THE SEED GRANT PROGRAM
For Cardiovascular-Related Research
Competitive Renewals
TTUHSC Center for Cardiovascular Disease and Stroke

DESCRIPTIVE TITLE:  Ischemic escape mechanisms and injury in rat hippocampus
INITIATOR  John C. Fowler, PhD  DEPARTMENT  Physiology

A. Progress report 09/01/2005 to 04/12/2006 (2 pages)

Brief summary.

Stroke in the brain is commonly caused by a clot in a cerebral artery. Downstream from the clot are two regions that are distinguished by blood flow: (1) the core with no blood flow, and (2) the surrounding penumbra with heterogeneous regions of varying levels of blood flow. The significance of distinguishing between core and penumbral conditions is that penumbral neurons are widely accepted as being salvageable while core neurons are thought to suffer a catastrophic death. Preclinical research must focus on experimental conditions modeling of penumbral conditions.

Much research directed toward a greater understanding of the contribution of electrophysiological responses to ischemic injury has focused on the role of a prominent ischemic depolarization (also termed anoxic depolarization (AD) or ischemic spreading depression). We argue that the ischemic depolarization is a terminal depolarization more applicable to the core. Therefore, there is need to identify conditions more relevant to penumbra.

Frequently, in electrophysiological recordings from the CA1 region of the rat hippocampus, the ischemic depolarization is preceded by an initial suppression of evoked synaptic transmission followed by an ischemic return of synaptic transmission. Under most conditions, the ischemic return of synaptic transmission appears to be cut short by an ischemic depolarization. Under these conditions, the ischemic return of synaptic transmission is considered to be an epiphenomenon of the ischemic depolarization.

We argue that the ischemic return of synaptic transmission is an ischemic escape phenomenon that is directly responsible for a significant portion of acute ischemic injury. The significance of the ischemic return is that it more accurately reflects the response of penumbral neurons and, as such, deserves more detailed examination for possible routes of benefic intervention. If we extrapolate to the whole animal response, our argument is that action potential-dependent synaptic activity that originates from healthy brain and enters ischemic brain (penumbra) progressively injures neurons.

Hypothesis. Our hypothesis is that ischemic escape phenomena contribute to acute ischemic neuronal injury in a way that differs from the depolarization-dependent mechanisms linked to glutamate/Ca\(^{2+}\) excitotoxicity. The ischemic return of evoked synaptic transmission is one of the two escape phenomena we proposed to examine.

Specific Aim (1). We will use simultaneous field and whole cell patch clamp electrophysiological recordings to characterize the two escape phenomena. We will examine experimental manipulations that are likely to selectively affect the two escape mechanisms.

Progress on Specific Aim (1). (The Figures cited below are linked to my Departmental web page http://www.ttuhsc.edu/SOM/physiology/Fowler/fowler.htm)

In experiments performed over the past few months, we have focused on one of the two escape phenomena: the ischemic return of evoked synaptic transmission. Our objectives have been to show that the ischemic return of synaptic transmission: (1) can be separated from the ischemic depolarization, and (2) imposes its own burden of neuronal injury.

We have used rat hippocampal slices to model neuronal responses to the energy deprivation. To mimic conditions of the core, we exposed slices to complete oxygen- and glucose-deprivation (OGD). To mimic conditions more representative of penumbra, we exposed slices to modified OGD containing varying amounts of glucose.

Using extracellular electrophysiological recordings from CA1 pyramidal cell region, we have shown that the ischemic return of evoked synaptic transmission can occur in the absence of the ischemic depolarization (Compare Figures 1(A)-1(C) of CV-Related Research Renewal - figures).

We have also shown that the ischemic return of evoked synaptic transmission is linked to neuronal injury as determined by impaired recovery of evoked synaptic transmission (See Figures 2 and 3).

The ischemic return of evoked synaptic transmission is a form of escape from the beneficial suppression of neuronal synaptic activation. In Figure 4, we show that synaptic activation during this escape from ischemic suppression contributes to neuronal injury.

The most widely accepted mechanism of acute ischemic neuronal injury is glutamate/Ca\(^{2+}\) excitotoxicity. In Figure 5, we show that the ischemic return is also acting through excitotoxicity despite the absence of the powerfully permissive environment provided by the ischemic depolarization. It is the absence of this permissive environment that raises questions as to how the ischemic return of evoked synaptic transmission triggers excitotoxicity.
**Difficulties, limitations and pitfalls.**

Our most notable failure has been in not obtaining whole cell patch clamp recordings to complement the extracellular recordings. We expected to collaborate with Dr. Tryba in our Department to obtain patch clamp recordings. Unfortunately, Dr. Tryba left TTUHSC after only a few collaborative efforts. Fortunately, he left behind a working patch clamp rig probably worth close to $150,000. Our alternative plan, going forward, is for my Research Associate, Gloria Martinez, and myself to learn to patch clamp. I have experience with other forms of voltage- and current-clamping techniques. And, we will have help available from Drs. Nathan and Escobar in the Physiology Department, as well as from my mentor at UNM, Dr. Partridge. Dr. Partridge and I have collaborated successfully in the past. All of these investigators have working familiarity with patch clamp technique.

