I. Cover Sheet

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Title of Proposal:

A Cellular Analysis of the Effects of Methamphetamine on the Neuroimmune System

End Dates of Proposed Activities:

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II. Narrative

Methamphetamine (METH) is one of the most commonly abused drugs in the US. METH is a psychostimulant, which acts as a powerful indirect agonist of dopamine in the nervous system. The resulting “dopamine dumping” produces overactivation and oxidative damage at dopamine terminals, both of which are upstream events leading to apoptosis (i.e., programmed cell death) and help to explain METH’s neurotoxic profile.

Microglia are phagocytic cells found in the CNS and are descendant from the same myeloid lineage as macrophages found in the immune system. They serve as both a line of defense against foreign agents, which cross the blood brain barrier, as well as a clean-up system for cellular debris. An additional function of microglia is one of cellular regulation. This is mainly done through the regulated release of protein messengers (cytokines and chemokines), which either inhibit or stimulate the recruitment and activation states of other support cells in the cellular milieu (e.g., other microglia and astrocytes). Microglia hyperactivation is a hallmark of several neurodegenerative diseases and disorders (e.g, Alzehiemer’s disease, Parkinson’s disease, Amyothrophic Lateral Sclerosis, Multple Sclerosis, NeuroAIDS, etc.). Microglia hypoactivation is of similar concern given the protective, immune functions they serve and may make the CNS

vulnerable to opportunistic infection and exacerbated secondary damage after primary focal insults. It is of no surprise that microglia activation states and cytokine/chemokine profiles are altered by neurotoxic doses of METH in the striatum (a region in the brain which receives a dense dopamine innervation from the midbrain substantia nigra). Is the effect of METH on microglia the result of indirect dopaminergic toxicity or the result of direct actions of METH on the microglia? If so, what are the mechanisms by which this is occurring? Finally, are microglia in other dopaminergic and non-dopaminergic regions of the brain also affected?

1. **Analysis of the effect of METH on Microglia Using *In Vitro* Techniques.**

To create a cell line of a non-cancerous cell, one must isolate the cell type of interest (e.g., microglia) and use molecular techniques to alter the native cell cycle. This now immortal cell line has the advantage of self-renewal, while also maintaining many of the characteristics of the primary cell from which it was developed (e.g., phagocytosis, migration, and cytokine and chemokine release). BV-2 is a murine microglia cell line useful for studying the direct effects of METH on microglia in the absence of other cell types. Our lab, under CURCA funding, has previously demonstrated a direct, dose-dependent, inhibitory effect of METH on BV-2 phagocytosis as well as an alteration in the microglia cytokine/chemokine profile. Importantly, these effects were the result of functional alterations and not an effect on cell viability. Based on these initial observations, several additional studies logically follow:

A. Are other characteristic microglia functions also altered by METH?

Microglia have multiple pseudopodia, which “feel” their extracellular environment. When stimulated they actively recruit other nearby microglia to also respond to the stimulus through chemical signals (i.e., chemokines). The motility of the microglia can be assessed using a migration assay, whereby “clumps” of BV-2 cells are counted and overall activated movement is measured in the presence or absence of METH and cellular debris. Under the right conditions, BV-2 exist as a uniform layer of cells. Cellular debris (i.e., homogenized mouse brain), mimicking METH induced neuronal damage, is added to some plates, which stimulates BV-2 cells and in turn stimulates active migration toward the debris. Will the addition of a drug found to be inhibitory to phagocytosis (i.e., METH) also demonstrate inhibition of a different microglia function (i.e., migration)? Inhibition of migration

would indicate that METH not only inhibits function (i.e., phagocytosis) but also recruitment of microglia in the presence of neuronal damage thereby potentially exacerbating the METH induced neurodegeneration.

