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The LSU Neuroscience Center is a comprehensive, multidisciplinary, and trans-departmental entity that unites fundamental neurobiology and the clinical neurosciences in the common goal of elucidating the workings of the brain and contributing to the treatment of currently incurable diseases of the nervous system. The objective of this program is to find solutions to neuroscience-related problems of interest to the US Army Medical Research and Development Command. The program is focused on exploiting novel neuroprotective strategies that lead to prevention of and repair after neural injury. Converging approaches using state-of-the-art tools of cell biology, neurochemistry, neuroimmunology, neurophysiology, neurop[harmacology, molecular biology and virology are ongoing. Over the four years covered in this proposal, this program aims to: 1) carry out seven research projects in the basic and clinical neurosciences; 2) expand central, shared facilities with the addition of highly specialized instrumentation not currently available to our scientists; 3) develop laboratory space to permit the physical consolidation and coordination of this research effort; and 4) institute a coordination unit to monitor, facilitate, and administrate the cooperative research programs, as well as to meet the associated budgetary, human resources, facilities, and communications needs for the attainment of the program goals.

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Participating Scientists: John England, M.D.; Leo Happel, Ph.D.; Daniel Kim, M.D.,; Cheryl Weill, Ph.D.

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John Hurst, Ph.D.
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Annual Technical Report 1995

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Project Director

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Neuroscience Center of Excellence

Volume 1 of 8

Neuroscience Core Research Facilities

Project Directors:
R. Ranney Mize, Ph.D.
Nicolas G. Bazan, M.D., Ph.D.

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PROGRESS REPORT SUMMARY

1. Specific Aims

Chapter 3 of the DoD Agreement proposed to establish a **Neuroscience Core Research Facility** consisting of three confocal microscopes. One is a laser scanning confocal microscope (LSCM, Noran Odyssey system) to be located at the Medical Education Building designed for calcium imaging, together with assorted equipment for electrophysiological studies. The second is a more general confocal microscope (Noran Odyssey system) to be located in the Eye Center within the cell biology section. This equipment configured for a conventional microscope, which utilizes only visible light laser radiation, will be used for real-time analysis of intracellular calcium changes and high resolution fluorescence microscopy and autoradiographic analysis. A third confocal microscope, also located at the Eye Center in the electron microscope core facility, is designed for viewing cells in the living eye and uses conventional white light (no laser). In addition to the confocal microscopes, the Core research facility will have the following instruments located at the Eye Center in the cell biology laboratory: a conventional compound microscope (Nikon Optiphot-2), an inverted microscope (Nikon Diaphot-200), a dissecting microscope (Nikon SMZ-U), and a carbon evaporator. The facility would be available to serve the needs of Neuroscientists throughout the LSUMC campus and provide support for several of the DoD funded projects.

2. Progress during Year 2

Core Research Facility equipment at the Medical Education Building

The Calcium Imaging Facility (CIF) has been operational for the past 10 months under the management of Dr. John Cork. Much of the early part of the year was spent installing the LSCM. Room 6156 in the Anatomy Department was remodeled to house the CIF and the majority of the equipment was set up during September 1994. By April 1995 most of the various pieces of ancillary equipment and the electrophysiology rig had been purchased and set up. At present the Facility consists of a Coherent multiline laser

and Noran Odyssey confocal laser scanning unit attached to a Nikon Diaphot inverted microscope. Image acquisition and analysis is controlled with Noran's InterVision software running on a Silicon Graphics Indy workstation. Other equipment available includes a temperature controlled cover-slip chamber, hydraulic manipulator and picospritzer. The electrophysiology equipment consists of an AxoClamp 2A amplifier, oscilloscope, and a 486 PC running Axon Instruments Clamp software.

Early testing of the calcium imaging equipment was done in conjunction with some exploratory experiments for Dr. Mize, on making calcium measurements in brain slices. There are currently several different types of experiments underway in the CIF. Approximately 20% of the time is spent in maintaining the Facility's hardware and software, and developing methods for utilizing the equipment. Priority usage of the Facility is provided to support DoD-funded researchers, of which there are currently two using the equipment. The remaining time on the machine is available for other Neuroscientists and Anatomy Faculty. Details of the progress on the specific projects in these categories are detailed below.

