solid line) or 24-h (dotted line) postinsult period. Slices were harvested and samples were assessed in parallel by immunoblot. Integrated optical density levels of immunoreactivity (y-axis) were determined by image analysis (mean $\pm$ SEM), and the results obtained for spectrin BDP$_N$ (A) and GluR1 (B) were plotted for 5-h (n = 6 groups of seven to eight slices each) vs 24-h (n = 7) postinsult data. Immunoblot lanes represent slices treated with TMT for 1 h and harvested 24 h later (lane 1) and with TMT for 4 h and harvested 5 (lane 2) or 24 h later (lane 3).

that the tissue can exhibit recovery from short toxin exposure. However, longer TMT treatment of 4 h caused sustained pathogenic activation of the protease that continued to increase over the 24-h recovery period. Similar evidence of sustained neurodegeneration after a 4-h TMT exposure was found by measuring the postsynaptic marker GluR1 (Fig. 1B) and the presynaptic marker synaptophysin (see Fig. 2B).

Positive Modulation of AMPA Receptors Promotes Cytoskeletal and Synaptic Repair

Next, we tested positive modulators of AMPA receptors for neuroprotection in hippocampal slice cultures previously exposed to TMT for 4 h. Allosteric modulators of the glutamate receptors, termed Ampakines, have been previously shown to

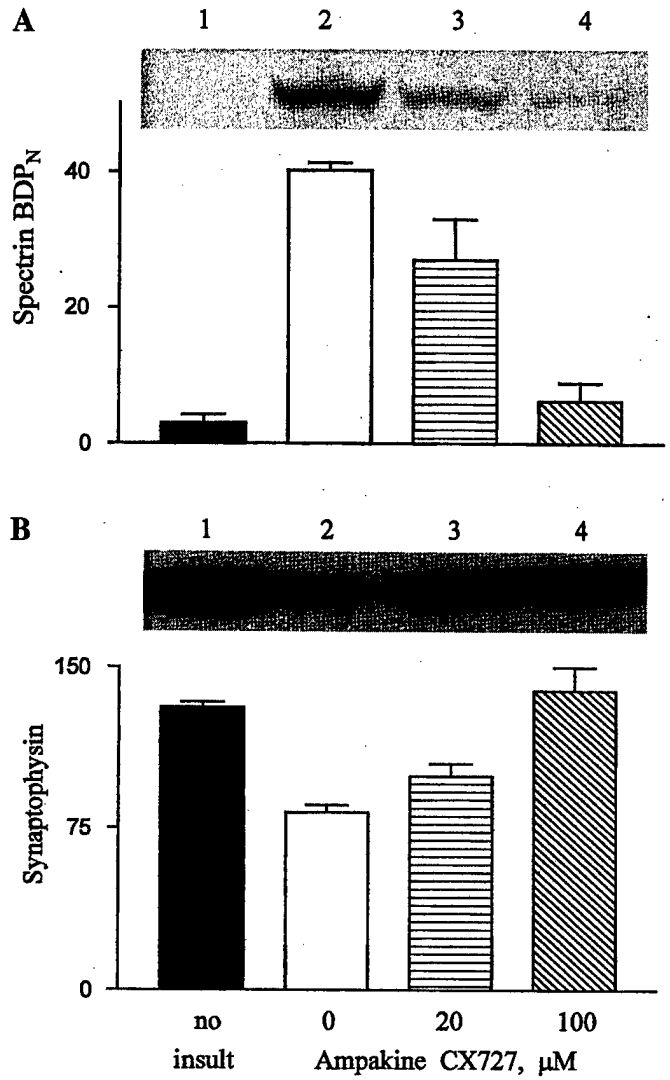


FIG. 2. Positive modulation of AMPA receptors promotes cytoskeletal and synaptic repair. Hippocampal slice cultures were untreated or exposed to 100 μM TMT for 4 h followed by rapid quenching of glutamate receptors. The 24-h postinsult recovery period was conducted in the presence of 0–100 μM CX727. Harvested slice samples were analyzed by immunoblot to determine integrated optical density levels of immunoreactivity for calpain-mediated spectrin BDP$_N$ (A) and the presynaptic marker synaptophysin (B). Immunoblot lanes 1–4 correspond to the treatment order of the graphs. Each bar represents the mean integrated density ± SEM. Analyses of variance: (A) $p < 0.0001$ and $F = 125$; (B) $p < 0.0001$ and $F = 49.3$.

