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PRINCIPAL INVESTIGATOR: Stephen M. Lasley, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois at Chicago
Chicago, IL 60612-7227

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6. AUTHOR(S)

Stephen M. Lasley, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois at Chicago
Chicago, IL 60612-7227

E-Mail: SML@uic.edu

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13. ABSTRACT (Maximum 200 Words)

This project is designed to test the hypothesis that chronic exposure to depleted uranium (DU) impairs neuronal processes underlying cognitive function via alterations induced at hippocampal glutamatergic synapses. As prescribed by the Statement of Work, efforts in year 3 concerned achievement of substantial progress on Technical Objective 2 (defining integrity of hippocampal glutamate release) and initiating Technical Objective 3 (identifying changes in glutamate receptor subtypes). Acute exposure to U *in vitro* diminished K^+-stimulated glutamate and GABA release in a concentration-dependent fashion in hippocampal synaptosomes, but the inhibitory effect on evoked glutamate release was substantially more potent (IC_{50} = 2.77 μM) than that on GABA release (IC_{50} = 3.20 mM). Depolarization of superfused hippocampal synaptosomes with high K^+ in Ca^{+2}-free buffer evoked modest increases in glutamate and GABA release, but acute uranium exposure *in vitro* across a range of concentrations did not exhibit Ca^{+2}-mimetic properties on transmitter release. Studies are currently underway examining the effects of chronic exposure on transmitter release *in vivo* and investigating the actions of acute U on NMDA receptor function. Given the similarity of effects of U on transmitter release to those of other multivalent metals and the fact that exposure in military scenarios is continuing, additional studies are warranted on uranium actions in several experimental settings.

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Introduction

The chemical properties of depleted uranium (DU) render the metal well suited for military purposes. The U. S. Army utilizes DU for tank armor and for kinetic energy penetrators in munitions, and has deployed such weapons in the Gulf War, in Kosovo, and in Iraq. Use of the metal in future military arenas is a virtual certainty, but knowledge of its toxicity is lacking. Gulf War veterans who retained fragments of DU shrapnel over several years have exhibited lowered performance on neurocognitive tests (1). Moreover, research in chronically exposed rats has indicated alterations in hippocampal synaptic transmission, suggesting DU-induced decreases in neuronal excitability (2). *This research proposal will therefore test the overall hypothesis that chronic exposure to DU impairs neuronal processes underlying cognitive function via alterations induced at hippocampal glutamatergic synapses that directly modulate* Ca^{+2}*-mediated cellular processes.* Glutamatergic function will be assessed in rats exposed for 12 months via intramuscular implants of varying amounts of DU pellets in order to identify the bases for the impaired cognition and diminished neuronal excitability. Components of depolarization-evoked glutamate release will be measured in the presence of acute *in vitro* or after extended *in vivo* exposure to the metal (Technical Objective 2). Determination of the actions of uranium on glutamatergic NMDA and AMPA receptors will be performed via approaches employing analogous *in vitro* and *in vivo* exposures (Technical Objective 3). Other studies will determine the concentrations of DU produced in blood and brain tissue as a result of exposure (Technical Objective 1). These results will be of critical importance to U. S. armed forces in defining risk and establishing treatment modalities for DU exposures sustained in recent conflicts and in future battlefield situations.

Body

Project activities in year 3 addressed completion of Technical Objective 1 and substantial progress on Objective 2. Currently work is being initiated on Technical Objective 3. A description of these efforts and the progress toward completion of each Objective is provided below.

Technical Objective 1 concerned establishment of the chronic DU exposure protocol, and this work was completed in year 3 with resolution of the question of whether an effect of chronic exposure was observable on the somatic rate of growth. Rats were exposed to 0 (controls), 300, or 600 mg DU by implantation of 30-mg (2 mm × 1 mm diameter cylinders) pellets in the gastrocnemius muscles of their hindlimbs. Tantalum pellets of the same size were used in control animals and to balance the total metal mass implanted across groups with an inert metal. Body weights of all animals were recorded at regular intervals during exposure. Several criteria were adapted to guide the statistical analyses and insure the validity and integrity of the findings.

Since the capacity for somatic growth varies as a function of age and since age at the time of DU pellet implantation was not uniform across groups, a range for pre-implantation body weights was identified to insure similar potential for growth. Thus, only animals weighing at least 250 g at the time of implantation surgery but not more than 400 g were included in the analyses. This criterion resulted in the inclusion of 15-19 growth records for each exposure group. A linear mixed model was chosen for the analysis to permit data to exhibit correlated and non-constant

variability, and so that growth records utilizing unequal intervals or having missing values could be included in the analyses (3).

Somatic growth for each group as a function of exposure duration is summarized in Figure 1. The test of fixed effects within the linear mixed model uncovered a significant effect of exposure ($p = 0.007$), manifested as a diminished rate of growth in the both DU groups. Compared to the control tantalum pellet group, the 300 mg ($p = 0.002$) and 600 mg ($p = 0.033$) DU groups both exhibited diminished rates of growth. The impaired rate of growth observed in this work is remarkably similar to that reported by Pellmar *et al.* (4) who used a similar exposure regimen. The agreement in these findings indicates the reproducible nature of the effect of DU exposure on the somatic rate of growth.

