IN VITRO STUDIES OF NUCLEAR CHANGES IN MAMMALIAN CNS NEURONS

SUBJECTED TO RAPID ACCELERATION IMPACT INJURY

THESIS

Presented to the Graduate Council of the

University of North Texas in Partial

Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Amy Wolf, B.A.

Denton, Texas

May, 1995

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An _in vitro_ model of Rapid Acceleration Impact (RAI) Injury was used to study the
effects of multiple impact (220 g/impact, 3-5 sec intervals) trauma on cultures of murine
CNS cells. Investigations with spinal cord cultures showed that 1) multiple impacts
delivered tangential to the plane of cell growth caused neuronal death (12% after 3 impacts
to 46% after 10 impacts); 2) multiple impacts delivered normal to the plane of cell growth
were much less effective (8% dead after 10 impacts); 3) most neuronal death occurred
within 15 minutes after injury 4) morphological changes observed included increased
nuclear prominence and somal swelling; and 5) pretreatment with ketamine (0.1mM)
reduced cell death from 51 to 14% and reduced somal swelling. Identical studies
performed on cortical cultures revealed minimal differences between the two tissues in their
response to multiple tangential impacts.

Of two hypotheses that can be proposed to explain the neuronal sensitivity to
impact injury (calcium leakage through N-methyl-D-aspartate [NMDA] receptor associated
channels and membrane perturbations severe enough to rupture the neuronal membrane
resulting in cell death due to non-specific Ca^{++} entry), the former is supported by the data.
Because of the protection by ketamine, it is suggested that an influx of Ca^{++} via NMDA-
related channels contributes to neuronal death in a manner similar to "excitotoxicity." It is
not clear whether NMDA channel activation results from stretch- induced paroxysmal
activity or from intracellular damage due to nuclear movement.

ACKNOWLEDGMENTS

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Introduction

Rapid acceleration impact (RAI) injury to the brain can result in varying levels of injury to the brain: subconcussive, where there are no functional disturbances; concussive, involving transient functional disturbances; and severe, resulting in immediate neuronal death, irreversible damage, and sustained functional disturbances (Frankowski et al. 1985). The incidence of RAI injury to the brain is approximately 200-400 cases/100,000 persons in the U.S. per year (Frankowski et al. 1985). A study by Kraus et al. (1984) determined that in San Diego County for the year 1981, the incidence of reported brain injury was 131/100,000 persons. The investigators concluded that 50% of all injury related death stems from brain or spinal cord trauma and the trauma rate for males was twice that for females with a peak rate found in males 15-24 years of age.

Gennarelli (1983) and Frankowski et al. (1985) found that rapid acceleration or deceleration (with or without impact) caused by vehicle accidents, falls, and assaults is the leading cause of injury to the brain. Because of inconsistencies in the classification and reporting of CNS injury, the numbers reported may be an underestimate of the actual incidence of head trauma.

The need for experimental models designed to study RAI injury to CNS tissues has been addressed in the form of *in vivo* applications (Genneralli and Thibault 1985). Unfortunately, these *in vivo* applications for experimental RAI are not always well suited for the study of cellular and subcellular events. The 3-dimensional quality of *in vivo* applications make it difficult to deliver a uniform physical trauma. Also, there is virtually no way to view cells before injury and then compare the same cells after injury.

Most *in vivo* studies of CNS injury have focused on axonal changes and not on perikaryal changes after injury. The studies looking at axonal changes supported the idea that the majority of neuronal injury occurred as a result of diffuse axonal injury in traumatic brain injury (Adams et al. 1982, 1983; Gennarelli et al. 1981; Gennarelli and Thibault 1985; Povlishock 1986). While an *in vitro* laser cell surgery model of CNS injury (developed in this laboratory) focuses on perikaryal and process changes after neurite transection it is still unable to address the perikaryal or dendritic changes after *in vitro* RAI injury to CNS cells. The model of RAI injury developed in this laboratory (Lucas and Wolf 1989, 1990, 1991) allows for the investigation of possible dendrosomatic components which may be responsible for the neuronal degeneration found as a result of traumatic brain injury (Hayes et al. 1992).

There is ample evidence from cellular studies to support the hypothesis that as a result of CNS trauma, there is an increase in intracellular calcium levels (Schlaepfer and Bunge 1973; Adams et al. 1982; Gennarelli et al. 1981; Choi 1985; Young 1985) which may activate cellular processes that lead to cell death. In *In vitro* studies of CNS injury, the effects of injury can be examined at the single cell level to determine specific morphological changes resulting from the injury. Recent studies of ultrastructural damage to cultured spinal cord neurons after dendrite transection also attributed dendrite transection injury to an increase in intracellular calcium levels (Gross et al. 1979; Emery et al. 1987; Lucas et al. 1990). The authors reported cytoskeletal changes including the disassemby of neurotubules and other perikaryal changes such as mitochondrial swelling and smooth endoplasmic reticulum dilation. In many instances, these changes led to cell death and may have been due to the increased free cytoplasmic calcium levels after neuronal injury (ibid.). The mitochondrial swelling that occurs as a result of increased calcium levels, can impair the metabolic activity of the mitochondrion, thus rendering it unable to undergo normal

respiration processes which may deplete the cell of its ATP levels and contribute to cell death (Young 1985).

Studies in this laboratory of the effects of rapid acceleration injury (RAI) on spinal cord neurons in culture have indicated that morphological changes may occur in both the perikaryon and the nucleus (Lucas and Wolf 1989, 1990, 1991). Increased nuclear prominence and lateral nuclear shifting suggested that the cytoskeleton was disturbed and that DNA could have undergone significant perturbations. As the number of tangential cumulative acceleration impacts increased, there was an increase in neuronal death and in the incidence of these morphological changes (ibid.). Comparison of the control (0 impacts) and experimental (10 impacts) groups revealed an increase in nuclear prominence (0% v. 37%), lateral nuclear shifting (1% v. 12%) and lateral nucleolar shifting (3% v. 13%). Preliminary electron micrographs (EM) of somal sections also showed clumping of the chromatin. These studies on spinal cord cultures also showed that cell death did not increase substantially 48 hours post-RAI. The present study examined whether delayed neuronal death (DND) occurs in cultured cortical neurons after RAI. Such delayed neuronal death has been reported to occur in the CA1 region of the hippocampus as a result of an ischemic insult (Kirino 1982).

The increase in intracellular calcium levels seen after injury can also activate calcium endonucleases (Cohen and Duke 1984; Jones et al. 1989; McConkey et al. 1990) which can cause DNA damage. Siddiqi and Bothe (1987) have observed that a gamma-irridation-induced increase in OH· radicals can cause DNA damage in eukaryotic cells. It is possible that these mechanisms may cause enough DNA damage to induce poly(ADP-ribosylation) in traumatized neuronal cells. ADP-ribosylation is the postsynthetic modification of protein by the covalent attachment of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD^+). NAD^+ serves as the sole substrate for

poly(ADP-ribose) polymerase. It occurs in the nuclei of all eukaryotic organisms (Purnell et al. 1980). Poly(ADP-ribose) polymerase serves as a catalyst in the formation of ADP-ribose units linked by 1'- 2" glycosidic alpha anomeric (ribose-ribose) bonds and 5'-5" pyrophosphate linkages. In the presence of DNA, the enzyme adds ADP-ribose units onto the original ADP-ribose residue forming an acid-insoluble product. Studies by Ahnstrom and Erixon (1973) and Ahnstrom (1988) indicated that DNA is very sensitive to shear forces and so it is possible that a rapid acceleration trauma, like that occurring in mild head injury (Tedeschi 1945; Nilsson and Ponten 1972; Adams et al. 1982; Gennarelli et al. 1981), may be able to damage the DNA backbone. This damage suffered may be enough to cause double stranded DNA breaks which is a prerequisite to induce poly(ADP-ribosylation).

The overall goal of this study was to evaluate RAI-induced neuronal death and changes both the perikarya and nuclear morphology of cortical cultures using light microscopic (LM) evaluations. The results from this study compared the RAI-induced changes in cortical cultures to data obtained previously from spinal cord cultures (Lucas and Wolf 1991).

A pilot study was also performed using MNNG (1-Methyl-3-nitro-1-nitrosoguanidine), which is known to induce poly(ADP-ribosylation) in C3H10T1/2 at concentrations above 20µg/ml (Jacobson et al. 1985a, 1985b), in an attempt to induce cell death in our spinal cord and cortical cultures. This could provide preliminary indirect evidence that poly(ADP-ribose) may be produced in our cell culture systems. However, physical injury and poly(ADP)ribose production have not yet been linked. It would be important for our understanding of cellular reactions to injury if it can be determined whether poly(ADP)ribosylation can be triggered by RAI.

Objective

A new *in vitro* injury model of RAI was used as the mode of injury to study the responses of neurons in cortical cultures to impact injury, and to compare earlier data on spinal cord cultures with that of cortical cultures. In addition, pilot experiments were conducted to determine whether poly(ADP)ribosylation occurs in neuronal cell culture.

Specific Aim 1 - To develop primary cortical cell culture procedures in this laboratory for use in rapid acceleration impact injury experiments.

Previously this laboratory has been using primarily spinal cord cultures for experiments. The desire to use cortical cultures for experiments necessitated the development of procedures that would allow cortical neuronal survival for at least 4 weeks.

Specific Aim 2 -To evaluate the effects of RAI at varying cumulative accelerations on the survival of neurons in cortical cultures and compare these results to those obtained in spinal cord cultures. The cortical cultures were also evaluated over a 72 hour period after trauma in order to investigate delayed neuronal death (DND).

Studies on spinal cord cultures showed that cell death increased with an increase in cumulative accelerations reaching approximately 50% and that cell death did not increase substantially up to 48 hours post-RAI (Lucas and Wolf, 1990; 1991). This could be an indication that there was no delayed neuronal death. However, such delayed death has been reported to occur in the CA1 region of the hippocampus after ischemic insult (Kirino, 1982). This study examined the response of cortical cultures to RAI at varying accumulations and whether DND occurs in cortical neurons after RAI.

Specific Aim 3 - To evaluate the following morphological changes in somata of cortical neurons as a result of multiple impact RAI: (a) somal swelling, (b) increased nuclear prominence, (c) lateral nuclear displacement, and (d) lateral nucleolar displacement.

In our preliminary studies on spinal cord cultures (Lucas and Wolf, 1991), these perikaryal changes have been observed to result from RAI. Additional data were added to previously published results (Lucas and Wolf, 1991) to increase the number of observations. Further, the same parameters were evaluated for cortical cultures to determine possible tissue specificity.

Specific Aim 4 - To determine whether neuronal death and morphological changes in cortical cultures after RAI can be prevented by pretreatment with ketamine.

Morphological changes observed in spinal cord cultures after RAI were reduced by pretreament with ketamine (ibid.). The same procedures were used on cortical cultures in an attempt to prevent or decrease neuronal death and morphological changes.

Specific Aim 5 - To determine whether MNNG, known to induce poly(ADP-ribosylation) in C3H10T1/2 at concentrations above 20μg/ml (Jacobson et al. 1985a: 1985b), can cause cell death in our spinal cord and cortical cell cultures.

Poly(ADP)ribose is produced as a result of hyperthermia, UV-irradiation, gamma-irradiation, carcinogens and other forms of stress. The MNNG toxicity data presented here provides preliminary, but indirect evidence, that poly(ADP-ribose) may be produced in our cell culture systems.

