1. CATEGORY JUSTIFICATION
I am a first-year tenure-track Assistant Professor in the College of Pharmacy-Duluth, and thus, qualify for Category #1. I am asking for financial assistance to develop an independent research program and to fund critical experiments for a NIH grant proposal focused on over-coming drug resistance in epilepsy.

2. ABSTRACT
More than 20 million epileptics worldwide do not respond to antiepileptic drugs. This is in part due to overexpression of the drug efflux transporter, P-glycoprotein, in capillaries of the blood-brain barrier. However, the mechanism signaling P-glycoprotein upregulation is unknown. Lack of this knowledge is an important clinical problem, because it limits successful treatment of patients with drug-resistant epilepsy. The goal of this proposal is to unravel the mechanism signaling P-glycoprotein upregulation in epilepsy. Our preliminary data show that glutamate, a neurotransmitter released during seizures, upregulates P-glycoprotein in brain capillaries. This effect is blocked by the COX-2-specific inhibitor, celecoxib. Importantly, using an animal model of epilepsy, COX inhibition abolished P-glycoprotein upregulation in brain capillaries in vivo. Thus, we hypothesize that glutamate signals through COX-2 to upregulate P-glycoprotein at the blood-brain barrier during epileptic seizures. We anticipate that the outcome of this proposal will improve treatment of patients with drug-resistant epilepsy.

3. PRESENT STATUS OF KNOWLEDGE
Epilepsy is the most common neurological disorder, affecting more than 50 million people worldwide. Although most epileptics can be treated with antiepileptic drugs, up to 40% of patients do not respond to pharmacotherapy (1, 2). The consequences for epileptics resistant to treatment are severe. A high incidence of uncontrolled seizures elevates the risk of brain damage and increases mortality rates (3-5). Thus, patients with antiepileptic drug resistance experience a low quality of life (1, 2, 6).

Increasing evidence suggests that therapeutic failure in epilepsy is partly due to overexpression of drug efflux transporters in capillaries of the blood-brain barrier (7-9). The most prominent of these transporters is P-glycoprotein that protects the brain from neurotoxicants, but also limits therapeutic drugs from entering the brain. Thus, P-glycoprotein is a primary obstacle for treatment of brain disorders (10-12). In epilepsy, seizures upregulate P-glycoprotein expression at the blood-brain barrier and brain uptake of antiepileptic drugs can be significantly improved by inhibition of P-glycoprotein (13-17). These findings and its role at the blood-brain barrier suggest P-glycoprotein as a key contributor to drug resistance in epilepsy (18, 19). However, the mechanism by which seizures signal P-glycoprotein upregulation is unknown. Understanding this mechanism is of high clinical significance. It could identify therapeutic targets to prevent seizure-induced P-glycoprotein overexpression and holds the promise of improving pharmacotherapy for patients that suffer from drug-resistant epilepsy.

This proposal is concerned with mechanistic links that connect seizure activity to increased P-glycoprotein expression at the blood-brain barrier. The following observations from recent publications provide a basis for a testable hypothesis concerning the mechanism of seizure-induced P-glycoprotein upregulation. First, glutamate released during seizures accumulates in brain interstitial fluid (20, 21). Second, glutamate upregulates P-glycoprotein expression in brain endothelial cells (22). Third, following seizures, enhanced glutamatergic signaling increases levels of the proinflammatory enzyme, cyclooxygenase-2 (COX-2), in neurons (23). Finally, activation of COX-2 induces P-glycoprotein expression in tumor cells (24, 25).

In support of our experimental approach, we have generated preliminary data using a combined ex vivo/in vivo approach focused on glutamate and COX-2 signaling in rat brain capillaries.

Preliminary Data
In the following experiments we used freshly isolated, intact brain capillaries from male Sprague-Dawley rats, an established ex vivo model of the blood-brain barrier (26-30). We exposed brain
capillaries to glutamate for 15-30 min and measured P-glycoprotein expression and transport activity after 6 hours. This protocol was designed to mimic epileptic seizure conditions in vivo where glutamate reaches interstitial brain concentrations of 10-100 μM for a short period of time (20, 21). P-glycoprotein expression was measured by Western blotting of isolated brain capillary membranes. P-glycoprotein transport activity was measured by an assay we previously developed (26, 29, 31). This assay is based on the P-glycoprotein-mediated accumulation of the fluorescent, P-glycoprotein-specific substrate, NBD-Cyclosporine A (NBD-CSA), in the capillary lumen. Accumulation of NBD-CSA in capillary lumens is measured by confocal microscopy and live cell imaging. Capillary images are analyzed using digital imaging software. The assay measures transport specific to P-glycoprotein and thus, indicates transporter activity in the intact brain capillary (26, 29, 31).