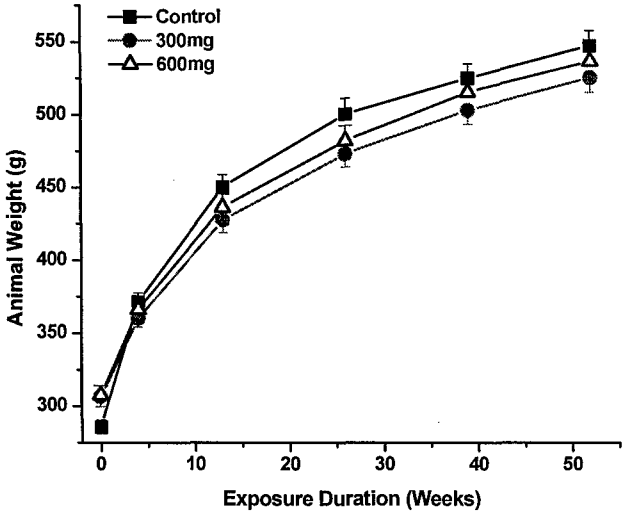

Figure 1. Increases in whole rat body weight as a function of amount and duration of exposure to implanted DU pellets. Control and low dose (300 mg) DU groups received tantalum pellet implants so that all animals received the same total mass of metal. Data were evaluated using a linear mixed model comparing growth rate across exposure groups using pre-implantation body weight as a starting point. This resulted in a significant effect of exposure ($p = 0.007$). Values are expressed as mean $\pm$ SEM with N = 15-19 for each exposed group and duration. An overall significant decrease in body weight was uncovered in both DU groups.

Technical Objective 2 concerned definition of the integrity of hippocampal glutamate release as a result of acute or chronic uranium exposure. The acute exposure studies, which utilize a superfusion procedure that permits measurement of endogenous glutamate and GABA release from hippocampal synaptosomes, are nearing completion. A range of uranium concentrations was applied to the synaptosomes via the superfusing buffer to permit estimation of the inhibitory/stimulatory potency on the transmitter release component under study. Hippocampi from eight 60-70 day old male Sprague Dawley rats were collected and homogenized in a glass vessel with 0.2-0.25mm total clearance (Kimble Kontes, Vineland, NJ) containing 10 mM HEPES-0.32M sucrose buffer (pH 7.4). The homogenate was centrifuged for 2 min at $3000 \times g_{max}$, and the supernatant removed and transferred to a clean tube. This supernatant was centrifuged again for 12 min at $14{,}600 \times g_{max}$, resulting in isolation of a synaptosomal pellet. The pellet was resuspended in HEPES-sucrose buffer and stored on ice for 30 minutes until protein concentration was determined using the bicinchoninic acid assay (5). Synaptosomes were then diluted to 1.0 mg protein/ml with an isotonic HEPES buffer (containing in mM: NaCl 132, KCl 1, $MgCl_2$ 1, $CaCl_2$ 0.1, glucose 10, HEPES 10, and 0.1% BSA; bubbled in 99.9% O_2, pH 7.40) and incubated for 30 min at 37°C.

The synaptosomes were centrifuged for 30 sec at 15,800×g_{max} and the supernatant removed prior to resuspension to 4.0 mg/ml in the above HEPES-buffer (without BSA, and containing a glutamate reuptake blocker - 0.5 mM DL-threo-β-hydroxyaspartic acid). Exposure solutions also contained uranium oxynitrate (Noah Technologies, San Antonio, TX) or uranium (VI) oxide (Ultra Scientific, North Kingstown, RI) at concentrations ranging from $10^{-3.5}$ to 10^{-8} M. 200µl of synaptosomes were then pipetted into each chamber (Model SF-12, Brandel, Gaithersburg, MD), and superfusion initiated with the same HEPES buffer at 0.6 ml/min. Flow was maintained for 30 min and then baseline samples were collected at 2 min intervals. During perfusate collection superfusion was switched for 2 min to the same HEPES buffer containing 31 mM KCl (Na^+ reduced to maintain isotonicity) and then returned to the normal buffer to re-establish the baseline.

After superfusion was complete, each sample was prepared for HPLC analysis by derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Tag, Waters Corp., Milford, MA). The derivatives were then analyzed using binary gradient liquid chromatography with fluorescence detection (excitation - 250 nm and emission - 395 nm). Eluent A consisted of Na acetate, pH 5.70 containing triethylamine, while eluent B was a 60:40 mixture of acetonitrile:water. Detector output was analyzed by EZChrom Elite software (Scientific Software, Pleasanton, CA).