B. What cellular mechanisms are affected by the METH?

Our data suggest that the effects of METH on microglia function are receptor mediated. Receptor-ligand interactions can initiate a cascade of intracellular signaling, which direct cellular function. Determining which signaling pathways are activated provides insights into the downstream effects of a drug as well as potential therapeutic targets. There are several key signaling inhibitors which we will systematically use to deduce the intracellular signaling pathways altered. For example H89 inhibits Protein Kinase A, which is an upstream activator of transcriptional regulation (e.g., CREB) and subsequent downstream functional activities. We will use the model system previous developed in the lab (i.e., the observed inhibition of BV-2 phagocytosis in the presence of METH) to measure BV-2 function. BV-2 phagocytosis is easily measured. If stimulated by cellular debris in the presence of small iron beads, the BV-2 will engulf both the debris and the beads. The iron beads are visible using a light microscope. The average number of beads per BV-2 cell provides an index of phagocytic activity. If a given inhibitor is applied to the culture system and the BV-2 cells fail to show reduced phagocytosis in the presence of METH, the signaling pathway must be important to this cellular function. Similar techniques can be applied until the signaling pathway/mechanism has been elucidated. Elucidation of the signaling pathway/mechanism will provide potential targets for inhibition of METH induced neurodegeneration exacerbated by microglia disfunction.

C. Does oxidative stress play a role in the microglia response to METH?

Perioxiredoxins (PRX) is a family of antioxidant enzymes, which reduce reactive oxygen and reactive nitrogen species. Oxidative stress triggered by high levels of ROS/RNS or low levels of antioxidants are associated with numerous neurodegenerative diseases. Further, PRXs are expressed in neurons and glia in different proportions in different regions of the brain. Their expression is particularly high in some dopaminergic nuclei, which are rescued from neurotoxic insults by PRX supplements. Does METH affect the level of PRX expression in BV-2 cells? We will use Real Time Reverse Transcriptase- Polymerase Chain Reaction (Real Time RT-PCR) to amplify the mRNA of PRX isoforms against a known, internal control to determine if METH affects the expression levels of these key antioxidants.

2. Analysis of the effect of METH on Microglia Using *In Vivo* Techniques.

D. Are there region specific expression changes in PRX isoforms after METH?

We have begun collaboration with Dr. Islam Khan at Wake Forest Baptist Medical Center to look at the levels of PRX proteins using Western Blotting in several areas of the brain. Given the connections between METH-induced dopamine dumping, dopamine's role as a dangerous free radical in high concentrations, and the protective nature of the PRX antioxidants, we will collect dopamine terminals in the brain from mice exposed to either chronic METH or saline. We will microdissect the ventromedial hypothalamus, striatum, and prefrontal cortex, which all receive dopamine innervation from elsewhere in the brain and are likely subjected to large quantities of extracellular dopamine in the presence of METH. We will use these samples to determine if the degree of PRX expression is related to the neurotoxicity or neuroprotection observed.

E. Are alterations in microglia functions also noted in *in vivo* models of METH exposure?

We will collect dopamine terminals in the brain from mice exposed to either chronic METH or saline. We will microdissect the ventromedial hypothalamus, striatum, and prefrontal cortex, which all receive dopamine fibers from elsewhere in the brain and are likely subjected to large quantities of extracellular dopamine in the presence of METH. The total RNA from this tissue will be extracted and the mRNA for several cytokines and chemokines will be analyzed using Real Time RT-PCR to determine if METH alters the expression of these proteins in a regionally-specific manner. Since cytokines and chemokines are directing important cellular events through both stimulatory and inhibitory mechanisms, alterations in either direction have potentially serious consequences. Therefore, the pattern of their expression throughout brain regions will be useful to more completely describe the effects of METH as well as provide valuable information that will guide future planned experiments.

Student involvement & Goals and Products: We have detailed our scientific goals and rationale above. Below we will detail our goals for student achievement, development and education.

Our lab uses a "see one, do one, teach one" approach to student development. Students new to the lab begin their involvement as helpers (see one) on their way to developing the skills they need (do one) which is facilitated by more seasoned students (teach one).

These student leaders practice high-level science while developing leadership skills in the lab through personnel and project management. The projects detailed above will involve 5 student leaders in the Fall semester, each responsible for their own project. Furthermore, other students have chosen to pursue scientific evaluation of novel labs they have developed and run in the classroom using the knowledge and skill sets developed while working on these studies. These educational research projects have been planned for both the fall and spring semesters and will offer invaluable teaching experience for our students interested in education while enhancing the overall academic experience at NGCSU. Additional student leaders will carry on these projects in the Spring. Student leaders must submit detailed project proposals before they begin their work that include an extensive literature review, detailed protocols related to their project and a timeline for completion. Student leaders manage all aspects of their projects and receive as much attention and help from PIs as necessary. All projects and proposals are developed in collaboration with the PIs, who are careful to ensure that each project will have a definitive and realistic product at the end of the semester. For example, the 5 projects detailed above will be presented at at least one regional conference (either the Southeastern Psychological Association (SEPA) or the Association for Southeastern Biologist (ASB)) and one local conference (the North Georgia Academic Research Conference (NARC)). Each student will be expected to and encouraged to apply for available internal and external travel awards and research grants. Our students have been successful in obtaining both internal and external funding in the past as they are able to draw heavily from the research proposals they have already developed.