Exposing isolated brain capillaries for 30 min to 50-100 μM glutamate increased P-glycoprotein expression and activity in a concentration-dependent manner (measurements made 5 ½ hours after exposure, Fig. 1). Time course experiments showed that 30 min glutamate exposure to 100 μM glutamate resulted in maximal stimulation of P-glycoprotein expression and activity (Fig. 2). From these observations we designed a standard protocol where we exposed brain capillaries to 100 μM glutamate for 30 min, removed the capillaries to glutamate-free medium, and then assayed for P-glycoprotein expression and activity 5 ½ hours later (6 hour experiment); controls were incubated in glutamate-free medium. In 3 separate experiments using this protocol, glutamate increased P-glycoprotein transport activity by 135 ± 37% and P-glycoprotein expression in Western blots by 101 ± 23%. These data indicate that glutamate upregulates P-glycoprotein at the blood-brain barrier.

A downstream target of glutamate signaling is the proinflammatory enzyme, cyclooxygenase-2 (COX-2), which converts arachidonic acid to prostaglandins (32, 33). Following seizures, brain levels of COX-2 and prostaglandins increase causing inflammation. Thus, COX-2 is a therapeutic target in conditions where glutamate causes neuroinflammation (34). Furthermore, COX-2 is involved in P-glycoprotein induction in tumor cells (24, 25). We detected COX-2 expression in brain capillaries and brain capillary membranes by Western blotting (Fig. 3). We also found that specific COX-2 inhibition with celecoxib abolished glutamate-mediated upregulation of P-glycoprotein (Fig. 4). These observations indicate that COX-2 plays a key role in glutamate signaling upregulation of P-glycoprotein.

We conducted one study to provide initial in vivo proof of principle to see if COX-2 inhibition could block P-glycoprotein upregulation in brain capillaries. Rats were pretreated with the anti-inflammatory COX-1/2 inhibitor, indomethacin, and then received pilocarpine i.p. injections to induce seizures. P-glycoprotein expression in brain capillaries was determined 2 days later by immunochemistry. Brain sections of animals with seizures showed increased P-glycoprotein staining in cerebral cortex compared to controls (Fig. 5). Analysis of staining showed that seizures significantly increased P-glycoprotein expression (Fig. 6). Importantly, indomethacin blocked seizure-induced P-glycoprotein upregulation to control levels in cerebral cortex (Fig. 6), hilus and the CA3 region (data not shown). These findings suggest that COX-2 plays a key role in seizure-induced P-glycoprotein upregulation in vivo and provides a unique target for therapy.

4. PLAN OF WORK
Understanding the seizure-induced mechanism that signals P-glycoprotein upregulation at the blood-brain barrier holds the promise to improve pharmacotherapy of drug-resistant epilepsy. Thus, the goal of this proposal is to elucidate the mechanism that signals P-glycoprotein upregulation at the blood-brain barrier in epilepsy. Our Central Hypothesis is that in epilepsy glutamate signals through COX-2 to upregulate P-glycoprotein at the blood-brain barrier. To provide a comprehensive assessment of our central hypothesis and to accomplish the goal of this proposal, we will pursue three specific aims:

Specific Aim 1: Demonstrate glutamate-induced upregulation of P-glycoprotein in vivo.
Our Preliminary Data suggest that glutamate upregulates P-glycoprotein in isolated brain capillaries ex vivo (Figs. 1 and 2). The Working Hypothesis for Specific Aim 1 is that glutamate initiates the mechanism signaling P-glycoprotein upregulation at the blood-brain barrier. To test this hypothesis, we will:

Determine the effect of glutamate microinjections on P-glycoprotein in vivo
We will microinject glutamate (5, 10, 20 nmol) into the hippocampus of rats (n=10 per group). Control animals will receive vehicle injections (n=10). 24 hours after
injection, rats will be decapitated; brains will be removed and frozen. Brains will be cut and sections will be stained for P-glycoprotein. P-glycoprotein staining of brain capillaries will be measured in the hilus and CA3 region of the hippocampus using confocal microscopy and digital image analysis. Data will be compared with data from the contralateral hippocampus (no injection) and from control rats (vehicle injection). We expect that glutamate will induce P-glycoprotein expression in the hilus and CA3 region. Together with our in vivo data, these in vivo findings will support that glutamate upregulates P-glycoprotein at the blood-brain barrier.