Materials and Methods

<u>Spinal Cord Culture</u>

Mice at day thirteen to fourteen of pregnancy were used as the source of neuronal tissue. After cervical dislocation, the mouse fur was washed with 70% ethanol followed by a rinse with ultra pure water. A medial longitudinal incision was made exposing the abdominal muscles, and the opening was rinsed with D_1SGH [0.8% NaCl, 0.04% KCL, 0.009% $Na_2H_2PO_4$-$7H_2O$, 0.003% KH_2PO_4, 0.2346% HEPES buffer, 0.3% glucose and 0.75% sucrose (or sufficient sucrose to bring the final osmolarity up to 320 mOsm) pH 7.3-7.4]. The muscle layer was cut exposing the abdominal cavity, the underlying viscera, and the uterus. Forceps were used to grasp the uterus at the uterine horn while surgical scissors were used to free the structure. The uterus was rinsed twice with D_1SGH before being placed in a 120mm culture dish (Corning) for further dissection. The embryos were removed and placed in a 60mm culture dish (Corning) of cold D_1SGH.

The culture procedure was based on the method of Ransom and coworkers (1977) with the addition of an enzymatic dissociation step [15-20 min in 20 units/ml papain (Boehringer Mannheim) and 0.05% DNase (Boehringer Mannheim)]. The cells (1×10^6/ml culture medium) were seeded in a $25cm^2$ tissue culture flask (Bellco) which was previously coated with poly-L-lysine [2.5 ml of $25\mu g$/ml, 70-150 kD (Sigma)] for one hour and then removed and allowed to air dry.

With the aid of a stereomicroscope and fine forceps, the spinal cords were removed from the embryos and placed in a 60mm dish of cold D_1SGH. The cords were then transferred into another sterile 60mm dish to be rinsed with cold D_1SGH before being

dissociated. Using a sterile 5 3/4" siliconized[1] pasteur pipette, the tissue was transferred to a fresh dish of D_1SGH, minced with #4 scapels, and rinsed with D_1SGH. The tissue and D_1SGH was collected with a sterile 5 3/4" siliconized pasteur pipette and transferred to a 15.0 ml centrifuge tube. The solution was centrifuged at approximately 800 rpm for 2 minutes as a final precaution against contamination. The resulting pellet was transferred to a sterile 60mm culture dish and a solution of 20units/ml papain (Boehringer Mannheim) with 0.05% DNase (Boehringer Mannheim) [3.0 ml/7-10 cords] was added. This enzymatic dissociation step continued for 15-20 minutes at $37^{\circ}C$ in an atmosphere of 10% CO_2.

The enzymatic actions were arrested by the addition of a high protein source in the form of MEM 10/10 [Minimum Essential Medium (MEM) + 10% by volume Horse Serum (HS) and 10% by volume Fetal Bovine Serum (FBS)]. The tissue was then transferred to a new 15.0 ml centrifuge tube and spun at 800 rpm for 2 minutes. The pellet was extracted and placed in a new 15.0 ml tube and the supernatant was discarded.

Approximately three milliliters of the MEM 10/10 were added to the tube to aid in the trituration process. The tissue was then gently disrupted by drawing it repeatedly (15-20 times) into a siliconized pasteur pipette with a wide opening (the rough edges were previously smoothed by flaming to prevent shearing of the cells) to break down large tissue clumps. This solution was allowed to settle (1-2 minutes) and then the upper suspension of single cells was removed and placed in a 15.0 ml centrifuge tube. The remaining cell pellet was triturated again with 3.0 ml MEM 10/10 as described above and allowed to settle. The single cell suspension was again added to the cell pool.

---

[1]DNA released from ruptured cells can clump and cause the tissue mass to become sticky. Siliconized pasteur pipettes were used to prevent tissue loss due to the adhesion of the DNA clumps to the glass. The pipettes were siliconized by soaking them for a total of 3 min in a solution of 10ml of the organosilicon concentrate Prosil-28 (PCR Incorporated) and 990 ml of ultrapure H_2O. The pipettes were rinsed in ultra pure H_2O for 30 min and heat sterilized.

Using a hemacytometer, the cell density of the suspension was determined. The suspension was diluted with sufficient MEM10/10 to obtain a final cell density of 1×10^6 cells/ml for seeding. Three milliliters of the final cell pool were added to each of the prepared culture flasks for a final cell density of 3×10^6 cells/flask.

The cultures were incubated at $37^{\circ}C$ in an atmosphere of 10% CO_2. The cultures were maintained in Minimum Essential Medium (MEM, GIBCO) containing 10% by volume horse serum (MEM10). The cultures underwent a complete medium change 24 hours after seeding. At day 5-6 (when the glial carpet was near confluency) a complete medium change was performed with the addition of an anti-mitotic agent, Fluorodeoxyuridine + uridine 13μg/ml (FduU) for 48 hours, to retard non-neuronal proliferation. A complete medium change to MEM10 and routine feeding was begun every 3-4 days involving a 1/3 medium change with MEM10. The spinal cord cultures were utilized between 21 and 28 days for these studies.

Cerebral Cortex Cultures

Cortical cultures used for the initial NAD^* determinations were prepared as follows. Cerebral cortices from 1-2 day murine neonates were removed and prepared for enzymatic dissociation. The neonates were rinsed with ethanol before being decapitated. The skull was gently opened revealing the cerebral cortex. The brain was removed from the skull and the meninges were removed. The brainstem was excluded from the dissection and the upper portions of the corticies were removed including the hippocampus. The pieces of cortex were then collected and placed in a dish of 0.25% trypsin at $37^{\circ}C$ in an atmosphere of 10% CO_2 for 13 minutes. The dissociated tissue was collected in a 15.0 ml Corning centrifuge tube and MEM 10/10 was used to stop the

reaction. The tissue was then centrifuged 3 min at 800 rpm. The resulting pellet was then extracted and placed in the following medium: 50% conditioned MEM (Cerebral Cortex), 50% fresh MEM and 10^{-5}M 5-Bromo 2-Deoxyuridine-Thymidine (BDuT). The pellet was then triturated 15-20 times with a 5 3/4" sterile siliconized pasteur pipette and the suspension was allowed to settle. The suspension of single cells was collected. The remaining pellet was triturated again as described above and the single cell suspensions were pooled. Four milliliters of the cell suspension containing 3×10^6 cells were seeded onto the surface of a 25cm^2 tissue culture flask which had been previously coated with poly-L-lysine (25µg/ml, 70-150 kD for one hour followed by air drying). On day 4 or 5 after seeding, there was a 1/2 medium change to fresh MEM10. The cultures were then fed every 3-4 days with a 50% medium change until considered mature at 2-3 weeks.

The cortical cultures used for the RAI studies were prepared according to the culture method described for spinal cord except for the use of embryonic tissue, the substitution of papain (Boehringer Mannheim) for trypsin, and the use of MEM supplemented with a higher glucose concentration (6g glucose/L) than that used for spinal cord cultures (5g glucose/L). Also, the seeding density was increased to 4×10^6/5-6.0 ml culture medium and the freshly seeded flasks were allowed to settle undisturbed until the carpet was confluent, usually 5-6 days, before FduU treatment. The cells were used between days 21-28.

RAI Apparatus

The apparatus used to deliver rapid acceleration impacts was modeled after the ballistic pendulum where one arm (m_2) holds the tissue culture flask while the second arm (m_1) delivers the impact. A schematic of the ballistic pendulum apparatus is shown

in Figure 1. By varying either the weight or the angle of m_1, one can achieve various accelerations that are measured as g-forces. For the experiments in this study, the weight of m_1 and the height from which it was dropped remained constant to achieve a constant acceleration/impact. An operator dropped m_1 from the selected height and allowed it to impact m_2. The operator stopped m_2 at the height of its arc when the velocity had fallen to zero. This procedure was repeated as many times as necessary to achieve the
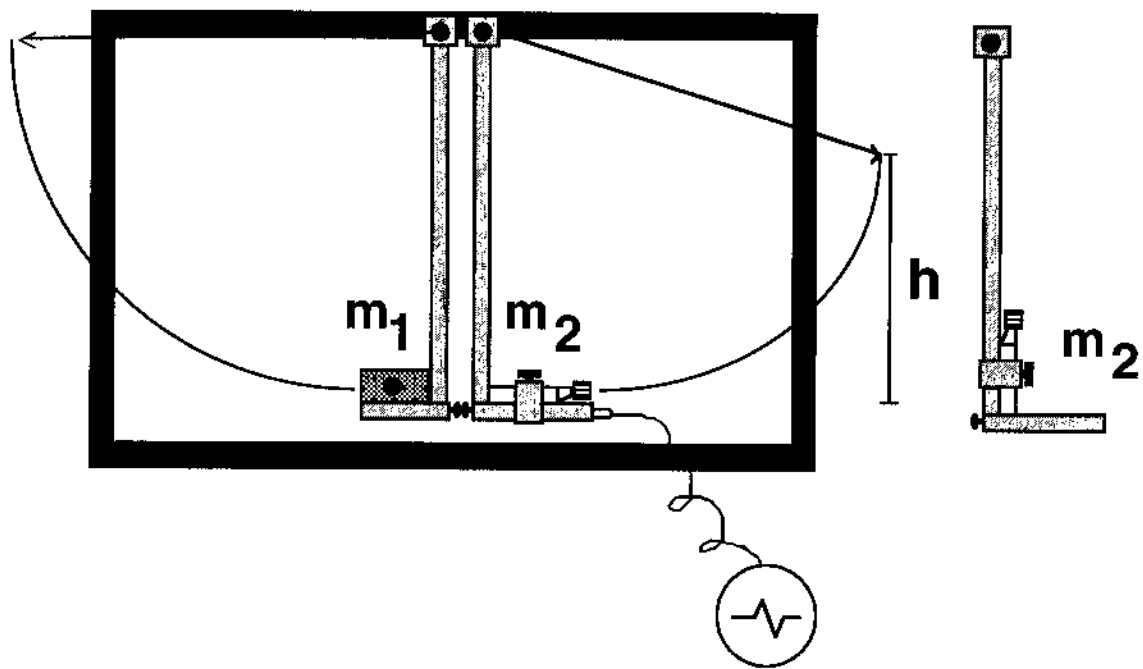
Fig. 1: Diagram of the ballistic pendulum apparatus used for these studies. M1 is dropped from its maximum height and allowed to impact M2. M2 is arrested by the operator just after reaching its maximum height - h. While the orientation of the flask in this diagram is for a tangential impact, the inset to the right of the apparatus shows the setup for an impact normal to the plane of cell growth. (From Lucas and Wolf, 1991)
desired cumulative accelerations.