Each of the student projects described above is a piece of a larger on-going project whose data will be submitted to a peer-reviewed neuroscience journal (e.g., Brain Research). And one project (the cell signaling inhibition project) will address reviewer criticisms for a journal article currently in revisions. Each of the 5 student leaders will work with newer lab students who are at various stages of development. Currently there are 3 students who are also involved in these projects. They will be authors on the presentations listed above and are working toward becoming a student leader in the lab.

The model of science and student development described above has been extremely successful in the past. Last year our students received 3 Psi Chi Regional Research Awards at the SEPA conference, 3 Best Presentation Awards at the Georgia Academy of Sciences conference, and a second place finish at the NARC poster session. In addition, two of our students were selected to present at

the CUR-sponsored Posters on the Hill event in Washington, DC. We expect our students to accumulate similar accolades this year in recognition of their hard work and scientific achievements. However, it is critical to note that these students would not have had the opportunities described above without the financial support of CURCA. I hope you see that the CUCRA money received went to good use.

III. Budget and Projected Timeline

In the past year, our lab has been extremely productive. We anticipate the completion and publication of several of the projects outlined above within the next year. However, as we empirically test some hypotheses, others arise to provide further avenues of research for our students. With research data supported by previous CURCA support, Drs. Shanks and Lloyd have been awarded one external grant and have submitted an additional NSF grant (pending review). We anticipate submitting several more applications this year using CURCA-supported preliminary data. Given our focus on the undergraduate research experience we are considering REU awards as well as foundation, NSF (TUES) and NIH (R15, AREA) basic science awards. The expansion of student participation and research capabilities necessitates that we obtain external funding support. However, continued research until that funding is obtained will serve to enhance our ability to do so. We have included the following budget to conduct this research over the next academic year.

Category	Description	Project	Price	Total
Mice	*Per diem for *in vivo* studies for 3 months (food, bedding, caging supplies, and animal facility maintenance fees)	D-E ~30 mice	3.82 per diem (3 months)	343.80
Cell Culture	**Per diem for *in vitro* studies for 9 months (media, flasks, slides, sterilization equipment, CO2, plates, pipette tips, gloves)	A-C	1.92 per diem (9 months)	518.40
Iron Beads	Phagocytosis assay beads	A-B	150.00	150.00
Chemicals	Cell signaling inhibitors (H89, Oakadic Acid, LY, Forskolin, IBMX)	B	~400.00	400.00
Western-blot Analysis	Antibodies, acrylamide gels, membrane	D	750.00	750.00
Real Time RT-PCR	PCR primers, tubes and PCR expression kit, tips, RNA isolation kit	C & E	297.00 (ea)	150.00
Equipment	Surgical equipment (scalpels, forceps, scissors, brain matrix)	D-E	~150.00	150.00
TOTAL				**3203.20**

*** Per diem costs** - inclusive of all costs associated with maintaining the animals including food, bedding, caging supplies, cleaning supplies, and other animal facility maintenance fees. Please note that this per diem is well under national averages.

**** Per diem costs** - inclusive of all costs associated with maintaining the BV-2 cell line including media, flasks, slides, sterilization equipment, CO2, plates, pipette tips, gloves. Please note that this per diem is well under national averages.

Timeline:

As described above, all student leaders submit their own, individual project proposal complete with a project timeline. We work closely with the students to ensure that their project and the product it produces are completed within a given semester. These student

projects are a part of a larger line of experimentation that will continue indefinately. Therefore, students work often work on their projects in subsequent semesters while training new students to carry on their line of questioning. For most projects the student begin by generating the necessary animals and/or practicing cell culture techniques. They then engage in their manipulation (drug exposures) followed by data collection and analysis and a formal write-up for presentation. Each step is carefully planned with the student leader to avoid other time conflicts (school, holiday, work, etc.) and to be completed within a given semester.

Additional Core Equipment/Supplies needed, but already obtained by Drs. Lloyd and Shanks:

1. Cell Culture Facility
2. BV-2 Cell Line
3. Real Time RT-PCR