promote cell survival through the link between AMPA receptors and MAPK signaling (Bahr *et al.*, 2002). The Ampakine CX727 was infused into the slices for the 24-h postinsult period, after which slice samples were prepared and analyzed for the calpain BDP response (Fig. 2A), synaptophysin (Fig. 2B), and GluR1 (Fig. 3A). The molecular markers indicated protection when comparing immunoblot samples from TMT-exposed slices ($n = 18$–27 groups of seven to eight slices each) with those that received CX727 after the insult ($n = 12$ groups). The Ampakine CX727 reduced the TMT-mediated spectrin breakdown in a dose-dependent manner, completely eliminating the calpain activation event at the higher dose ($p < 0.001$, post-hoc Tukey test). Correspondingly, the neuroprotectant action restored the pre- and postsynaptic proteins to levels comparable to those found in control slices ($n = 17$–24 untreated groups). Similar to the cytoskeletal protection, Fig. 2B shows that the higher dose of CX727 also caused complete restoration of the presynaptic marker ($p < 0.001$). At the lower CX727 dose of 20 μM, TMT-induced spectrin proteolysis was reduced by 37%, synaptophysin exhibited 39% recovery, and GluR1 exhibited 51% recovery ($p < 0.001$, $p < 0.05$, and $p < 0.001$, respectively, post-hoc tests).