Figure 2 includes a photograph of the actual RAI apparatus. An accelerometer (Entran Devices) was attached to m_2 (Fig. 2a) for measuring the acceleration and impact duration. The resulting signal from the accelerometer was amplified and displayed on a storage oscilloscope (Tektronix) (Fig. 2c). These signals were photographed and used to
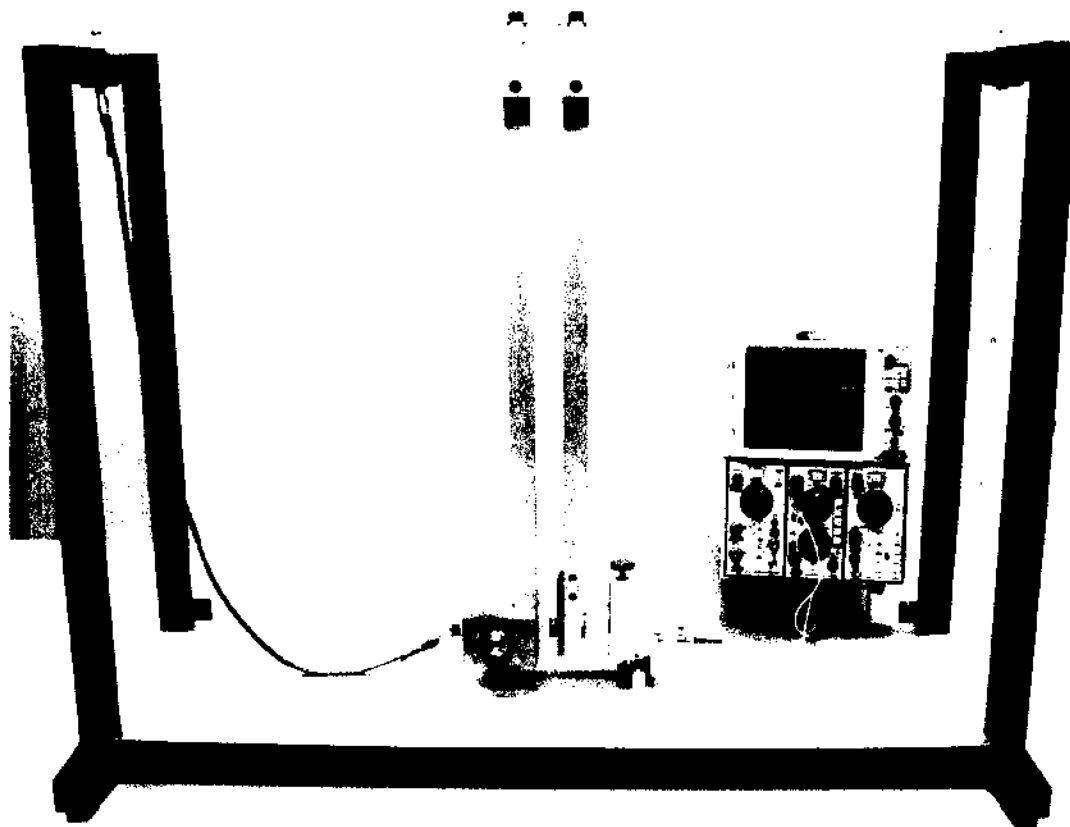
Fig 2a: Actual ballistic pendulum apparatus used for these studies.

Fig. 2b: Closeup of a culture flask arranged for a tangential impact.

Fig. 2c: Oscilloscope trace of the amplified signal from a g-meter showing impact duration. The sweep speed was 1msec/div.

determine both the impact duration (T) and the peak acceleration (A) achieved by m_2.
Since the initial momentum of m_2 is zero, the impulse (J) is proportional to the peak
momentum achieved by m_2 (1339g) after impact by m_1 (3165g). Therefore, $J=m_2 (gh)^{1/2}$
where g is determine both the peak acceleration (A) achieved by m_2 and the time to peak
acceleration (T). The impulse (J), the force (F), and the acceleration (A) can also be
calculated. Since acceleration due to gravity (9.78 m·s^{-2}) and h is the maximum height (34
cm) achieved by m_2 (Fig 1). The force (F) is equal to the impulse (J) divided by the time
to peak acceleration (T) or $F=J/T$. Thus, acceleration (A) = F/m_2 (Lucas and Wolf, 1991).

Small Network Selection

A grid design was scratched onto the bottom of each flask to divide it into three
areas. Within each area, two culture regions were chosen and photographed using a video
copier processor (Tektronix). Figure 3 shows the grid design scratched onto the bottom of
the flask to aid in the relocation of the photographed networks. The number of neurons in
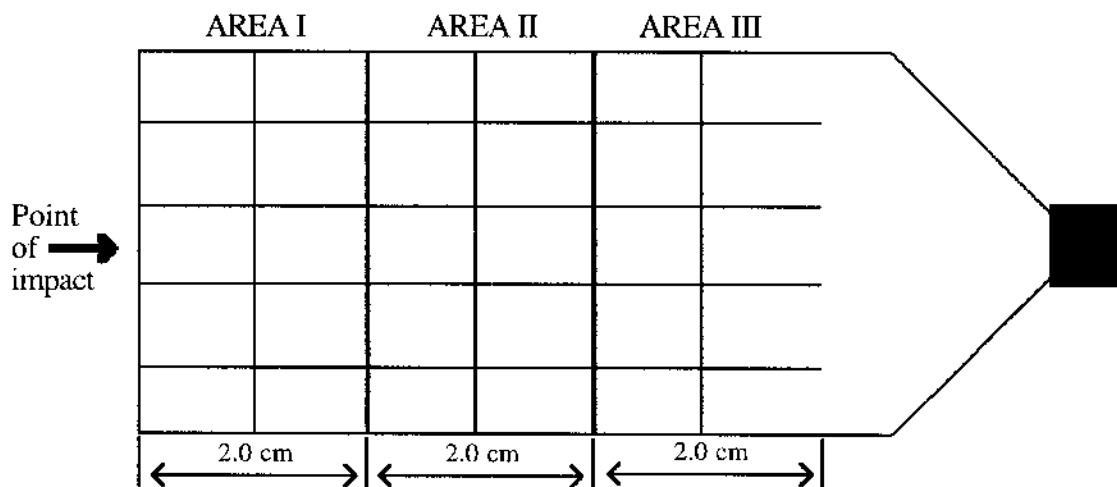
Fig. 3: Diagram of the style of flask used in these studies and the grid design scratched
onto the outside bottom surface to serve as a relocation aid.

each group chosen for an observation usually ranged from 5-15 cells. The cells chosen had prominent features i.e. distinct perikarya, nuclei, nucleoli, and processes to allow for a study of morphological changes. Following RAI, the photographs were used for relocation of the selected areas. For the studies of morphological changes, photographs were taken of the same areas after RAI and compared to those taken prior to RAI. This procedure was followed for all preliminary and current RAI experiments including toxicity studies except where a radiolabel was used. An early study performed showed that the distance from point of tangential impact to cell location did not influence cell survival (Lucas and Wolf, 1991).

RAI Procedure

For the RAI experiments, 3-4 cultures were selected from the same seeding date. Six regions (2 per area) were chosen in each culture and photographed for later relocation. In all experiments, the culture medium was carefully removed prior to RAI in order to minimize hydrodynamic shear stress during impact. The control culture (medium removal with no impacts) underwent a period of medium deprivation equal to the experimental cultures $\leq$ 45 seconds. The experimental cultures were subjected to impacts ranging from 1 - 10 impacts. After RAI or medium deprivation, a mixture of 2/3 conditioned and 1/3 fresh medium was added to the flasks (Fig. 4). The fresh medium was necessary for studies looking at long term effects of RAI and therefore, it was necessary to treat all cultures with the same mixture ratio to keep experimental parameters the same. For the poly(ADPR) studies (Figs. 5 and 6), the cultures were incubated prior to experimentation with a 3.0 ml mixture of 2/3 conditioned and 1/3 fresh medium containing 20 μCi/ml ^{3}H-Adenine. After RAI or medium manipulation the cultures were used for either assessing

Select 3-4 cultures from the same seeding date

↓

Choose 6 regions (2 per area) in each flask and photograph for relocation

↓

Remove the medium under sterile hood

Control

Experimental

Medium removal with no impacts. Period of deprivation ≤45 seconds.

With the RAI apparatus, deliver tangential or normal impacts; number of impacts variable.

Under sterile hood, add 4 ml mixture of 2/3 conditioned + 1/3 fresh MEM10 to flasks.   Return all flasks to incubator.

↓

At 15 min, 2 hours, 24 hours or 48 hours, assess viability of preselected neurons with phase contrast light microscopy (PCLM).   Verify with erythrosin-B dye exclusion (ERB).

Fig. 4: Flowchart of RAI experimental protocol.

Select cultures from the same
seeding date

↓

Remove the existing medium and replace with 3 ml of a 2/3
conditioned & 1/3 fresh MEM10 mixture with 3H-Ade at a
final concentration of 20μCi/ml and let it incubate overnight

Control ↙   ↘ Experimental

Medium removal with no impacts

With the RAI apparatus, deliver 1-10
tangential or normal impacts

↘   ↙

At desired time i.e. immediately, 2, 24, 48 hours, add
100% TCA to a final concentration of 20%. Scrape
cells off the flask bottom and collect TCA precipitate.

↓

Manipulate the acid-soluble and
acid-insoluble products to be put over
respective column for biochemical analysis.

Fig. 5: Flowchart showing the procedure for radiolabeling cultures.

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Take TCA extract (1 ml) and spin at 1K/10 min
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Acid-insoluble product | Acid-soluble product

| Dissolve pellet in 200µl ice cold 88% formic acid + 3 ml H20 + 0.8 ml 100% TCA | Dilute TCA sample supernatant up to 11 ml with 88% Ammonium formate [AF]pH 8.6 |

| incubate 30 min on ice spin 3K/10 min | adjust pH to 8.6 with NH40H |

count 0.5 ml

| dissolve pellet in 1 M KOH/100mM EDTA (1 ml) | put over DHB-S column |

| incubate at 37° C/2 hr | wash with 10 ml AF pH 8.6 |

| add 1 M GAE up to 10 ml (pH 9.0) adjust to 9.0±0.2 | wash with 2 ml H2O |

count 0.5 ml

| put over DHB-B column | elute with AF pH 4.5 2 ml 2X |

| wash with 25 ml 1 M GAE pH 9.0 until negligible counts detectable | take 1 ml of elutant and count - use rest for NAD determinations if necessary |

wash with 10 ml 1 M Ammonium Bicarbonate/10mM EDTA

elute with 1, 1, then 3 ml aliquots of H20, take 1 ml of total 5 ml and count

Fig. 6: Flowchart of protocol for DHB-B and DHB-S procedures.

viability and/or morphological changes (Fig. 4) or column chromatography to collect any acid-soluble or acid-insoluble products (Fig. 6).