Core Research Facility equipment at the Eye Center

The imaging and cell biology facilities at the Lions building have been operational for the past 11 months under the direction of Dr. Mark A. DeCoster and Dr. William C. Gordon, respectively. The laboratories are located in rooms 4B17 and 4B18 of the Lions Building. Approximately 33% of the imaging room is equipped with electrophysiology instrumentation. In addition to the originally purchased Noran Instruments laser scanning confocal microscope (LSCM) system, the imaging facility has quickly advanced in obtaining hardware and software that strengthen the capabilities of the laboratory. Below is a summary of the significant upgrades to the facility:

--A second **Silicon Graphics Indy workstation** has been added with Noran Instruments analysis software to provide another analysis workstation for Neuroscience Center scientists.

--The **Noran LSCM** now has 3 photomultiplier tubes (PMTs) and a transmitted light detector. The 3 PMTs will allow simultaneous measurement of multiple fluorescent

probes, or measurement of one probe and the ratio of two other probes, expanding the possible ionic events that can be measured. The transmitted light detector will allow fluorescence and visible light overlays of neuronal preparations, valuable when cellular and tissue morphology cannot be fully distinguished by fluorescence alone.

--Both the Noran and the second Indy workstation have been upgraded with additional random access memory for more efficient data analysis. An external 2 Gigabyte hard disk and a 24-bit video card upgrade have been added to the second Indy, and a 1.2 Gigabyte rewritable optical disk drive has been added to the Noran Indy.

--Both Indy workstations can be linked to a **color dye sublimation printer** and slide maker located in the imaging facility lab at the Lions Eye building.

Maintenance and Instrument Development.

The focus of Dr. Cork's research interests is digital video microscopy and techniques for making intracellular calcium measurements. In conjunction with setting up, maintaining, and developing applications for the equipment, he has undertaken the following projects.

a) Preliminary trials of the CLSM and InterVision software revealed several problems with the Noran system. Some of these were simple bugs in the programs but others were more serious omissions due to a lack of understanding on the part of the Noran programmers. Ongoing discussions with the software engineers at Noran have resolved many of these problems and some have been addressed in new versions of the InterVision software.

b) A major problem with the software is its inability to keep track of the photomultiplier gains and to adjust the ratio values derived from the images according to the gain settings. Dr. Cork has calibrated the PMT gain controls and written a small c-shell script to calculate gain adjustments. It will be necessary to use this program to obtain correct calcium concentrations and to make any quantitative fluorescence measurements. One of the future goals of this project is to further develop this idea and, with the cooperation of Noran, incorporate these calculations into the InterVision software.

A portion of Dr. DeCoster's time is spent working with optical, imaging, and computer/data storage problems. Noran Instruments has been forthcoming with software and hardware upgrades, but problems still remain. One upgrade scheduled for late this fall will add memory capabilities for data collection, and will allow for time lapse experiments to be set up. These will alleviate some current problems and aggravations. Another problem that Noran is still working on is certain program parameters that appear to take up large amounts of memory to function. These parameters can stall, or crash the system under certain circumstances. However, the technical staff from Noran is easy to reach by phone and has been helpful in working on these difficulties.

Dr. Gordon has devoted some time to install a software to control a video capture computer board. The image generated with the **Nikon Optiphot-2** upright microscope and viewed in video form in the attached Sonny Triniton digital color monitor, can now be transfered to the computer for subsequent analysis.

DoD-Funded Research.