The observed neuroprotection associated with postinsult

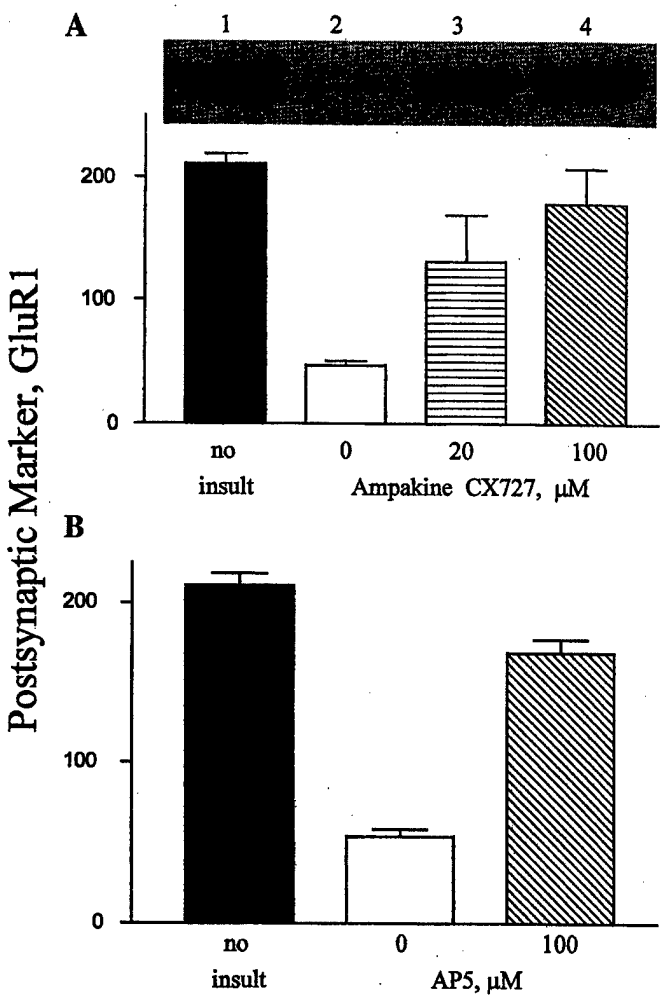


FIG. 3. Ampakine CX727 and AP5 elicit similar synaptic protection against TMT exposure. Slices were untreated or exposed to 100 μM TMT for 4 h with the 24-h postinsult period including 20 or 100 μM CX727 (A). Alternatively, both the TMT exposure time and the postinsult period were conducted in the absence or presence of AP5 (B). Slice samples were analyzed by immunoblot to determine the integrated optical density levels for the AMPA receptor subunit GluR1. Immunoblot lanes 1–4 correspond to the treatment order in A. GluR1 immunoreactivity is expressed as mean integrated density ± SEM. Analyses of variance: (A) $p < 0.0001$ and $F = 61.7$; (B) $p < 0.0001$ and $F = 183$.

TABLE 1

Ampakine Modulators and NMDA Receptor Antagonist Produce Similar Levels of Protection against TMT-Induced Cytoskeletal Damage and Synaptic Decline

Neuroprotectant	Reduction of spectrin breakdown (%)	Restoration of GluR1 (%)
Ampakine CX516		
20 μM	99	44
100 μM	100	70
Ampakine CX727		
20 μM	37	51
100 μM	93	79
AP5		
100 μM	95	74

Note. Levels of neuroprotection were determined from slice cultures previously exposed to TMT for 4 h. Neuroprotectants were infused into the slices for a 24-h postinsult period, after which the slices were harvested and assessed for changes in BDP and GluR1 by immunoblot.

Ampakine treatment was compared to that produced by the known blocker of excitotoxicity AP5. AP5 is an NMDA receptor antagonist that has previously been shown to block pathogenic calpain activation induced by TMT (Bahr *et al.*, 1995b). When 100 μM AP5 was present during both the 4-h insult and the recovery period, the TMT-induced spectrin breakdown measure of 36 $\pm$ 2.2 was significantly reduced to 4.4 $\pm$ 2.1 ($p < 0.0001$). This is the same level of BDP reduction as that produced by the postinsult application of the Ampakine CX727 (Table 1). AP5 also caused pronounced restoration of the synaptic marker GluR1 (Fig. 3B). Similar levels of synaptic protection were elicited by CX727 (see Fig. 3A; $p < 0.001$, post-hoc test) when applied during the recovery period.

Positive Modulation of AMPA Receptors Promotes Cell Survival

CX516 appears to be more effective than CX727 with regard to protecting against TMT-induced calpain activation and resultant spectrin breakdown. Evidence of spectrin proteolysis was completely abolished by Ampakine CX516 at 20 μM in TMT-treated slices, whereas 100 μM CX727 was required for such a level of neuroprotection (Table 1). Note that Arai *et al.* (1996) have demonstrated that 20 μM CX516 is comparable to the concentration needed to increase glutamatergic communication in hippocampal circuitries. Blocking cytoskeletal damage after excitotoxic protease activation is an important factor for cellular recovery since a correspondence exists between the extent of calpain-mediated spectrin breakdown and cell death (Bahr *et al.*, 2002). Due to its potent action against spectrin breakdown, CX516 was tested for its ability to prevent neuronal loss in vulnerable regions. In addition to cytoskeletal damage and synaptic decline, hippocampal slice cultures exposed to TMT for 4 h exhibited regionally selective cell death. Cresyl violet-stained sections revealed a marked decrease in CA3 pyramidal neurons and dentate gyrus granule cells (Fig. 4, middle) compared to sections from control slices (Fig. 4, top). Pyknotic alterations are evident in the remains of the CA3 subfield, and the dentate gyrus consists almost entirely of pyknotic nuclei in TMT-treated slices. The Ampakine modulator CX516 was applied postinsult, resulting in dramatic prevention of the pyknotic changes and cell death (Fig. 4, bottom).