Specific Aim 2: Assess the role of COX-2 in glutamate-mediated P-glycoprotein upregulation.
Our Preliminary Data suggest that glutamate signals through COX-2 to upregulate P-glyco-protein in brain capillaries ex vivo (Figs. 3 and 4). The Working Hypothesis for Specific Aim 2 is that COX-2 is involved in glutamate-mediated upregulation of P-glycoprotein. To test this hypothesis, we will:

Determine the role of COX-2 in glutamate-mediated P-glycoprotein upregulation ex vivo
To assess the role COX-2 plays in glutamate-mediated P-glycoprotein upregulation, we will use brain capillaries isolated from COX-2 knockout mice. We will do these experiments in collaboration with Dr. David Miller, NIEHS/NIH, who will provide the mice. In a first study we will expose brain capillaries isolated from COX-2 knockout mice (n=20 per treatment) to glutamate with and without the COX-2 inhibitor, celecoxib. In a second study we will expose brain capillaries to arachidonic acid (COX-2 substrate) and prostaglandin E2 (COX-2 product). Parallel experiments will be conducted with isolated brain capillaries from wild-type mice (n=20 per treatment). We will measure P-glycoprotein expression by Western blotting of capillary membranes. Transporter activity will be determined by measuring P-glycoprotein-mediated accumulation of NBD-CSA in capillary lumens. In brain capillaries from wild-type mice we expect to see P-glycoprotein upregulation with glutamate and anticipate that this effect will be blocked by COX-2 inhibition with celecoxib. In brain capillaries isolated from COX-2 knockout mice, we expect to see no increase in P-glycoprotein levels with glutamate and thus, no effect with celecoxib. We expect that arachidonic acid and prostaglandin E2 upregulate P-glycoprotein in capillaries from wild-type mice, whereas in capillaries from COX-2 knockout mice we expect to see P-glycoprotein upregulation by prostaglandin E2, but not arachidonic acid. These results will define the role of COX-2 in glutamate-mediated P-glyco-protein upregulation.

Specific Aim 3: Establish the role of COX-2 in seizure-induced P-glycoprotein upregulation in vivo.
Our Preliminary Data suggest that COX-2 inhibition blocks seizure-induced P-glycoprotein upregulation in vivo (Fig. 6). The Working Hypothesis for Specific Aim 3 is that COX-2 plays a key role in seizure-induced P-glycoprotein upregulation at the blood-brain barrier in vivo. To test this hypothesis we will:

Evaluate COX-2 as therapeutic target in seizure-induced P-glycoprotein upregulation in vivo
We propose an in vivo experiment using an animal model of epilepsy. In this experiment, we will induce seizures in rats by i.p. injections of pilocarpine (10 mg/kg, every 30 min until onset of seizures, maximum of 10 injections, n=10); control rats receive vehicle injections (n=10). A third group of rats will receive the COX-2 inhibitor, celecoxib (10 mg/kg, oral gavage, n=10), one day before seizure induction with pilocarpine. Two days after seizures, rats will be decapitated; brains will be removed, frozen and cut. Brain sections will be stained for P-glycoprotein. P-glycoprotein staining will be analyzed in cerebral cortex, hilus and the CA3 region using confocal microscopy and digital image analysis. In animals with seizures we expect to see an upregulation of P-glycoprotein, which is blocked in animals that received celecoxib. This finding will emphasize the important role of COX-2 in seizure-induced P-glycoprotein upregulation in vivo and provide a target for the therapy of drug-resistant epilepsy.

Innovation
The proposed research is innovative. It is of high significance and due to its translational character has a direct clinical application. The experimental approach includes a novel ex vivo blood-brain barrier model (isolated brain capillaries), use of modern technology (confocal microscopy, live cell
imaging, digital image analysis), new pharmacological tools (specific inhibitors), and genetically modified animals (COX-2 knockout mice). We have developed and tested the tools necessary to undertake the proposed research and have the expertise and experience needed to obtain definitive outcomes.

**Expected Outcomes**
Accomplishing the specific aims outlined in this proposal will for the first time provide a mechanistic link between drug-resistant epilepsy and P-glycoprotein upregulation at the blood-brain barrier. Accomplishing these aims will identify a general mechanism causing drug resistance in epilepsy (Specific Aim #1), it will identify COX-2 as a key player in drug-resistant epilepsy (Specific Aims #2 and #3), and it will evaluate COX-2 as a new therapeutic target in overcoming drug-resistant epilepsy (Specific Aim #3). These results are expected to have an important positive impact on the pharmacotherapy of patients with drug-resistant epilepsy.