Current research projects at the Medical Education Building

Currently, Drs. Mize and Bobbin are the principal users of the Facility at the Medical Education Building. The following research projects are either completed or in progress.

a) Distribution and Function of Calcium Binding Proteins in the Rat Superior Colliculus. (R.J. Cork, F-S. Lo and R.R. Mize)

The distributions of the calcium binding proteins, Calbindin (CB) and Parvalbumin (PV), in the neurons of the rat superior colliculus have been mapped using fluorescently labeled antibodies. This project makes extensive use of the mutilabel protocols in the InterVision system and customized look-up tables have been developed to display double-labeled images in pseudocolor. Results to date show that these calcium binding proteins are expressed in complimentary cell tiers. They are largely separate cell groups, although both CB and PV cells vary in size and morphology. Exploratory electrophysiology experiments, done by Dr. Lo, have characterized some of the membrane properties of cells in the optic layer, some of which we know contain CB. These include low threshold

calcium spikes, an inward h-current at hyperpolarizing potentials and a unique high frequency burst firing mode. Methods are being developed to use the CLSM to localize CaBPs in the cells that have been recorded from. Two abstracts detailing the results of these experiments are in press.

Combining the electrophysiological experiments with calcium measurements has proved to be more problematic. Initial trials using ester loading or indicator injections in brain slices, showed that the preparation requirements for the electrophysiology experiments were largely incompatible with the requirements for imaging. Efforts are currently underway to develop a dissociated cell preparation for calcium imaging. If this is successful then a goal of the CIF will be to combine calcium measurements with studies of the membrane properties of acutely dissociated superior colliculus neurons.

b) NMDA Receptor Localization and Cytoplasmic Calcium Measurements in NOS-containing Cells of the Rat Superior Colliculus. (C. Scheiner, R.J. Cork, C. Leblanc and R.R. Mize) There are several discrete populations of NOS- containing cells in the rat superior colliculus. We plan to test the hypothesis that NOS is activated by calcium influx through NMDA receptor channels, by making calcium measurements in dissociated cells from the superior colliculus that have been manipulated pharmacologically with NMDA agonists and antagonists. Preliminary experiments for this project are underway.

c) Distribution of ATP Receptors in Isolated Cochlear Hair Cells from Guinea Pig and Rat. (C. Leblanc, P. Barnes, R.P. Bobbin and R.J. Cork)

ATP receptors were labeled using the fluorescent probe TNP-ATP. Fluorescence and bright-field images of the labeled cells were collected using the video capture functions of the InterVision software. The distributions of label were then analyzed using the 2D Analysis package. The results are still being analyzed but it appears that rat hair cells have very few, if any ATP receptors while guinea pig cells have receptors concentrated at the apical end. These studies have confirmed studies obtained from patch-clamp experiments done in Dr. Bobbin's lab, and a publication detailing the results is in preparation.

d) Calcium Transients in Response to Extracellular ATP in Isolated Cochlear Hair Cells. (C. Chen, R.J. Cork, and R.P. Bobbin)

To extend their patch-clamp studies Drs. Chen and Bobbin wish to record calcium transients in the cells of their in vitro cochlear preparation. Preliminary experiments have shown that these cells can be loaded with indo-1-AM and transient elevations of cytoplasmic calcium have been recorded in response to extracellularly applied ATP. These measurements will be extended to different cochlear cell types to determine if ATP functions as an extracellular signaling molecule. It is then hoped to combine patch-clamp experiments with simultaneous calcium measurements.

Current research projects at the Eye Center

a) Role of lipid mediators in neuronal signalling processes. (M. A. DeCoster and N. G. Bazan).

Platelet activating factor (PAF) has been tested in a complex set of experiments to address the immediate, long term, independent, and modulatory roles of this potent lipid mediator. The calcium imaging aspects of the LSCM at the Lions eye building have been used to monitor calcium dynamics in primary neuronal cultures treated with PAF. Application of PAF was found to have some immediate effects on neuronal calcium, and potentiated responses to glutamate stimulation. Long-term treatment with PAF, however, desensitized neurons to subsequent glutamate stimulation.

b) Phospholipases A_2 as factors in neuronal toxicity. (M. A. DeCoster, M. Kolko and N. G. Bazan).

Phospholipases A_2 (PLA_2s) from bee and snake venoms have been tested for both toxicity and calcium modulatory effects on primary rat cortical neuronal cultures. While these PLA_2s are toxic, it was found that some actually decreased basal levels of calcium in neurons, in contrast to the toxic action of glutamate, which increased neuronal intracellular calcium.

c) (Drs. D. Zhang, P. Homayoun, W. C. Gordon, and N. G. Bazan). Pathophysiological events triggered during light-induced damage to the retina.