DISCUSSION

The data presented show that, for a prolonged period following TMT treatment, the exposed hippocampal tissue exhibited neuronal damage, including cytoskeletal and synaptic deterioration. The latter correlates with previous *in vivo* studies in which the toxic action of TMT was shown to cause a marked loss of synaptic components (Harry *et al.*, 1985; Brock and O'Callaghan, 1987; Whittington *et al.*, 1989). TMT-treated hippocampal slice cultures provide a convenient model of selective neurodegeneration. The regional selectivity of the TMT-induced damage in the slice model was similar to that found in adult rats (Andersson *et al.*, 1997). Interestingly, TMT action, *in vitro* and *in vivo*, targets the same subfield found to be particularly vulnerable to kainate-induced seizure activity (Nadler *et al.*, 1980; Bi *et al.*, 1996).

As with seizure responses, the TMT toxicity in the slice model involves glutamate since an NMDA receptor antagonist elicited cytoskeletal and synaptic protection. Glutamate excitotoxicity has been suggested to play a role in the toxic action of TMT (Patel *et al.*, 1990; Bahr *et al.*, 1995b; Dawson *et al.*, 1995; Patterson *et al.*, 1996; Ishida *et al.*, 1997). However, TMT appears to express dual mechanisms of neurotoxicity with differential involvement of excitotoxic events. Low concentrations of TMT caused apoptotic death of cerebellar granule cells through oxidative signaling, and higher TMT levels initiated necrotic death mediated by glutamate receptors (Gunasekar *et al.*, 2001). Excitotoxicity was found to contribute little to the TMT-induced apoptosis in the granule cells. The concentration of TMT used in the present report suggests that the necrotic mode of cell death occurs in the hippocampal slice model. This is consistent with the pyknotic changes found in the dentate gyrus granule cell layer and in the CA3 pyramidal zone. NMDA receptors are a primary focus of studies on excitotoxicity (Lynch and Guttmann, 2002), and blocking these receptors in the slice model caused a marked reduction in neurodegeneration. In contrast, blockage of NMDA receptors did not reduce TMT-mediated apoptosis in cerebellar granule cells (Gunasekar *et al.*, 2001).

Results from the slice model indicate that cellular repair is elicited by the positive modulation of glutamatergic activity through AMPA receptors. Enhanced glutamatergic activity has previously been shown to increase synaptic resilience and neuronal survival *in vitro* and *in vivo* (Bambrick *et al.*, 1995; McKinney *et al.*, 1999; Bahr *et al.*, 2002; Limatola *et al.*,