Determinations of Cell Viability

Cell viability after RAI or medium deprivation was determined by erythrosin-B dye exclusion tests (ERB). ERB uptake by cells is considered indicative of a lethal loss of membrane integrity. Cells stained by ERB have pink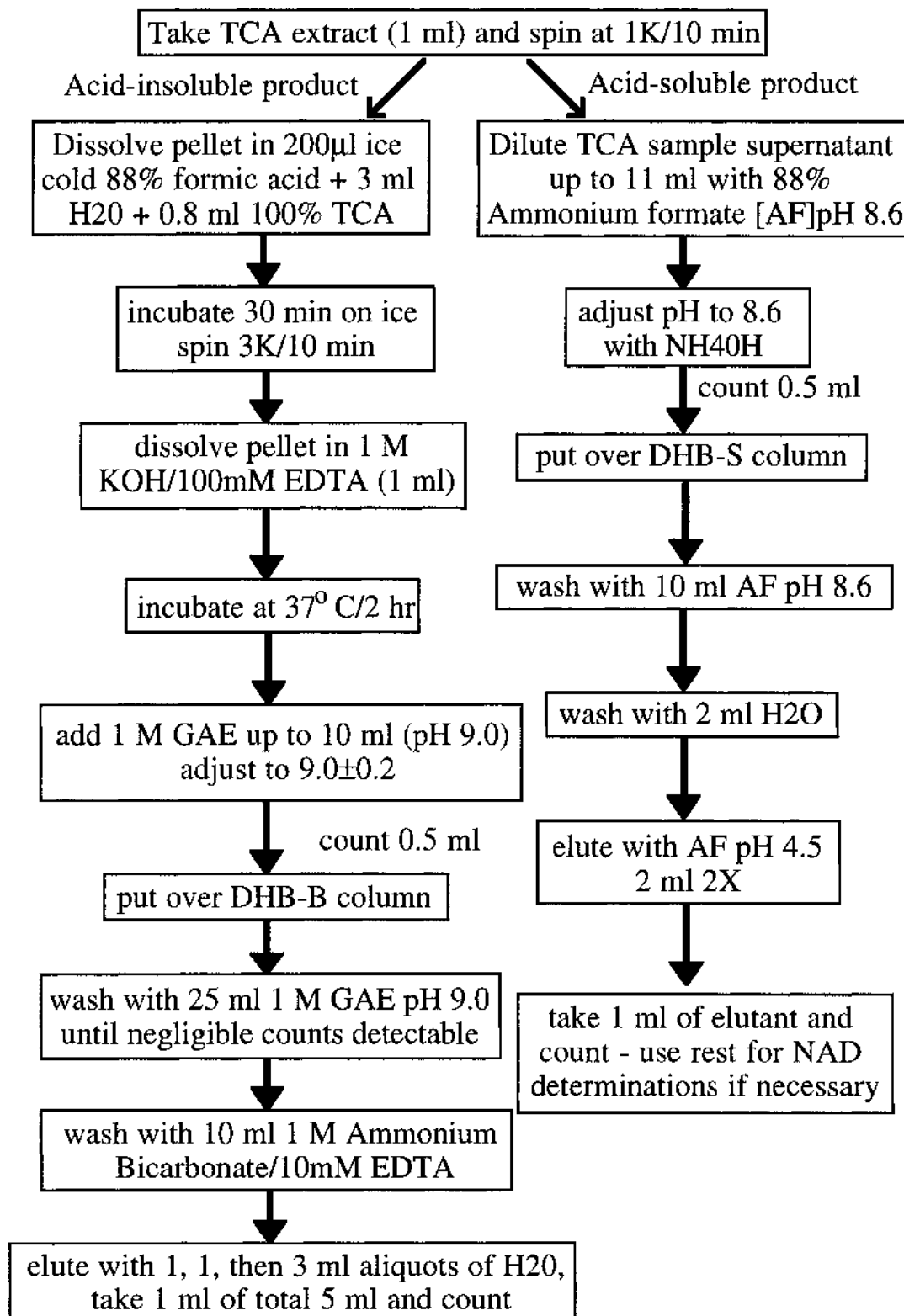 or red somal cytoplasm. ERB dye was applied according to the method of Phillips (1973) with the modifications by Emery and coworkers (1987). Briefly, a 0.4% stock solution of the dye was made and from this, 0.5 ml ERB/ml of medium was added to the culture. The culture was incubated for 5 min under 10% CO_2 before being rinsed twice with warm D_1SGH. The preselected areas were then relocated and neuronal viability was evaluated by LM.

Morphological Evaluations using Light Microscopy

For the studies of morphological changes observed after RAI the cultures were evaluated using LM prior to ERB determination of neuronal viability. Following the RAI protocol, six separate locations were chosen in each flask and photographed. The individual cells were observed and numbered. Immediately after the RAI injury, the cultures were then evaluated under LM. The selected locations were relocated and photographed again. The before and after photographs and the LM evaluation were used to determine which cells underwent any morphological changes due to RAI. After an extensive study of morphological changes in spinal cord neurons subjected to RAI (Lucas and Wolf, 1991), it was determined that the most prevalent and important changes included

somal swelling, increased nuclear prominence, lateral nuclear shifting, and lateral nucleolar shifting. These morphological changes were evaluated for additional experiments and also for the cerebral cortex cultures for a comparison of tissue differences after RAI.

Determinations of Average Cell Counts

In order to determine whether as a result of RAI there was an increase or decrease in poly(ADP) ribose production it was necessary to have an approximate cell count for each assay. Since we used culture flasks for our studies, it was important to obtain an average number of cells per culture flask/week in an effort to determine whether there was an increase, decrease or no significant change in the amount of acid-insoluble product i.e. poly(ADP)ribose polymers as a result of RAI injury. Therefore, it was necessary to devise a way to remove the cells from the substrate and count them. To remove the cells from the substrate, 1.0 ml of a solution (26.0 ml D_1SGH, 0.05g EGTA, and 1.6mg DNase) was added to 1.5 ml of papain (20units/ml). This mixture was immediately added to the culture flask and the flask was incubated at 37°C under 10% CO_2 for 15 min. The enzymatic reaction was stopped by the addition of 600μl horse serum. The cells were gently shaken off the substrate and counted with a hemacytometer. A triplicate count was performed on random flasks from the same culture date and the average cell number per flask was determined.

Tissue Culture Uptake of Label and Conversion to NAD*

Because NAD^+ is the sole substrate for poly (ADP-ribose), it was necessary to determine the optimum incubation time required for the conversion of ^3H-Ade into

^{3}H-NAD. Three to four week old spinal cord or cerebral cortex cultures containing $1\text{-}2\times10^6$ neurons/dish were incubated with 20μCi /ml ^{3}H-Ade for 0, 3, 6, 9, 12, 15, 18, or 21 hours. The procedure of Aboul-Ela et al. (1988) was used to determine the conversion of ^{3}H-Ade to ^{3}H-NAD on triplicate cultures at each time interval. This procedure is described below.

First, the unlabeled medium was removed; then the cultures were washed 2X with a phosphate buffered saline (PBS) solution, and medium containing ^{3}H-adenine at a final concentration of 20μCi /ml was replaced on the cultures. At three hour time increments from 0-21 hours, the cultures were taken from the incubator and the medium was removed. The triplicate dishes were then rinsed 3X with ice cold PBS and aspirated to dryness with a drawn out 9" pasteur pipette between washes. Ice cold 0.5M $HClO_4$ (200μl) was added and the dishes were scraped with a disposable cell scraper (costar). The resulting homogenates from the triplicates were transferred to separate 15.0 ml centrifuge tubes. The dishes were rinsed with 100μl ice cold 0.5M $HClO_4$ and the residue was transferred to the appropriate tube and then put on ice for 15 minutes. Next the sample was centrifuged at 1500 rpm for 5 minutes and the supernatant transferred to another tube that was neutralized with a predetermined volume (by titration) of 1 M $KOH/0.33M$ K_2HPO_4. The sample was centrifuged a second time and the supernatant was saved in another tube.

From the extract, we were able to determine counts taken up by the cells, counts in NAD, and the specific activity of the labeled NAD pool. The DHB-Sepharose column binds the acid soluble fractions thus allowing for the determination of NAD in the cell pool (Aboul-Ela et al., 1989). This involves bringing the sample extract up to a 10.0 ml volume with 0.25M ammonium formate (AF) (pH 8.5) and applying it to a DHB-Sepharose column which had been prewashed with 5.0 ml H_2O and 10.0 ml of the AF solution. The

column was then washed with 10.0 ml of 0.25M AF (pH 8.5). After washing the column with 2.0 ml H2) the sample was eluted off with 4.0 ml 0.25 M AF (pH 4.5). A 500μl aliquot was counted from each sample.

The pmol concentration of the total NAD in our cell pool/1×10^6 cells was determined by the microtiter NAD assay. Using varying concentrations of three components, the sample or standard, diluent, and the premix, one can obtain a standard curve from NAD concentrations of 0-50 pmol. The microtiter wells are filled and allowed to incubate at $30^\circ C$ for 30 min before being read through an ELISA reader.

MNNG Toxicity

Mature spinal cord cultures and cerebral cortex cultures were exposed to various concentrations of MNNG (1-Methyl-3-nitro-1-nitrosoguanidine) for 30 minutes to 2 hours at $37^\circ C/$ 10% CO_2. ERB exclusion tests were performed to determine cell death. This alkylating agent, known to be a mutagen at concentrations above 20μg/ml (Jacobson et al. 1985a; 1985b), was used to induce the production of poly(ADP-ribose). In our spinal cord cultures, cell death was observed at concentrations above 20μg/ml as well. To date, the ability of this agent to enhance poly(ADP-ribosylation) had not been tested on *in vitro* spinal cord or cerebral cortex cultures.

Results

Light Microscopic Evaluations of Cerebral Cortex Cultures

It is essential that neurons can be positively identified in the living state in order to characterize neuronal morphological changes after injury. The morphological characteristics of the spinal cord monolayer cultures obtained with the same methods used in the present studies have been previously reported (Lucas et al., 1985). The following light microscopic morphological criteria were used by Lucas and coworkers to identify spinal cord neurons: (1) presence in the uppermost stratum of the culture; (2) multiple processes which are branched and long; (3) large, phase-bright cell bodies and; (4) well-defined nuclei and nucleoli (Fig. 7). As reported by Lucas et al., at least 78% of the selected cells were electrically active. An additional study was performed recently with Dr. M. Wilk (CNNS postdoctoral fellow) which showed that approximately 96% (23 of 24 cells) of the visually selected spinal cord cells were electrically active.

Fig. 7: Phase contrast light micrographs of neuronal networks found in monolayer spinal cord cultures used in this study. Bar = 10μm.

Until this study, cerebral cortex cultures were not used by this laboratory for extensive studies. The methods described in the previous section resulted in robust cortical cultures. The cultures were used between three and four weeks of age when they were considered to be mature. The cortical cultures resemble the spinal cord cultures in that the neurons lie over a glial carpet. From observations reported for this study, this was never violated: neurons were never seen growing in regions without glial cells.

While there tended to be some reaggregation of the cortical neurons, the cultures were dispersed enough to view many small networks of neurons as well as individual cells. Overall there was more process fasciculation between the reaggregates in the cortical cultures than in the spinal cord cultures (Fig. 8). The neurons in the cerebral cortex cultures were classified according to the guidelines presented by Kriegstein and Dichter (1983) in their report on the classification of rat cortical cells *in vitro*. Basically, the investigators identified the cortical cells depending on their morphology. They classifed the cells as pyramidal-like (triangular soma with one distinct apical process and more basilar processes emerging from the soma), multipolar (multiple processes of fairly uniform lengths projecting from various sites of the soma) and fusiform (usually a spherical perikaryon with two major processes emerging from opposite ends of the soma). Kriegstein and Dichter suggested that all cortical neurons *in vitro* fall into one of these categories and can be classified as early as 24 hours after initial seeding. The individual cells tended to maintain their morphology but after time *in vitro*, the glial cells began to cover the processes and thus made the assessment more difficult (ibid). We supplemented phase contrast microscopy with histology for further characterization. A Loots modified Bodian stain (Loots et al., 1979) was employed to help classify the cortical cells used in these studies (Fig. 9) and to observe the early stages of neuronal development (Fig. 10).
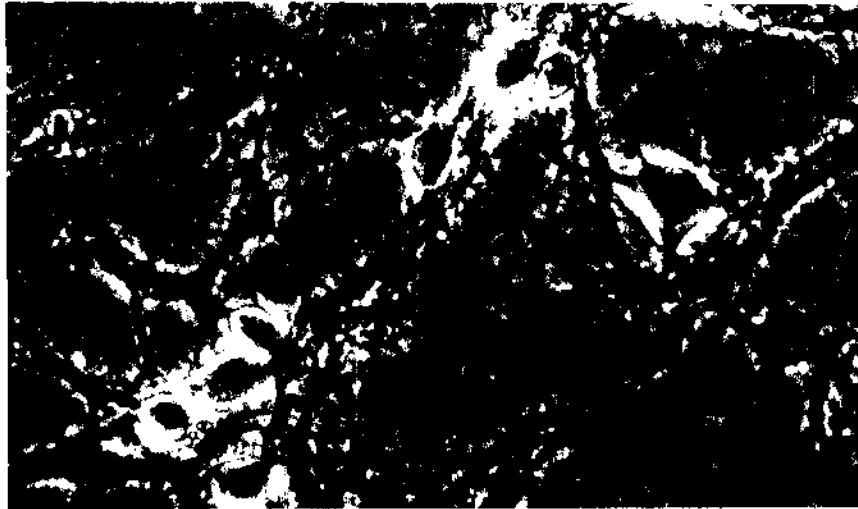
Fig. 8: Phase contrast light micrographs of neuronal networks found in monolayer cerebral cortex cultures used in this study. Bar = 10µm.