**Specific Aim (2).**

Obtain fluorescent live/dead measurements (Molecular Probes) with confocal microscopy to complement the electrophysiological measures of neuronal injury and to further localize the area of injury.

**Progress on Specific Aim (2).**

This is a critical Aim, to show that the ischemic return is linked to structural injury. Originally, we proposed to use the LIVE/DEAD® Viability/Cytotoxicity Assay Kit from Molecular Probes. However, this method is a rather a non-specific assay of neuronal death providing little information about localization of injury between neuronal compartments. Instead, we have successfully begun using immunofluorescence labeling of the structural protein, microtubule-associated protein-2. The advantage of MAP-2 is that it is a protein known to be limited to neuronal dendrites in healthy preparations. The dendrites are one of the structures involved in supporting evoked synaptic activity. Another advantage of MAP-2 is that a loss in MAP-2, as determined from Western blot, is widely accepted as an indicator of irreversible neuronal injury. We have identified a very early MAP-2 response of redistribution from dendrite to soma (as opposed to loss of MAP-2). We have nice data correlating loss of function with redistribution of MAP2 fluorescence *(See Figure 6 of Progress Report-Figures).*

**Figure 6** extracted from the Table of Content, Figures showing a comparison of microtubule-associated protein-2 (MAP-2) distribution observed in control and following 20 min exposure to modified OGD (2 mM glucose/95% N₂). Measurement of fluorescent intensity was obtained along a line in the cell body layer (upper left panel). In control slices, immunoreactivity was localized predominantly to the dendrites of the pyramidal cells (n=3). Slices exhibiting IR+, AD- (n=3) showed increased fluorescence along the cell body layer when compared to control. Finally, slices with IR+, AD+ (n=4) showed an additional significant increase in fluorescence along the somata as seen by Buddle *et al.* Results were expressed as mean±S.E.M of arbitrary unit of fluorescence (a.u.f.). (*, **, *** significantly different, p<0.001).

These results strengthen the link between the ischemic return and neuronal injury in the absence of anoxic depolarization.
B. Research Plan (2 pages)

We are still following the Research Plan of the original proposal (see Table of Contents http://www.ttuhsc.edu/SOM/physiology/Fowler/fowler.htm).

I feel that demonstrating the skill to obtain whole cell patch clamp data is vital to preparing a competitive proposal for extramural funding (see link Table of Contents, Summary Statement for R21 submission and Extramural funding section below). Patch clamp data will allow us to identify specific membrane channel proteins that underlie the paradoxical ischemic return of neuronal excitability and subsequent neuronal injury. With whole cell patch clamp we will search for drugs that will modulate the ischemic return and then use functional recovery and MAP-2 redistribution to determine whether, for example, suppressing the ischemic return can reduce injury.

We believe that MAP-2 redistribution is one of the earliest expressions of penumbral-associated glutamate/Ca²⁺ excitotoxicity. We have already shown that excitotoxic antagonists can block loss of function following ischemic return (see Figure 5). This data strengthens our argument that the ischemic return induces injury along a continuum of neuronal damage with the greatest injury occurring with the terminal depolarization characteristic of the core.

Extramural funding.

I submitted an R21 proposal for meeting date 10/06/2005 entitled, “Pathological synaptic transmission in ischemic hippocampus.” The proposal was triaged. The Summary Statement is linked to the Table of Contents at my Departmental web page http://www.ttuhsc.edu/SOM/physiology/Fowler/fowler.htm.

Responses to the Summary Statement:

1) Make a compelling case for the causal relationship between the ischemic return and ischemic damage. In Figure 6 of the Renewal we show that the ischemic return, in the absence of the ischemic depolarization, results in a redistribution of MAP-2 fluorescence from dendrite to soma. To strengthen a causal relationship, we need to show whether modulation of the ischemic return affects the magnitude of MAP-2 redistribution.

2) Show that the ischemic return is separable from the ischemic or anoxic depolarization. We now have a strong case that these two phenomena are separable (especially see Figure 1c, showing the ischemic return in the complete absence of the ischemic depolarization.

3) Defend the relevance of this phenomenon in vivo. We frequently saw the ischemic return in our past in vivo recordings (See the link on my web page, Figure 1, Gervitz, L. M., D. Nalbant, S. C. Williams, and J. C. Fowler. Adenosine-mediated activation of Akt/protein kinase B in the rat hippocampus in vitro and in vivo. Neurosci. Lett. 328: 175-179, 2002.

4) Provide a more extensive description of the collaboration with Dr. Tryba and whole cell patch clamp recording. The focus of this competitive renewal is to gain expertise in whole patch clamp with Dr. Tryba’s setup. I strongly believe that with this data we will have a competitive proposal.

Plan A.