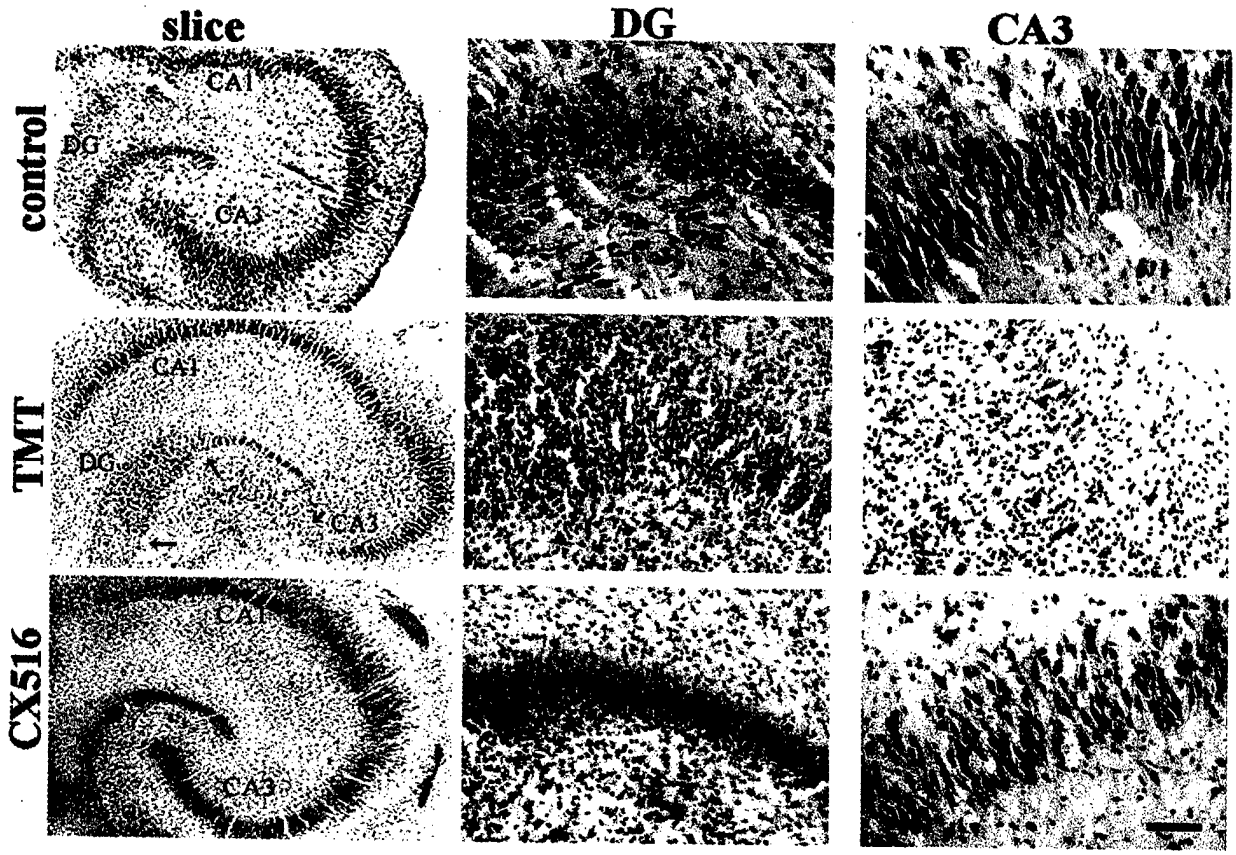


FIG. 4. Positive modulation of AMPA receptors promotes cell survival. Cultured hippocampal slices were untreated (top) or exposed to 100 μM TMT for 4 h followed by a 24-h postinsult period in the absence (middle) or presence (bottom) of 100 μM CX516. Slices were then fixed, sectioned, and stained by Nissl. Low-power photomicrographs show all neuronal subfields including the dentate gyrus (DG) and CA3 that are targeted by TMT (see arrows). High-power photomicrographs are shown to illustrate neurodegeneration induced by TMT. Bar represents 400 μm for leftmost panels, 90 μm for remaining panels.

2002). Our current results clearly show that, through allosteric modulation, AMPA receptor responses can be intensified in such a way as to strengthen survival signaling and to reduce hippocampal damage. The allosteric modulators used are known to selectively act on the AMPA class of glutamate receptors, leading to distinct changes in channel properties (see Staubli *et al.*, 1992; Suppiramaniam *et al.*, 2001). Previous studies identified a functional link between AMPA receptors and MAPK, and the work indicated that this pathway represents a compensatory response to CA1-targeting excitotoxins (Bahr *et al.*, 2002). The present work found that the survival system activated with Ampakine modulators also protects against CA3-targeting neurotoxin exposure. Thus, the glutamate-mediated survival response can be exploited to attenuate the pathogenic sequela set in motion by the action of neurotoxins. It stands to reason that, following toxin exposure, glutamatergic communication between affected neurons appears is vital for cellular maintenance signals.

Survival signaling through AMPA receptors may involve brain-derived neurotrophic factor (BDNF). AMPA receptor activation has been shown to increase BDNF mRNA (Hayashi *et al.*, 1999) in a manner that can be positively influenced by Ampakine modulation on the same time scale as shown here for neuroprotection (Lauterborn *et al.*, 2000). BDNF is a well-known component of injury-induced compensatory responses. In fact, the endogenous pathway that activates BDNF expression may explain why the CA1 region is not affected by TMT exposure (Andersson *et al.*, 1997; also see Xiao *et al.*, 1999). The CA3 region does not exhibit a compensatory change in BDNF production and, accordingly, is vulnerable to degeneration in the TMT-treated rat. It is conceivable that the AMPA receptor signaling pathway promotes those endogenous self-repair mechanisms that alone are not sufficient to protect vulnerable neurons against a toxic insult.