Fig. 9: Three cortical cell types found in our culture system. A Loots modified Bodian stain was used on the cortical cultures. a. A pyramidal-like neuron. b. A fusiform neuron. c. A multipolar neuron. Bar=10µm.

A comparison of light micoscopic evaluations of spinal cord and cerebral cortex cultures at various times after initial seeding showed similarities in early culture development. At various times ranging from three hours after initial seeding to maturity (three to four weeks), phase contrast light micrographs were taken of living spinal cord cultures or cerebral cortex cultures for comparison. Additionally, a representative culture from the same age was fixed for the Loot's modified Bodian stain procedure to visualize more clearly the perikarya and processes. These representative cultures were then
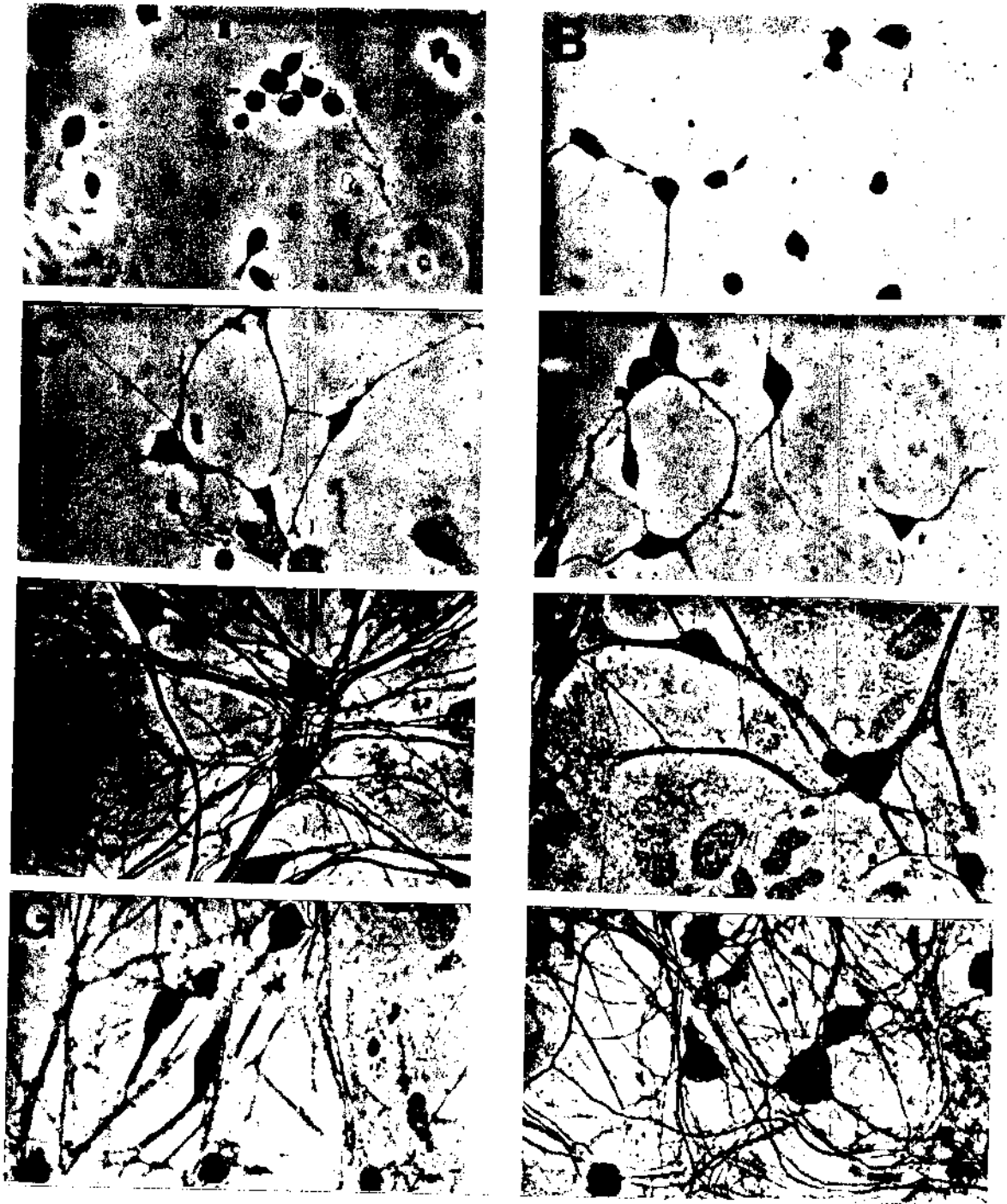
Fig. 10: Loots modified Bodian stained SC and CC cultures at various times after seeding.
A. SC-3 hrs; B. CC-3 hrs; C. SC-24 hrs; D. CC-24 hrs; E. SC-3 weeks old; F. CC-3
weeks old; G. SC 4 weeks old; H. CC 4 weeks old. Bar =10µm.

evaluated under a phase contrast light microscope (Zeiss) and photographed for comparison (Fig. 10).

In order to ensure that cortical neurons can be visually identified in the living state with phase contrast microscopy, a study was performed with Dr. M. Wilk to determine whether selected representative cerebral cortex cells for these experiments were electrically active. This study showed that 100% of the visually selected cortical cells (44 cells) were electrically active (Fig. 11). Cells which were believed to be non-neuronal, possibly astrocytes, had membrane potentials but produced no spike potentials (7 glial cells selected).
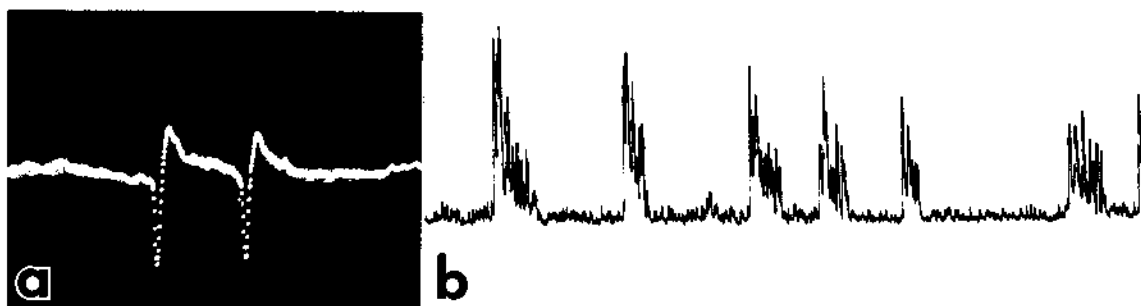
Fig. 11:. Oscilloscope tracing of extracellular recording (50mV/div and a sweep speed of 5ms/div) and printout of the integrated activity (100mm/min) from a single cerebral cortex neuron as determined by M. Wilk, M.D., PhD (CNNS Postdoctoral Fellow).

Viability Studies and Morphological Changes in Spinal Cord and Cortical Somata after RAI

A. Spinal Cord Culture

Preliminary studies of the effects of RAI on spinal cord monolayer cultures (Lucas and Wolf, 1991) have shown that 10 multiple impacts delivered tangential to the plane of

cell growth, cumulative acceleration (Acum) = 2200g, can cause maximum neuronal death of approximately 46%. This study added one additional experiment to each category of injury level (Table I) to the initial analysis. Almost all of the neurons died immediately or within the initial 15 min period after RAI. This information was the basis for choosing 10 multiple impacts delivered tangential to the plane of cell growth for the supplemental studies presented here.

The morphological changes observed after RAI were previously assessed for spinal cord neurons (Lucas and Wolf, 1991). An additional experiment was performed recently to add additional data to the initial observation to increase the number of observations per each group tested (Table II.).

Table I. Viability of spinal cord neurons after RAI expressed as mean ± sd.

	(mean±sd of cells/flask/	Neurons Viable mean±sd	(%) Dead mean±sd
Control (0 impacts)	(38±9.6/4)	91±3.8	9±3.8
Normal (10 impacts)	(36±8.4/4)	92±12.1	8±12.1
Tangential (3 impacts)	(35±6.4/4)	88±3.8	12±3.8
Tangential (5 impacts)	(36±10.2/4)	60±12.1	40±12.1
Tangential (10 impacts)	(39±11.6/4)	54±8.1	46±8.1

Table I was modified from studies by Lucas and Wolf (1991) with one additional experiment included in each category (N=3-7, 816 neurons observed). For each flask used, the mean ±sd of cells represents the average number of experimental cells per flask.

Table II. Morphological changes in spinal cord neurons after RAI.

	(mean±sd of cells/flask/)	Somal swelling*	Increased nuclear visibility*	Lateral nuclear shifting*	Lateral nucleolar shifting*
Control (0 impacts)	(38±9.6/4)	6	1	2	2
Normal (10 impacts)	(36±8.4/4)	15	2	0	3
Tangential (3 impacts)	(35±6.4/4)	24	9	7	5
Tangential (5 impacts)	(36±10.2/4)	23	23	16	2
Tangential (10 impacts)	(39±11.6/4)	37	38	13	13

*% of surviving cells only

Table II was modified from studies by Lucas and Wolf (1991) with one additional experiment included in each category (N=3-7, 816 neurons observed). For each flask used, the mean ±sd of cells represents the average number of experimental cells per flask.

B. Cortical Cultures

The effects of RAI on cerebral cortex cultures indicated that the maximum neuronal cell death can also be achieved after 10 impacts [Acum=2200g] delivered tangential to the plane of cell growth (Table III). As with the spinal cord cultures, the cerebral cortex cultures exhibited the maximum amount of cell death within the first 15 minutes post-RAI. And also similar to the spinal cord cultures, the cerebral cortex cultures exhibited the same sort of "plateau" which peaked at approximately 55% cell death post-RAI. This maximum amount of cell death observed occurred within the first 15 minutes after RAI in spinal cord cultures and did not increase with time up to 48 hours post -RAI (Lucas and Wolf, 1991). The cortical cultures exhibited a similar peak within 15 minutes post-RAI and the amount of cell death did not increase substantially up to 72 hours post-RAI (Fig. 12).

Table III. Viability of cortical neurons immediately after RAI.