5. **BUDGET JUSTIFICATION**

**Personnel**
I am requesting partial salary support for one Graduate Student for 2 semesters, 25% time (CUFS codes 7005-10, 7102-10); the remaining 25% will be covered with my start-up money. Assistance from a Graduate Student will allow me to advance this project to a level where it will be externally fundable. The student will conduct the experiments outlined under Specific Aim #2 and assist me in all in vivo studies (Specific Aims #1 and #3). Specifically, the student’s duties will include brain capillary isolations, transport assays, brain capillary membrane isolations, Western blot analyses, assistance with the in vivo epilepsy animal model (induction of seizures, monitoring seizure occurrence), assistance with glutamate microinjections, immunochemistry of brain sections, and analyses of stained brain sections.

**Animals**
To obtain statistically robust data, the in vivo experiments proposed under Specific Aims #1 and #3 require a certain minimum number of rats. In addition, after pilocarpine injection about 30% of rats do not show continuous convulsive seizure activity and therefore, have to be excluded from the study. This also increases the starting number of rats required for these experiments. For the experiments proposed under Specific Aim #2 we will receive a large number of COX-2 knockout and corresponding wild-type mice from our collaborator, Dr. David Miller, NIEHS/NIH. After transportation, animals will first have to adapt to the new environment for at least one week before they can be used for experiments. This fact and the number of animals needed for our experiments explain the animal and housing cost (CUFS codes 7320-10, 7321-10).

**Supplies**
Conducting the proposed experiments requires general chemicals (buffers, BSA, Ficoll, etc), specific pharmacological agents (arachidonic acid, celecoxib, glutamate, pilocarpine, prostaglandin E2), and consumables (pipettes, pipette tips, tubes, flasks, meshes, filters, cell strainers, glass beads, culture dishes). We also need specific fluorescent compounds for confocal microscopy, supply materials and antibodies for Western blotting and immunochemistry (CUFS codes 7320-25, 7320-40).

**Equipment**
All Specific Aims require the use of confocal microscopy and image analysis (live cell imaging during transport assays, digital image analysis of capillaries and brain sections). The use of a confocal microscope and working station for image analysis will be charged by the hour. We expect to use this equipment for about 50 hours (CUFS code 8131-10).

6. **NEED JUSTIFICATION**
I have received a $247,000 start-up package, of which $7,000 were spent on computers and software since I started on this position in mid-August 2007. Thus, $240,000 are remaining in the account. As a new faculty member, I am currently in the process of setting up my laboratory and establishing an independent research program. I have no external funding yet and therefore, need the start-up money for the first 3 years until I have secured substantial external funding. I am currently writing on a large grant focused on my main project in the laboratory; this main project is different from the one in this proposal. I plan on spending the start-up money on my main project to generate crucial preliminary data for the above-mentioned grant. The start-up money will be spent as follows.

**Salaries**
A significant amount of the start-up money will be spent on a laboratory technician’s salary for 2 years (about $90,000 total). This technician will be working full-time on my main project. Start-up money will also be used to cover the remaining 25% of the Graduate Student salary requested in this
proposal (about $12,000). Total salary costs over two years will be about $100,000.

Insert #2 – Proposal Bjoern Bauer, PhD

Animals

My research is very animal intensive. For example, 10 rats are required to obtain enough brain capillaries for one ex vivo experiment (about $450 per experiment), or about 30 animals are required for one in vivo experiment (about $1350 per experiment). Crucial experiments for my main project also require the use of transgenic animals, which are expensive. Total animal costs will be about $60,000 over two years.

Equipment & Supplies

To fully setup my laboratory I will have to purchase several standard equipment items such as pipettors, glassware, tissue grinders and homogenizers, microcentrifuges, shakers, hotplate stirrers, electrophoresis chambers, power supplies, microscopes, surgical tools, and a specific rotor for an ultra-centrifuge (total about $30,000). The supply costs for consumables, chemicals, and pharmaceutical agents will be about $12,000 per year (about $35,000 for 3 years). Total costs for equipment and supplies will be about $65,000 over 3 years.

Miscellaneous

After subtracting from the start-up money the costs listed above for salaries, animals, equipment and supplies, about $15,000 remain. This money will be used for lab services, travel, and publication fees. It will also be used as a security cushion for maintenance and repair services as well as other unforeseeable expenses.

For the reasons outlined in the above spending plan, my start-up money cannot be used to finance the proposed project "Overcoming P-glycoprotein-Mediated Drug Resistance in Epilepsy". This project has high potential for external funding, because it has clinical significance, is translational and has a direct application in the clinic. It holds the promise to have an important, positive impact on the pharmacotherapy of patients suffering from drug-resistant epilepsy. However, more data are required for a strong and competitive proposal for an external funding agency. A Grant-in-Aid Award will help me to advance this recently-initiated research project to generate crucial data for a NIH grant proposal.

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REFERENCES

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