ACKNOWLEDGMENTS

The authors thank Dr. Jennifer Bendiske and Ms. Queenie Brown for their assistance. This work was supported by U.S. Army Medical Research Grant DAMD17–99-C9090 (B.A.B.) and NIH Grant 1R43NS38404–01 (B.A.B. and G.R.).

REFERENCES

Andersson, H., Wetmore, C., Lindqvist, E., Luthman, J., and Olson, L. (1997). Trimethyltin exposure in the rat induces delayed changes in brain-derived

neurotrophic factor, fos and heat shock protein 70. *Neurotoxicology* **18,** 147–159.

Arai, A., Kessler, M., Rogers, G., and Lynch, G. (1996). Effects of a memory enhancing drug on DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor currents and synaptic transmission in hippocampus. *J. Pharmacol. Exp. Ther.* **278,** 627–638.

Bahr, B. A., Abai, B., Gall, C., Vanderklish, P. W., Hoffman, K. B., and Lynch, G. (1994). Induction of β-amyloid-containing polypeptides in hippocampus: Evidence for a concomitant loss of synaptic proteins and interactions with an excitotoxin. *Exp. Neurol.* **129,** 81–94.

Bahr, B. A., Kessler, M., Rivera, S., Vanderklish, P. W., Hall, R. A., Mutneja, M. S., Gall, C., and Hoffman, K. B. (1995a). Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. *Hippocampus* **5,** 425–439.

Bahr, B. A., Tiriveedhi, S., Park, G. Y., and Lynch, G. (1995b). Induction of calpain-mediated spectrin fragments by pathogenic treatment in long-term hippocampal slices. *J. Pharmacol. Exp. Ther.* **273,** 902–908.

Bahr, B. A., Hoffman, K. B., Kessler, M., Hennegriff, M., Park, G. Y., Yamamoto, R. S., Kawasaki, B. T., Vanderklish, P. W., Hall, R. A., and Lynch, G. (1996). Distinct distributions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits and a related 53,000 M_R antigen (*GR53*) in brain tissue. *Neuroscience* **74,** 707–721.

Bahr, B. A., Hoffman, K. B., Yang, A. J., Hess, U. S., Glabe, C. G., and Lynch, G. (1998). Amyloid β protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. *J. Comp. Neurol.* **397,** 139–147.

Bahr, B. A., Bendiske, J., Brown, Q. B., Munirathinam, S., Caba, E., Rudin, M., Urwyler, S., Sauter, A., and Rogers, G. (2002). Survival signaling and selective neuroprotection through glutamatergic transmission. *Exp. Neurol.* 174, 37–47.

Bambrick, L. L., Yarowsky, P. J., and Krueger, B. K. (1995). Glutamate as a hippocampal neuron survival factor: An inherited defect in the trisomy 16 mouse. *Proc. Natl. Acad. Sci. USA* **92,** 9692–9696.

Bendiske, J., and Bahr, B. A. (2002). Lysosomal activation is a compensatory response against protein accumulation and associated synaptopathogenesis—An approach for slowing Alzheimer's disease? *J. Neuropathol. Exp. Neurol.* (in press).

Bendiske, J., Caba, E., Brown, Q. B., and Bahr, B. A. (2002). Intracellular deposition, microtubule destabilization, and transport failure: An 'early' pathogenic cascade leading to synaptic decline. *J. Neuropathol. Exp. Neurol.* **61,** 640–650.

Bi, X., Chang, V., Siman, R., Tocco, G., and Baudry, M. (1996). Regional distribution and time-course of calpain activation following kainate-induced seizure activity in adult rat brain. *Brain Res.* **726,** 98–108.

Brock, T. O., and O'Callaghan, J. P. (1987). Quantitative changes in the synaptic vesicle proteins synapsin I and p38 and the astrocyte-specific protein glial fibrillary acidic protein are associated with chemical-induced injury to the rat central nervous system. *J. Neurosci.* **7,** 931–942.