	(mean±sd of cells/flask/)	Neurons (%) Viable mean±sd	Dead mean±sd
Control (0 impacts)	(53±8.1/3)	76±3.0	24±3.0
Tangential (3 impacts)	(52±8.1/3)	64±4.3	34±4.3
Tangential (5 impacts)	(54±2.9/3)	56±2.9	44±2.9
Tangential (10 impacts)	(46±2.3/3)	45±2.2	55±2.2

Table III represents the immediate (15 min post-RAI) percent viability of cortical cells subjected to RAI injury expressed as the mean±sd. For each flask used, the mean ±sd of cells represents the average number of experimental cells per flask.
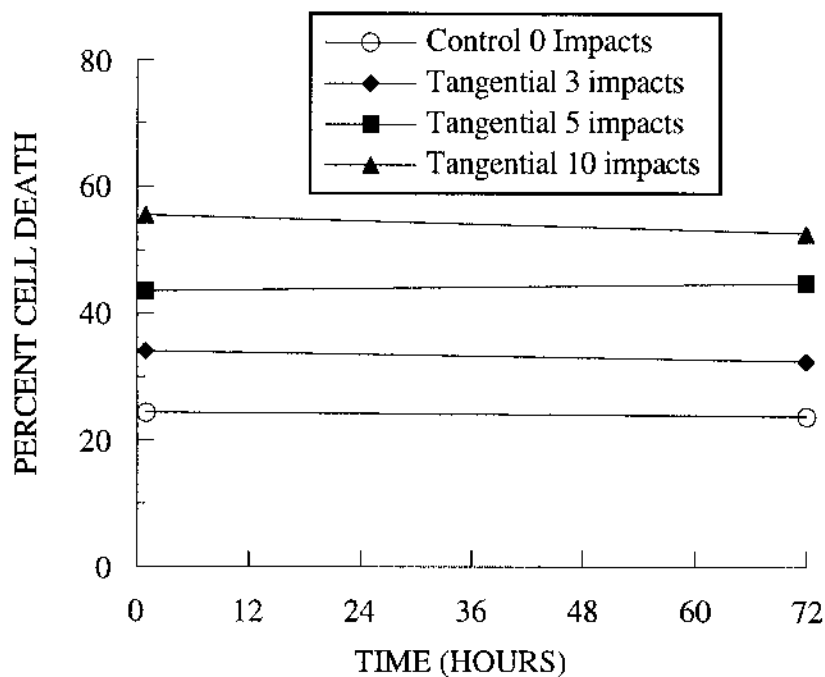

Fig. 12: Cerebral cortex neuronal death can be seen 15-min post-RAI after 10 impacts [Acum=2200g] and does not change over a 72 hr observation period.

Studies designed to look at delayed neuronal death involving *in vivo* gerbil hippocampus (Kirino, 1982) showed that the incidence of delayed neuronal death in the CA1 region of the hippocampus region occurs by 72 hours post-injury. The fact that we did not observe this phenomenon of delayed neuronal death in our cortical cultures could be due to the greater number of cerebral cortex cells than hippocampal cells used in our culture procedures. The hippocampal cells of the CA1 region have a very high N-Methyl D-Aspartate (NMDA) receptor concentration. The NMDA receptor/complex is a subclass of the glutamate receptors and is involved in excitotoxic responses to injury. The same experimental parameters were applied to both spinal cord and cortical cultures. The results indicate that both tissues react similarily to rapid acceleration impact injury. The morphological changes observed immediately after RAI in cerebral cortex neurons can be seen in Table IV.

Table IV. Morphological changes in cerebral cortex neurons immediately after RAI (N=3, 613 neurons observed).

	(mean±sd of cells/flask/)	Somal swelling*	Increased nuclear visibility*	Lateral nuclear shifting*	Lateral nucleolar shifting*
Control (0 impacts)	(53±8.1/3)	6	3	3	2
Tangential (3 impacts)	(52±8.1/3)	20	6	2	3
Tangential (5 impacts)	(54±2.9/3)	22	17	9	5
Tangential (10 impact)s	(46±2.3/3)	33	28	13	11

*% of surviving cells only

Table IV lists the morphological changes observed in the cortical cells within the first 15 min post-RAI. For each flask used, the mean ±sd of cells represents the average number of cells per flask.

Comparison of Neuronal Viability and Morphological Changes after RAI with and without Ketamine

Ketamine is a noncompetitive NMDA antagonist which binds at the phencyclidine site of the NMDA receptor/complex. Blocking this receptor can help prevent the excitotoxic influx of extracellular Ca^{2+} into the cells. The protection observed after pretreatment with ketamine supports the idea that Ca^{2+} may be involved in the morphological changes observed. A study was performed to determine if the maximum nontoxic concentration of ketamine (0.1mM; Lucas and Wolf, 1991) applied to spinal cord cultures before RAI could prevent or decrease neuronal cell death and/or morphological changes i.e. increased nuclear prominence, nuclear shifting and somal swelling. The additional studies performed for this thesis have strengthened the previous evidence that ketamine applied prior to RAI protects spinal cord neurons in culture. The combined data show that the presence of ketamine reduced death of spinal cord neurons subjected to 10 tangential impacts from 51% to 14% (Table V). Ketamine also decreased the incidence of somal swelling in spinal cord cells from 31% to 6% and reduced the incidence of increased nuclear prominence from 17% to 6% (Table VI). However, ketamine made virtually no difference to the incidence of lateral nuclear shifting and lateral nucleolar shifting in spinal cord cultures after RAI.

In this study, new data were collected on cerebral cortex cultures as a comparison to the spinal cord data. These suggest that the response to multiple impact RAI for cerebral cortex cells is very similar to that found with spinal cord cultures. Ketamine (0.1mM/ml) appeared to have a protective effect on the cerebral cortex cultures. The presence of ketamine reduced death of cerebral cortex neurons subjected to 10 tangential impacts from 53 % to 25 % (Table V). Pretreatment with ketamine also decreased the incidence of somal

Table V. Comparison of spinal cord and cortical neuron viability (mean±sd) with or without pretreatment with ketamine (0.1mM).

	Spinal cord neurons		Cerebral cortex neurons	
	%Viable	% Dead	%Viable	% Dead
Control				
0 impacts without 0.1mM ketamine	89±4.1	11±4.1	79±9.7	21±9.7
(mean±sd of cells/flask)	(52±8.7/4)		(48±10.1/5)	
Control				
0 impacts with 0.1mM ketamine	93±1.3	7±1.3	89±2.5	11±2.5
(mean±sd of cells/flask)	(50±2.9/4)		(50±6.5/5)	
Tangential				
10 impacts without 0.1mM ketamine	49±11.6	51±11.6	47±10.5	53±10.5
(mean±sd of cells/flask)	(40±7.3/4)		(50±9.7/5)	
Tangential				
10 impacts with 0.1mM ketamine	86±5.5	14±5.5	75±6.6	25±6.6
(mean±sd of cells/flask)	(49±12.7/4)		(53±9.2/5)	

Table V represents a comparison of spinal cord (N=4, 768 neurons observed) and cortical (N=5, 1026 neurons observed) neuron viability expressed as the mean±sd with or without pretreatment with ketamine (0.1mM). For each flask used, the mean ±sd of cells represents the average number of cells per flask.

Table VI. Morphological changes in spinal cord neurons described in Table V.

	(mean±sd of cells/flask/)	Somal swelling*	Increased nuclear visibility*	Lateral nuclear shifting*	Lateral nucleolar shifting*
Control 0 impacts w/o ketamine	(52±8.7/4)	13	5	2	0
Control 0 impacts w/ ketamine	(50±2.9/4)	10	3	1	1
Tangential 10 impacts w/o ketamine	(40±7.3/4)	31	17	4	4
Tangential 10 impacts w/ ketamine	(49±12.7/4)	6	6	4	2

*% of surviving cells only

Table VI lists the morphological changes in spinal cord neurons described in Table V after pretreatment with ketamine as modified from studies by Lucas and Wolf, 1991 with one additional experiment included (N=4, 768 neurons observed).

swelling from 29% to 9% and reduced the incidence of increased nuclear prominence from 15% to 6% (Table VII). Again, as in the spinal cord cultures, ketamine made no difference to the incidence of lateral nuclear shifting and lateral nucleolar shifting in cerebral cortex cultures .

Table VII. Morphological changes in cerebral cortex neurons described in Table V.

	(mean±sd of cells/flask/)	Somal swelling*	Increased nuclear visibility*	Lateral nuclear shifting*	Lateral nucleolar shifting*
Control 0 impacts wo/ ketamine	(48±10.1/5)	19	9	2	2
Control 0 impacts w/ ketamine	(50±6.5/5)	13	4	<1	1
Tangential 10 impacts w/o ketamine	(50±9.7/5)	29	15	3	2
Tangential 10 impacts w/ ketamine	(53±9.2/5)	9	6	2	2

*% of surviving cells only

Table VII lists themorphological changes in cerebral cortex neurons described in Table V after pretreatment with ketamine (N=5, 1026 neurons observed).

Tissue Culture Uptake of Label and Conversion to NAD*

Preliminary results investigating the time course of label uptake and NAD conversion for both spinal cord and brain cultures indicated that the optimum ^{3}H-Ade incubation time was from 18-21 hours. Therefore the incubation time used was approximately 18 hours at a concentration of 20μCi ^{3}H-Ade/ml medium to label the NAD pool in our culture system. NAD assays performed showed that in both spinal cord and cerebral cortex cultures, there is approximately 2000pmol of NAD per 10^6 cells.

Determinations of Average Cell Counts

Because it was necessary to know approximately how many cells were in each of our cultures, the cells were removed from the culture substrate and counted. Sets of flasks from various weeks were counted in triplicate and it was found that the average number of cells per flask was consistent within the same week. The number of cells per flask varied between weeks but were fairly consistent within a culture date. From one culture date, six representative cultures were counted. The range was from 2.87×10^6 - 3.07×10^6 cells with a mean$\pm$sd of $2.98 \times 10^6 \pm 8.20 \times 10^4$ cells. Similar results from a second culture date gave the following mean$\pm$sd.: $3.83 \times 10^6 \pm 2.75 \times 10^5$ cells. An additionl method using the fluorescent dye, acridine orange, was used to see that what was removed from the substrate and counted were actually whole cells. This dye stains nucleic acids labelling the DNA orange and the RNA green. It was used here to check the results from above to be certain that the cell counts were reflective of the true spinal cord and cortical cells for the poly(ADPR) studies. This method of determining cell numbers and the consistency of cell numbers/flask within culture dates allows for a determination of the amount of poly(ADP)ribose production/individual flask to determine whether there is an increase, decrease or no difference after cell injury.