Using light- and electron microscopy the time-sequence of events triggered by light damage in retinal structure was followed. Following our *in vivo* (2 hours, 7000 lux) and *in vitro* (6 hours, 66 $\mu E/m^2$ sec) protocols for retinal light damage, sections were

prepared for light- and EM microscopy. All retinal dissections and trimming were done with the aid of a **Nikon SMZ-U** stereo-zoom dissecting microscope. Retinal samples were sectioned at 5 μm thickness and the number and location of apoptotic (pink) photoreceptor nuclei observed in the light microscope by the Terminal dUTP Nick End Labeling (TUNEL) technique Light microscope sections were viewed, analyzed, and photographed with a **Nikon Optiphot-2** upright microscope and a **Nikon UFX-DX** automatic camera system which allowed 35mm photography of the sections. Ultra thin sections for EM were viewed with a Zeiss C10 transmission electron microscope (Carl Zeiss, Jena, Germany), and photographed on 3¼" x 4" Kodak electron microscope film.

These studies have shown that both *in vivo* and *in vitro* light damage-induced photoreceptor death occurs as a results of light triggering the process of apoptosis. The *in vitro* model will be used in subsequent studies to further explore the involvement of PAF in this type of cellular death. Using this model, PAF antagonists (i. e. BN50730) will have direct access to photoreceptors and will allow to determine their ability to rescue photoreceptors from apoptotic death.

d) (A. Morrison, W. C. Gordon, and N. G. Bazan). The kindling model of epileptogenesis and synaptic plasticity.

During kindling, an animals model of epilepsy, a series of initially subconvulsive stimulations leads to progressive intensification of seizure activity that culminate in generalized motor activity. Using the rat model of rapid kindling, accomplished over a 21-day period, we have as a primary objective of this project to elucidate the role of the second messenger platelet activating factor (PAF) in epileptogenesis and morphological alterations in the hippocampus (i.e. neuronal loss, mossy fiber axon sprouting, and synaptic reorganization). This studies were done in control (CSF and DMSO) and experimental (BN50730) treated rats following the experimental protocol of kindling. Animals were killed 24 hours after the last stimulation, the hippocampus dissected with the aid of a **Nikon SMZ-U** stereo-zoom dissecting microscope and prepared for histological analysis (**Nikon Optiphot-2** microscope). Cresyl violet staining was used for general tissue surveys and nuclear counts. TIMM's stain was used to visualize nerve

terminals. In kindled rats cresyl violet staining showed cell loss in the hilar region of dentate gyrus and TIMM staining indicate mossy fibers sprouting in the CA3 region. Moreover, the PAF antagonist BN50730 appears to have a protective effect on cell loss.

Other Projects.

Other LSUMC faculty currently have ongoing projects in the CIF at the Medical Education Building and Eye Center

a) (Dr. Cindy Linn) Calcium channels in catfish retinal cells. Dr. Linn is combining electrophysiology techniques with fluorescent calcium indicator measurements. Several successful experiments have been done using both ratio imaging and the continuous "Ratio Over Time" capability of the InterVision system.

b) (Dr.Mark Alliegro and Dr. John Cork) Dr. Alliegro is collaborating with Dr. Cork on some of the projects undertaken to test the capabilities of the MEB Facility. He is providing Sea Urchin eggs for these experiments into the roles of calcium in regulation of axis formation, cytokinesis and development.

c) (Drs. M. A. DeCoster and N. G. Bazan). To test the capabilities of the confocal aspects of the LSCM at the Lions Eye building, confocal 3-dimensional reconstructions and animated movies have been made of neurons and astrocytes from primary cell cultures. These studies will help develop methods for scanning and reconstruction needed for future interested scientists at the Neuroscience Center who may require 3-dimensional information about cells and tissues.

d) (Drs. M. A. DeCoster, D. Linn, and N. G. Bazan). Fluorescent nuclear dyes have been injected into intact retinal tissue and prepared slices from these tissues visualized using the LSCM. These preliminary experiments were important for testing methods to be used to localize cellular events. Thus, ionic (calcium) events may now be localized within (or outside of) structures such as the nucleus.