Brodie, M. E., Opacka-Juffry, J., Peterson, D. W., and Brown, A. W. (1990). Neurochemical changes in hippocampal and caudate dialysates associated with early trimethyltin neurotoxicity in rats. *Neurotoxicology* **11,** 35–46.

Buchan, A. M., Li, H., Cho, S., and Pulsinelli, W.A. (1991). Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats. *Neurosci. Lett.* **132,** 255–258.

Chang, L. W. (1996). Toxico-neurology and neuropathology induced by metals. In *Toxicology of Metals* (L. W. Chang, Ed.), pp. 511–535, CRC Press, New York.

Dawson, R., Jr., Patterson, T. A., and Eppler, B. (1995). Endogenous excitatory amino acid release from brain slices and astrocyte cultures by trimethyltin and other neurotoxic agents. *Neurochem. Res.* **20,** 847–858.

Dey, P. M., Graff, R. D., Lagunowich, L. A., and Rehul, K. R. (1994). Selective loss of the 180-kDa form of neural cell adhesion molecule in hippocampus and cerebellum of the adult mouse following trimethyltin administration. *Toxicol. Appl. Pharmacol.* **126,** 69–74.

Dyer, R. S., Deshields, T. L., and Wonderlin, W. F. (1982). Trimethyltin-induced changes in gross morphology of the hippocampus. *Neurobehav. Toxicol. Teratol.* **4,** 141–147.

Feldman, R. G., White, R. R., and Eriator, I. I. (1993). Trimethyltin encephalopathy. *Arch. Neurol.* **50,** 1320–1324.

Granger, R., Deadwlyer, S., Davis, M., Moskovitz, B., Kessler, M., Rogers, G., and Lynch, G. (1996). Facilitation of glutamate receptors reverses an age-associated memory impairment in rats. *Synapse* **22,** 332–337.

Gunasekar, P., Li, L., Prabhakaran, K., Eybl, V., Borowitz, J. L., and Isom, G. E. (2001). Mechanisms of the apoptotic and necrotic actions of trimethyltin in cerebellar granule cells. *Toxicol. Sci.* **64,** 83–89.

Hampson, R. E., Rogers, G., Lynch, G., and Deadwyler, S. A. (1998). Facilitative effects of the ampakine CX516 on short-term memory in rats: Correlations with hippocampal neuronal activity. *J. Neurosci.* **18,** 2748–2763.

Harry, G. J., Goodrum, J. F., Krigman, M. R., and Morell, P. (1985). The use of synapsin I as a biochemical marker for neuronal damage by trimethyltin. *Brain Res.* **326,** 9–18.

Hayashi, T., Umemori, H., Mishina, M., and Yamamoto, T. (1999). The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. *Nature* **397,** 72–76.

Ishida, N., Akaike, M., Tsutsumi, S., Kanai, H., Masui, A., Sadamatsu, M., Kuroda Y., Watanabe, Y., McEwen, B. S., and Kato, N. (1997). Trimethyltin syndrome as a hippocampal degeneration model: Temporal changes and neurochemical features of seizure susceptibility and learning impairment. *Neuroscience* **81,** 1183–1191.

Ishikawa, K., Kubo, T., Shibanoki, S., Matsumoto, A., Hata, H., and Asai, S. (1997). Hippocampal degeneration inducing impairment of learning in rats: Model of dementia? *Behav. Brain Res.* **83,** 39–44.

Kreyberg, S., Torvik, A., Bjorneboe, A., Wiik-Larsen, and Jacobsen, D. (1992). Trimethyltin poisoning: Report of a case with postmortem examination. *Clin. Neuropathol.* **11,** 256–259.

Lauterborn, J. C., Lynch, G., Vanderklish, P., Arai, A., and Gall, C. M. (2000). Positive modulation of AMPA receptors increases neurotrophin expression by hippocampal and cortical neurons. *J. Neurosci.* **20,** 8–21.