MNNG Toxicity Studies

The alkylating agent MNNG (1-Methyl-3-nitro-1-nitrosoguanidine) is known to induce poly(ADP-ribose) production in C3H10T1/2 cells at concentrations above $20\mu g/ml$ as reported by Jacobson et al.(1985a, 1985b). The toxicity studies performed for this thesis showed that MNNG can induce neuronal cell death in both spinal cord and cortical cell cultures at the same concentrations and incubation times (Figs. 13 & 14). The cultures
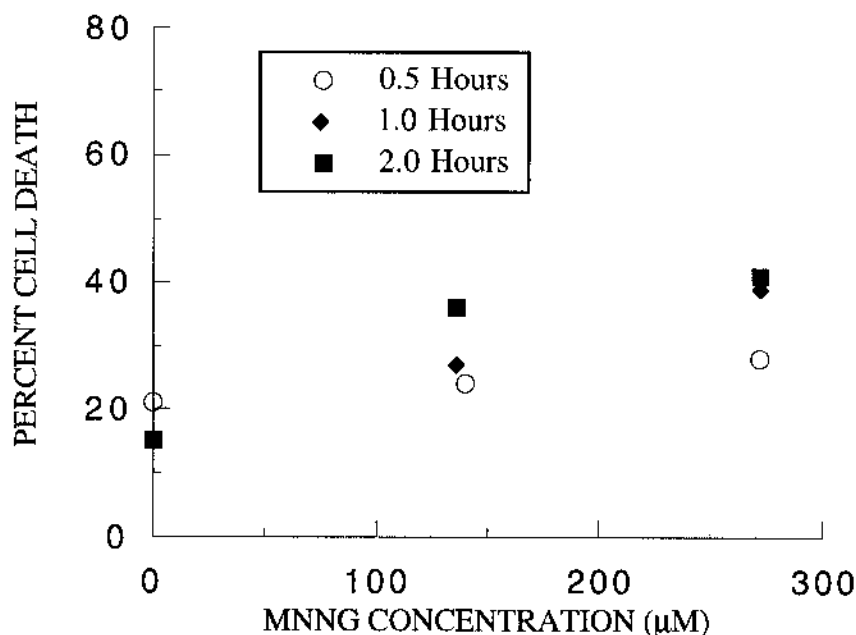
Fig. 13: Toxicity graph of spinal cord cell death due to MNNG at various concentrations. (N=3 cultures/data point; a total of 1948 neurons observed).

were treated with MNNG which was dissolved in no more than 160µl acetone (it was predetermined that this concentration of acetone was not detrimental to the cells) for times ranging from 30 minutes to 2 hours. The cell death totals were determined using ERB exclusion dye and evaluated under phase contrast light microscopy. Triplicate observations were done at each concentration of MNNG: 0µg/ml; 20µg/ml (136µM); 40µg/ml (272µM). Each data point on the graph represents the average of the three different observations.

Jacobson et al. (1984) reported that C3H10T1/2 cells treated with 5µg/ml MNNG for 20 minutes resulted in 34 - 42 % cell viability. We have found that our values for CNS
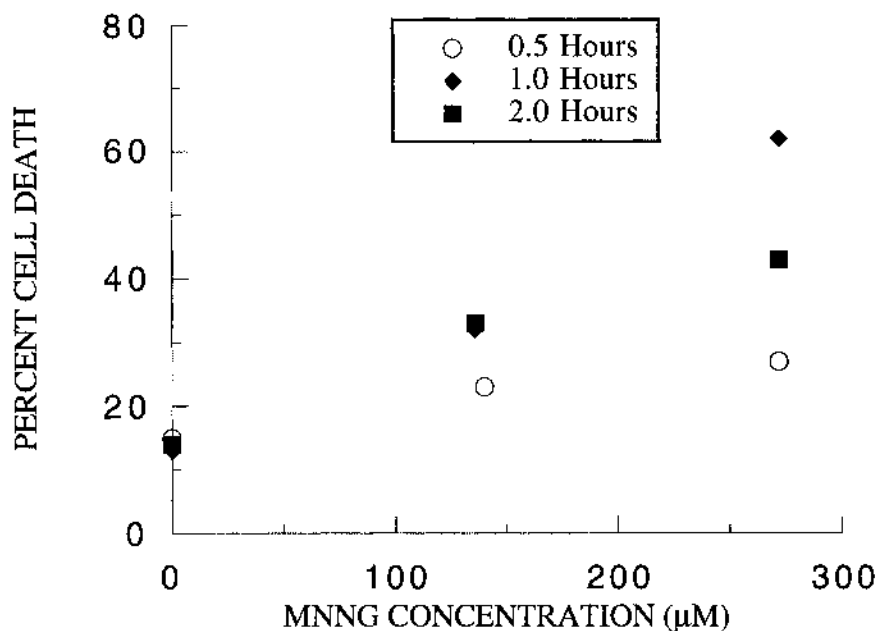
Fig. 14: Toxicity graph of cortical cells to MNNG at various concentrations.
(N=3 observations/data point, 1736 neurons observed).

cells in culture represent a higher viability rate than C3H10T1/2 at higher MNNG

concentrations. The difference in cell viabilty rates could be due to the method of cell

viability determinations since Jacobson et al. looked at colony-formation ability as an

indication of viabilty and our study looked at ERB dye cell uptake as an indication of cell

death.

Discussion

The *in vitro* model of rapid acceleration used in these studies was developed in this laboratory and used as an experimental model of RAI for spinal cord cultures (Lucas and Wolf, 1991). This model of mechanical injury allows the study of impact injury on the cellular level because it can deliver a uniform physical insult to all of the cells in a primary CNS cell culture. The three dimensional structure of *in vivo* preparations does not allow one to deliver a uniform physical injury to all the cells. In addition, studying RAI *in vitro* includes the ability to view cells before and after injury.

Initial investigations of spinal cord neuron and glial reactions to RAI (Lucas and Wolf 1991) showed that the cumulative acceleration threshold for neuronal death was 660 g delivered tangential to the plane of cell growth, and that the maximum mortality rate occurred at 1100 g to 2200 g (Lucas and Wolf, 1991). For the present studies we chose to deliver cumulative accelerations in excess of 660 g to cultures of cortical cells, and to compare the responses of cortical neurons and glia to the responses of spinal cord neurons and glia. As with spinal cord cultures, death of neurons in cortical cultures increased with increasing cumulative acceleration.

As there are many differences between spinal cord and cortical tissue (such as NMDA receptor distribution, Cotman et al. 1989), the development of cortical cell culture procedures for RAI studies was an important goal. The methods described in this report have produced robust cortical cultures which survive as long as three to four weeks in culture. Initially the cortical cultures resemble the spinal cord cultures in that they lie over the glial carpet, but after 5-6 days in vitro, the cortical cultures begin to reaggregate and form more process fasicles than the spinal cord cultures. The cortical cultures seem to have a much higher metabolic rate than the spinal cord cultures which may be due to an

higher rate of cell proliferation. This observation necessitated the need for supplemental glucose levels in the medium for the cortical cultures.

Overall, the spinal cord and cerebral cortex cultures behaved similarly in response to RAI. After 5 tangential impacts (1100g cumulative acceleration) maximum neuronal death (approximately 50%) was achieved in both culture preparations. One difference appears to be how the cortical cultures react to the medium manipulation (no impact). While only 12% of the spinal cord neurons died after medium deprivation, 21% of the cortical neurons died after medium manipulaton. Death due to medium manipulation may have been related to excitotoxicity because the addition of 0.1mM ketamine reduced both cortical and spinal neuronal death.

Neuronal death occurred within 15 minutes post-impact in both culture preparations. Earlier investigations showed that there was no additional spinal cord neuronal death up to 48 hours post-RAI (Lucas and Wolf, 1991). The studies presented here show that for cortical neuron cultures, there also was no increase in cell death for up to 72 hours post-RAI. These results imply that there is no delayed neuronal death in either spinal cord or cortical cultures following impact injury. This is at variance with studies by Kirino (1982) and Thilmann et al. (1986) who observed delayed neuronal death *in vivo* in an ischemic model of injury in the CA1 region of the hippocampus. While the hippocampus is included in our brain dissection for cortical cultures, most of the tissue is from the neocortex. Thus, there may not be a sufficient number of CA1 hippocampal neurons in our cortical cell population to allow for the detection of delayed neuronal death. This question could be addressed by observing the effects of RAI on pure hippocampal cultures.

Using this model we were able to look specifically at perikaryal or dendritic changes that occurred as a result of rapid acceleration impact injury. While most *in vivo*

and clinical studies (Adams et al, 1982, 1983; Gennarelli et al, 1981; Gennarelli and Thibault, 1985; and Povlishock, 1986) of CNS impact trauma have not shown much dendrosomatic damage or immediate neuronal loss, we observed perikaryal perturbations as well as immediate, (i.e. within the first 15 minutes post-RAI), neuronal death. These results suggest that multiple impact RAI may cause dendrosomatic changes as well as the axonal interruptions observed *in vivo* and clinically (Friede, 1861; Strich, 1961; Gennarelli, 1983).

The morphological changes observed in this study as a result of RAI to CNS neurons *in vitro* have been assessed and the results have indicated that there were changes in neuronal somata and especially in the nucleus. At the lethal injury level (5-10 impacts) we found perikaryal changes which could have been due to a weakening of the cytoskeleton. As the degree of injury increased, there was an increase in nuclear prominence. In some instances the nuclei were released from the perikarya and were observed floating in the medium during LM observations. The incidence of these morphological changes and neuronal death was substantially reduced when the cultures were pretreated with 0.1 mM ketamine, a non-competitive NMDA channel blocker. Thus, this level of injury may allow for an influx of calcium through the NMDA receptor governed ion channel which may have been activated due to the injury. This influx may alone be enough to cause cell deterioration which may allow even more calcium to enter leading to cell death (Lucas and Wolf, 1991). Perhaps this increase in calcium may be primarily due to the activation of the NMDA receptor because interestingly enough, these morphological changes and neuronal death were decreased with the pretreatment of the cultures with 0.1mM ketamine. Ketamine is an NMDA antagonist and blocking this receptor to help reduce the overall amount of Ca^{2+} from entering the cells also appears to prevent or reduce the incidence of the morphological changes observed as a result of RAI.

Perhaps the cytoskeleton is weakened by the Ca^{2+} influx, an effect that can be prevented by the addition of NMDA antagonists.

The hypothetical sequence of events leading to cell death in our impact injury model is shown in Fig 15 (from Lucas and Wolf, 1991). We hypothesize that as the level of injury increases the membrane is physically perturbed leading to changes in membrane protein function including activation of the NMDA receptor/ion channel complex. Activation of NMDA channels allows calcium to enter the cells. As the level of injury is increased the level of calcium current causes weakening of the cytoskeleton which allows for organelle shifting during subsequent impacts. In some cells the nucleus may shift and break the plasma membrane resulting in a catastrophic influx of calcium. Actual enucleation may occur.
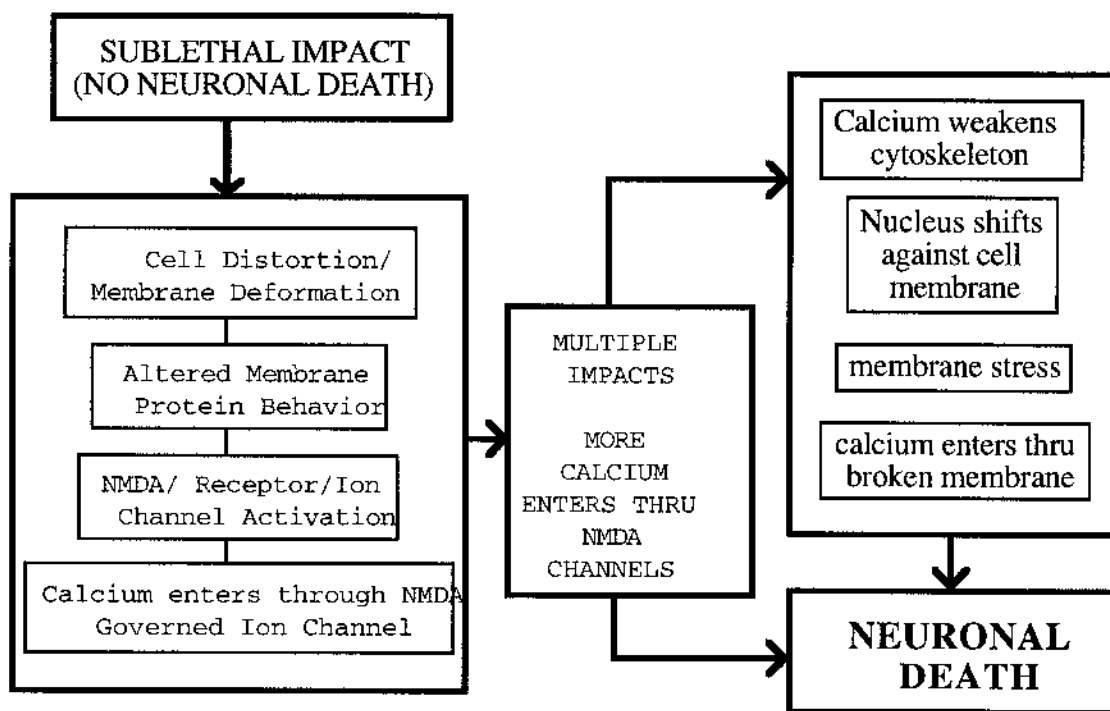

Fig. 15. Diagram of possible sequence of events leading to cell death after RAI injury. (From Lucas and Wolf, 1991.