My intention is to apply to NINDS and/or NIA for funding. Based on past experience gained while serving on NINDS study sections, I believe our first priority is to provide preliminary data demonstrating familiarity whole cell patch clamp technique. With Dr. Tryba’s departure, my Chair, Dr. Nathan, has kindly given me unrestricted access to a complete patch clamp rig. Over the last few months we have made progress in satisfying the need to show that electrophysiological indices of neuronal injury are supported by confocal morphological measures. Gloria Martinez has addressed this requirement by obtaining very nice images using MAP-2 immunofluorescence from slices exposed to specific conditions of energy deprivation and electrophysiological endpoints. With respect to basic stroke research, stroke-related injury is an area of emphasis for NINDS.

With respect to NIA funding, we have preliminary data showing that slices from aged animals exhibit poorer recovery from ischemic insult. We are currently obtaining baseline data from a long-lived rat strain, Fischer F344, that is suitable for aging research. We believe that electrophysiological data coupled to immunohistochemistry will strengthen any proposal to NIA. One of our goals is to generate sufficient additional preliminary data to respond to an NIA program announcement for basic research on: Age-related changes in tissue function: underlying biological mechanisms, PA number: PA-03-147, with an expiration date of July 30, 2006.

Plan B.

It is quite clear that funding from NIH, in general, and NINDS, in particular, is becoming increasingly competitive. My Plan B is to more aggressively pursue other sources of funding:

1) Pursue an area of scientific study that is applicable to other Institutes that have a more favorable funding history (e.g., NIAAA or NEI).

2) The whole cell patch clamp is a critical technique that can be applied to a variety of cellular preparations in order to pursue collaborations. My mentor (Dr. Partridge) has pursued a successful strategy of learning patch clamp, pursuing collaborations, and, ultimately, putting together a successfully funded R01.

3) Finally, I need to more aggressively pursue funding sources other than that from the NIH Institutes. The majority of my funding in the past has been from NIH but I think it is becoming more difficult to depend on NIH for a consistent source of research funds.
### SOM SEED GRANT PROGRAM

**BUDGET**

(For Period 09/01/2006 through 08/31/2007)

<table>
<thead>
<tr>
<th>Amount Requested</th>
<th>Overall Budget</th>
<th>From SOM Seed Grant Program</th>
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#### Supplies*

(Include description, numbers and unit costs where appropriate)

- **Gas tanks**: 95% N₂/5% CO₂, 1 per mo @ $88 each, 12 mo = $1056
- 95% O₂/5% CO₂, 1 per mo @ $44 each, 12 mo = $528

**$1584**

- **Electrophysiology supplies**: $1000
- **General lab supplies**: $800

**$1800**

- **Immunohistochemistry supplies**: Primary Ab (monoclonal Anit-MAP2, Alexa Fluor (e.g., 594, 488)

**$1000 $500**

- **Pharmaceutical/Drugs**: $1500

**$1500**

- **Animals (purchase and operating costs)**: 4 animals/wk, 48 wks
- **Euthanasia (ketamine, isoflurane)**

**$2700**

**Total Supplies**: $9084

#### Other

(Travel and publication costs will NOT be supported)

**Total Other**: $0

#### Equipment

**Total Equipment**: 0

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*Personnel:

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<th>Title</th>
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<th>Amount Requested</th>
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<td>Principal Investigator</td>
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<td>Gloria Martinez</td>
<td>Research Associate</td>
<td>100%</td>
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Total Salaries and Fringe Benefits: $0

**GRAND TOTAL (supplies, other, equipment, salaries)**: $9084

*Cost of supplies extrapolated from annual costs in past years.*
Biographical sketch.

NAME  John C. Fowler, PhD

POSITION TITLE  Associate Professor, Physiology

EDUCATION  (Begin with baccalaureate and include postdoctoral training.)

<table>
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<th>INSTITUTION AND LOCATION</th>
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<tr>
<td>UNM, Albuquerque, NM</td>
<td>BS</td>
<td>1975</td>
<td>Biology/Chemistry</td>
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<tr>
<td>UNM SOM, Albuquerque, NM</td>
<td>PhD</td>
<td>1982</td>
<td>Medical Sciences</td>
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<tr>
<td>UMB, Baltimore, MD</td>
<td>Post-doc</td>
<td>1985</td>
<td>Neurophysiology</td>
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RESEARCH AND PROFESSIONAL EXPERIENCE:  Concluding with present position, list (in chronological order) previous employment and experience. List (in chronological order) the titles, all authors, and complete references to all publications during the past three (3) years and to representative earlier publications pertinent to this application. Place refereed publications and meeting abstracts in separate lists.  **DO NOT EXCEED TWO (2) PAGES.**

1985-1987  Research Associate  Biophysics/Neurobiology Group, Los Alamos National Laboratory, NM
1987-1988  Scientific collaborator  Biophysics/Neurobiology Group, LANL, NM
1988-1990  Staff Scientist  Physiology Group, LANL, NM
1990-1996  Assistant Professor  TTUHSC, Lubbock, TX
1996-present  Associate Professor  TTUHSC, Lubbock, TX

**Extracted refereed publications relevant to use of hippocampal slice and study of ischemia:**


**Fowler, J.C.** Changes in extracellular adenosine levels and population spike amplitude during graded hypoxia in the rat hippocampal slice. *Naunyn-Schmiedeberg's Archives of Pharmacology* 347:73-78, 1993.


**Recent relevant abstracts:**