Lebrun, C., Pilliere, E., and Lestage, P. (2000). Effects of S 18986–1, a novel cognitive enhancer, on memory performances in an object recognition task in rats. *Eur. J. Pharmacol.* **401,** 205–212.

Limatola, C., Ciotti, M. T., Mercanti, D., Santoni, A., and Eusebi, F. (2002). Signaling pathways activated by chemokine receptor CXCR2 and AMPA-type glutamate receptors and involvement in granule cells survival. *J. Neuroimmunol.* **123,** 9–17.

Lynch, D. R., and Guttmann, R. P. (2002). Excitotoxicity: Perspectives based on *N*-methyl-D-aspartate receptor subtypes. *J. Pharmacol. Exp. Ther.* **300,** 717–723.

McKinney, R. A., Capogna, M., Durr, R., Gahwiler, B. H., and Thompson, S. M. (1999). Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat. Neurosci.* **2,** 44–49.

Nadler, J. V., Perry, B. W., Gentry, C., and Cotman, C. W. (1980). Degeneration of hippocampal CA3 pyramidal cells induced by intraventricular kainic acid. *J. Comp. Neurol.* **192,** 333–359.

Nellgard, B., and Wieloch. T. (1992). Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. *J. Cereb. Blood Flow Metab.* **12,** 2–11.

Noraberg, J., Gramsbergen, J. B. P., Fonnum, F., and Zimmer, J. (1998). Trimethyltin (TMT) neurotoxicity in organotypic rat hippocampal slice cultures. *Brain Res.* **783,** 305–315.

Patel, M., Ardelt, B. K., Yim, G. K., and Isom, G. E. (1990). Interaction of trimethyltin with hippocampal glutamate. *Neurotoxicology* **11,** 601–608.

Patterson, T. A., Eppler, B., and Dawson, R. (1996). Attenuation of trimethyltin evoked glutamate (GLU) efflux from rat cortical and hippocampal slices. *Neurotoxicol. Teratol.* **18,** 697–702.

Philbert, M. A., Billingsley, M. L., and Reuhl, K. R. (2000). Mechanisms of injury in the central nervous system. *Toxicol. Pathol.* **28,** 43–52.

Sheardown, M. J., Suzdak, P.D., and Nordholm, L. (1993). AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. *Eur. J. Pharmacol.* **236,** 347–353.

Staubli, U., Ambros-Ingerson, J., and Lynch, G. (1992). Receptor changes and LTP: An analysis using aniracetam, a drug that reversibly modifies glutamate (AMPA) receptors. *Hippocampus* **2,** 49–58.

Staubli, U., Rogers, G., and Lynch, G. (1994). Facilitation of glutamate receptors enhances memory. *Proc. Natl. Acad. Sci. USA* **91,** 777–781.

Suppiramaniam, V., Bahr, B. A., Sinnarajah, S., Owens, K., Rogers, G., Yilma, S., and Vodyanoy, V. (2001). Member of the Ampakines class of memory enhancers prolongs the single channel open time of reconstituted AMPA receptors. *Synapse* **40,** 154–158.

Tang, C-M., Shi, Q-Y., Katchman, A., and Lynch, G. (1991). Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. *Science* **254,** 288–290.

Vanderklish, P. W., and Bahr, B. A. (2000). The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. *Int. J. Exp. Pathol.* **81,** 323–339.

Wang, Y., and Durkin, J. P. (1995). α-Amino-3-hydroxy-5-methyl-4isoxazolepropionic acid, but not *N*-methyl-D-aspartate, activates mitogen-activated protein kinase through G-protein subunits in rat cortical neurons. *J. Biol. Chem.* **270,** 22783–22787.

Whittington, D. L., Woodruff, M. L., and Baisden, R. H. (1989). The time-course of trimethyltin-induced fiber and terminal degeneration in hippocampus. *Neurotoxicol. Teratol.* **11,** 21–33.

Xiao, Y., Harry, G. J., and Pennypacker, K. R. (1999). Expression of AP-1 transcription factors in rat hippocampus and cerebellum after trimethyltin neurotoxicity. *Neurotoxicology* **20,** 761–766.