In vivo studies of head injury performed by Jenkins and coworkers have also demonstrated a greater sensitivity of cells with a higher density of NMDA receptors. Hayes et al. (1992) also found that as a result of traumatic brain injury there was an increase in muscarinic and NMDA neurotransmitter activation. Hayes found that this phenomena could be prevented by the administration of the respective neurotransmitter antagonists within the first 30 minutes post-injury, the time he determine to be the "therapeutic" window. Thus, these *in vivo* studies also support the possibility of a dendrosomatic component of neuronal injury consequent to head injury.

An influx of calcium can also activate calcium endonucleases and damage the DNA of surviving cells. Some cultures used for these studies were fixed 15 min post-RAI for EM analysis of ultrastructural changes (separate study by Wolf, Lucas and Emery). The nuclear changes observed included what appeared to be aggregation of the chromatin. An early stage of apoptotic cell death includes the clumping of the chromatin and the dispersion of the clumps towards the nuclear membrane (Kerr et al. 1972). This phenomena can be induced by cytotoxic events such as the addition of tumor necrosis factor, gliotoxin, or hyperthermia (Laster et al., 1988; Waring et al., 1988; Takano et al., 1991) all of which induce DNA fragmentation. The DNA "ladders" produced by DNA fragmentation break the DNA up into subunits varying from 180-200kb in size. While the chromatic clumping seen may be evidence of DNA fragmentation such changes have been observed in apoptosis. Thus, additional studies must be done to determine if actual DNA fragmentation occurs as a result of RAI.

Hopefully, the development of this model of *in vitro* CNS trauma can aid in the ongoing search for new therapeutic interventions for decreasing the amount of cell damage or at least increasing the chance of neuronal survival immediately after head or spinal cord trauma (which seems to be the overall goal of CNS neurotrauma researchers). It can serve

as a basis for looking at injury on the cellular level to uniform insults without many of the problems inherent to *in vivo* studies. Tissue specificities, if any, can be determined depending on the purity of tissue cultures used. Also, while this model does not serve to (and cannot) substitute for other models of CNS injury, it helps provide a new method for characterizing cellular and nuclear events which may take place as a result of rapid acceleration impact injury.

Suggestions for Future RAI Studies

Neurons subjected to RAI may have increased membrane permeability which may allow for the influx of Ca^{2+}. This influx may activate Ca^{2+}-dependent endonucleases which act directly on the DNA. If DNA fragmentation can be induced by our model of injury it should be detectable by electrophoretic techniques. Electrophoretic gel experiments comparing a control (medium deprivation), experimental (10 tangential impacts), experimental (+ MNNG - no RAI) and a standard to determine whether these DNA "ladders" can be detected should be performed in the future.
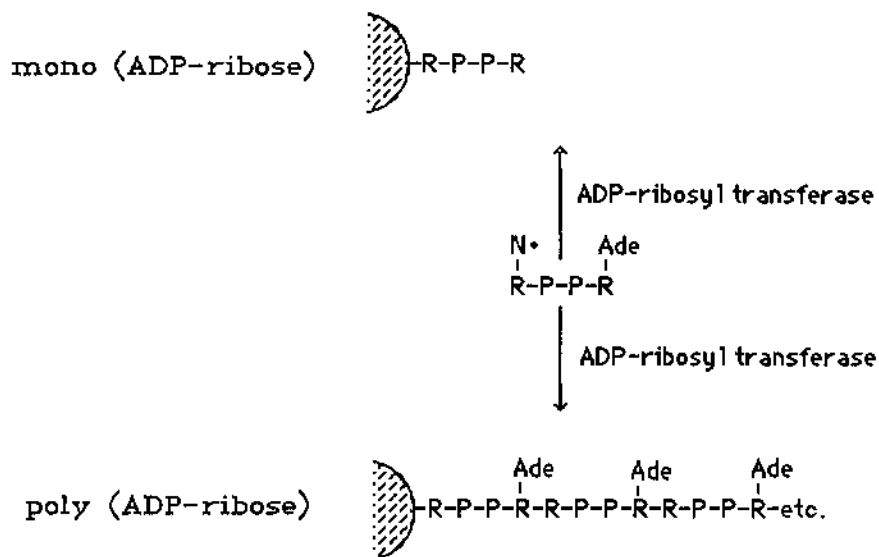
Fig. 16: Diagram of protein, NAD^+ and enzyme required for the formation of mono and poly(ADP-ribose).

Poly(ADP-ribose) exists in intact cells and was initially discovered and first reported by Paul Mandel's lab while using methods to prepare particulate fractions of nuclear extracts for what began as a study of RNA-polymerase (Chambon et al., 1963; 1966). In 1973, Colyer et al. reported successfully labelling intact synchronized L-cells with ^{3}H-adenosine for ADP-ribose studies without disrupting the cells. Because NAD^+ is too charged to traverse the cell membrane without permeabilizing the membrane, Colyer's

ability to label the NAD^+ cell pool with ^{3}H-adenosine allowed for the investigation of properties of poly(ADP-ribose) and the polymerase in intact cells. While much interest has been given to the post synthetic modification of protein via ADP-ribosylation as it relates to the regulation of the growth cycle (G2 phase), cellular differentiation and DNA repair, this study can investigate whether poly(ADP-ribosylation) occurs as a stress response in neuronal cells subjected to a defined mechanical injury.

Poly(ADP-ribose) has been found to be associated with the nuclei of cells while mono(ADP-ribose) is primarily located in the non-nuclear portion of the cell (i.e. cytoplasm). Following DNA strand breaks, approximately 95% of the total NAD^+ degradation is nuclear and its metabolism is due to the synthesis and degradation of ADP-ribose bound to protein (Shall et al., 1977). ADP-ribose [2'-(1"-ribosyl)adenosine-5',5'-bis(phosphate)] (MW 112,000-135,000) is synthesized from NAD^+ by an enzyme found in the chromatin of eukaryotic cells called ADP-ribosyl polymerase which is necessary for DNA repair (Zahradka and Ebisuzaki, 1982). The poly(ADP-ribose) polymerase and the product, poly(ADP-ribose), have been shown to be involved in cellular differentiation, cell growth and transformation, as well as in DNA repair as a cellular response to DNA damage due to hyperthermia, carcinogens, and other forms of DNA strand breaks.

There are various influencing factors that increase the activity of poly(ADP-ribose) polymerase such as DNA strand breaks, Mg^{2+}, polyamines, and histones. DNA fragmentation is the most significant stimulator that increases the enzyme's activity. Mg^{2+} and histones appear to activate the enzyme through DNA-enzyme interactions. Histones, specifically H1 in the initiation of poly(ADPR) chains, serve as acceptors of poly(ADP-ribose) and also stimulate the polymerase reaction while the absence of histones (as well as the absence of DNA) can inhibit the reaction (Zahradka and Ebisuzaki, 1982).

If the DNA fragmentation induced by RAI is enough to cause poly(ADP) ribosylation in CNS cultures, then the techniques described by Aboul-Ela et al. should be able to detect any poly(ADP)ribose residues (see appendix). The fact that we are able to successfully label NAD in our cultures should allow for the determination of poly(ADP) ribose production. The MNNG toxicity studies show that we are able to cause cell death in our cultures as well , thus enabling one to use an MNNG control culture to compare to an RAI injured culture to measure poly(ADP)ribose levels. Future studies should utilize the preliminary research presented here to determine whether a RAI induced injury can cause enough DNA damage to elicit poly(ADP)ribose production in spinal cord and cortical cell cultures.

APPENDIX

Determination of Poly(ADP Ribose)

Techniques developed by Aboul-Ela and coworkers (1989), to determine ADP-ribose polymers using a Dihydroxyl-Boronyl BioRex (DHB-B) column results in the elution of poly (ADP-ribose). These techniques were used to quantify the amount of poly (ADP-ribose) produced by CNS cultures as a result of multiple impact RAI as compared to a control (no RAI) cultures. These results can be compared to poly(ADP-ribose) production in cultures exposed to the potent mutagen and alkylating agent MNNG (1-Methyl-3-nitro-1-nitrosoguanidine).

Between 3-4 weeks of age the spinal cord cultures were incubated at $37^{o}C/10\%$ CO_2 overnight in a mixture of 2/3 conditioned medium and 1/3 fresh medium containing $20\mu Ci$ ^{3}H-Ade/ml. The eight cultures were divided evenly into an experimental group and a control group. The medium was removed and the flasks were experimentally manipulated: subjected to (a) multiple tangential RAI or (b) simply a medium manipulation (control). After RAI or medium manipulation the "hot" medium was replaced for 30 min at $37^{o}C$. The flasks were then washed 2X with cold PBS and then the reaction was stopped by the addition of 20% TCA. The resulting precipitate was scraped off of the bottom with a rubber policeman and all of the material was collected in a 15.0 ml centrifuge tube. The material from the experimental group of flasks was pooled as was the material from the control group. The resulting material was centrifuged at 1500 rpm for 5 min and the acid-insoluble pellet was dissolved in 0.5M KOH/50mM EDTA (2ml) and allowed to incubate at $37^{o}C$ overnight. For a final volume of 10.0 ml, 1M GAE pH 9.0 ±0.2 (1M Guanidine Hydrochloride, 0.25M Ammonium Acetate and 10mM EDTA) was added to the dissolved pellet. A 0.5 ml aliquot was counted and the rest of the sample was delivered to the DHB-B column, which was prewashed with H_2O and 1M GAE. The column was then washed

with 25.0 ml (10.0 ml, 10.0 ml & 5.0 ml) 1M GAE pH 9.0 ± 0.2 and the flow through was counted. The column was then washed twice with 10.0 ml 1M Ammonium Bicarbonate/10mM EDTA pH 9.0. The final product (poly(ADP-ribose) was eluted with H_2O (1.0 ml, 1.0ml, & 3.0 ml aliquots), pooled together and 1.0 ml was counted using a scintillation counter.

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