e) (V. L. Marcheselli, W. C. Gordon, and N. G. Bazan). Seizures-induced neuronal cell loss in the hippocampus. Histological sections of hippocampus from control and Kainic acid-treated rats are also analyzed using the **Nikon Optiphot-2** microscope. After tissue

fixation the hippocampal CA1 cell were radially oriented prior to embedding and sectioning under the **Nikon SMZ-U** microscope. Following different conventional staining techniques (i.e. hematoxilin/eosin and toluidine blue stained sections) changes in neuronal morphology and nuclei numbers, especially in the CA1 area of the hippocampus, are being analyzed during seizures to determine the time course of neuronal damage as well as the neuroprotective effect of the PAF antagonist BN50730.

f) (W. C. Gordon and N. G. Bazan). Using the estradiol-testosterone-induced experimental prostate enlargement in the rat the effect of new drugs that can prevent and/or ameliorate histological changes that occur during the process are analyzed. To that end, 1μm plastic sections are stained with toluidine blue and analyzed using the **Nikon Optiphot-2** microscope.

3. Goals for Year 3.

It is projected that the level of usage will continue to grow dramatically in the coming year. The equipment at the MEB is able to support four or five projects running concurrently. Several Neuroscience and Anatomy faculty have expressed interest in starting projects in the Facility. It is hoped that as the demand grows new equipment can be added to the existing system. We are presently planning to purchase a color printer to provide users with hard-copy output of their results.

With the integration of all the software and hardware upgrades to the imaging facility at the Neuroscience center in the Lions Eye building, the coming year should progress well for current and new projects. The role of PLA_2s and PAF in neuronal calcium dynamics should reach the stage of developed manuscripts. More work will be required on the interplay of these lipid mediators and excitatory amino acids. In this regard, a major goal for year 3 will be to extend these studies to a perfusion system that will allow LSCM ion monitoring with simultaneous perfusion control of on/off signal. A second goal will be to integrate these capabilities with the electrophysiological technology located in the same laboratory.

Studies at the histological level will continue during the coming year, specially looking at neuroprotective effects of drugs after retinal light damage and traumatic brain injury. Also alterations in [^{3}H]DHA uptake and metabolism in retinal cells induced by light damage will be followed by light- and EM autoradiography.

4. Publications

Cork, R.J., Baber, S.Z., and Mize, R.R. (1995) Calbindin$_{D28k}$ and parvalbumin are expressed in complementary patterns in the rat superior colliculus. Soc. Neurosci. Abstr. 21 (in press).

Lo, F-S, Cork R.J., and Mize, R.R. (1995) A high-frequency burst firing mode in neurons of the rat superior colliculus. Soc. Neurosci. Abstr. 21 (in press).

Chen, C., Barnes, P., Leblanc, C., and Bobbin R.P. (1995) Lack of ATP response in rat outer hair cells. (In preparation)

DeCoster, M. A. and Bazan, N. G. (1995). Modulation of intracellular calcium dynamics in rat hippocampal neurons by platelet-activating factor. XV th Washington International Spring Symposium Abstracts, 82.

DeCoster, M. A., Bazan, H. E. P., and Bazan, N. G. (1995a). Platelet-activating factor induced intracellular calcium oscillations in rat hippocampal neurons. Neurosci. Abst. (in press).

Kolko, M., DeCoster, M. A., and Bazan, N. G. (1995). Neurotoxicity and modulation of calcium dynamics in rat cortical neurons by phospholipases. XV th Washington International Spring Symposium Abstracts, 106.

Kolko, M., DeCoster, M. A., Lambeau, G., Lazdunski, M., and Bazan, N. G. (1995) Effects of secretory phospholipases A_2 on viability of rat cortical neurons and calcium dynamics. Neurosci. Abst., (in press).

Zhang, D., Hardy, M. N., Pecci-Saavedra, J., Homayoun, P., Gordon, W. C., and Bazan, N. G. (1994) Light induced cell death in *in vitro* retinas of the rat. IOVS Abst. 36, S917.