While it is more common to measure synaptosomal glutamate/GABA release employing ^3H-amino acids that are loaded into synaptosomes and then released by high K^+ stimulation, it is generally accepted that this form of release may not be drawn from the same intracellular pools as release of the endogenous transmitter. However, endogenous release is more analytically difficult to detect because of the small concentrations involved. The superfusion-liquid chromatography system described above possesses sufficient sensitivity to measure these latter concentrations.

The presence of uranium (VI) oxide or uranium oxynitrate in the superfusion buffers diminished depolarization-evoked synaptosomal glutamate and GABA release across the range of metal concentrations tested. Figure 2 shows the time course of the effects of two concentrations of uranium on stimulated glutamate release compared to that from a non-exposed (i.e., control) preparation. The high K^+ stimulus elicited a maximal response that represented an approximate 3-fold increase in non-exposed synaptosomes, while smaller increases were seen in the presence of superfused uranium. If the areas under the stimulation response curves are computed and compared across uranium concentrations to the control area, a percent inhibition of the response can be calculated for each level of uranium. These transformations result in the inhibition curves for stimulated glutamate release shown in Figure 3.

An analogous effect on the time course of K^+-stimulated hippocampal GABA release is shown in Figure 4. Again, the maximal response is an approximate 3-fold increase in release with smaller responses observed in the presence of concentrations of uranium. An inhibition curve for the effect of uranium on stimulated GABA release is shown in Figure 5. The effect of acute exposure to the metal is strikingly more potent with respect to glutamate release (IC_{50} = 2.77 µM) than it is for GABA release (IC_{50} = 3.20 mM).

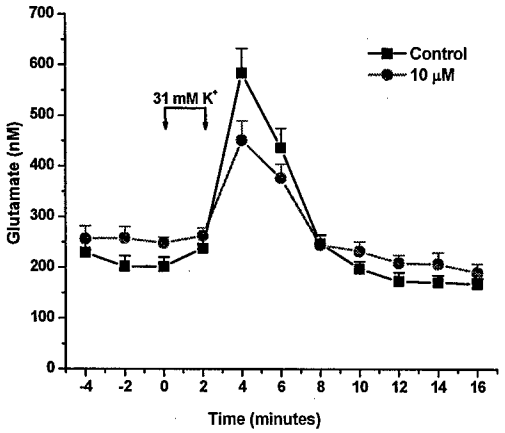

Figure 2. Time course of glutamate concentration in response to superfusion with 31 mM K^+ across hippocampal synaptosomes in standard 10 mM HEPES-sucrose buffer (pH 7.4) or in buffer containing 10 µM uranium. The stimulation-evoked increase in endogenous glutamate was diminished by the presence of uranium. Values are expressed as mean ± SEM of independent determinations – N = 10 for control conditions, and 4-5 for each uranium concentration) – conducted in triplicate.

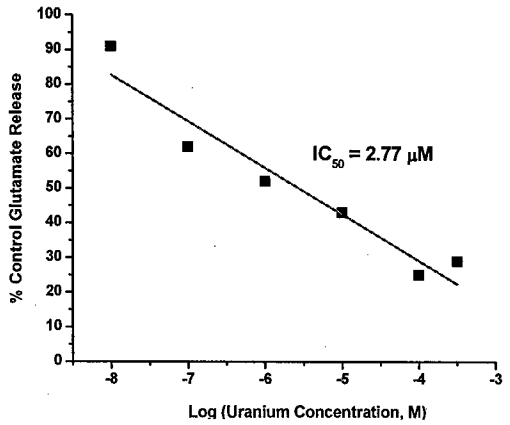

Figure 3. Percentage inhibition of stimulated glutamate release from hippocampal synaptosomes as a function of the concentration of uranium in the superfusing solution. Maximal inhibition is limited by the solubility of uranium in aqueous media at pH 7.4. The data were fitted by linear regression of the types of response inhibition shown in Figure 2.

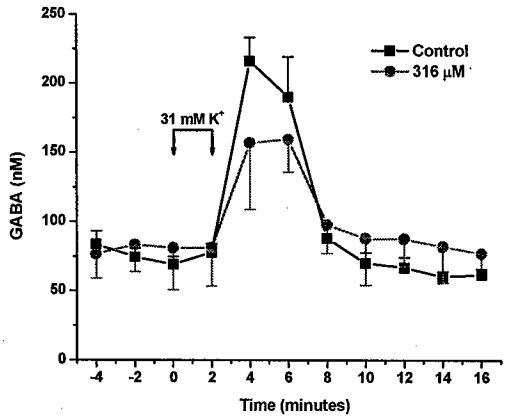

Figure 4. Time course of GABA concentration in response to superfusion with 31 mM K^+ across hippocampal synaptosomes in standard 10 mM HEPES-sucrose buffer (pH 7.4) or in buffer containing 316 µM uranium. The stimulation-evoked increase in endogenous GABA was diminished by the presence of uranium. Values are expressed as mean ± SEM of independent determinations – N = 10 for control conditions, and 4-5 for each uranium concentration – conducted in triplicate.

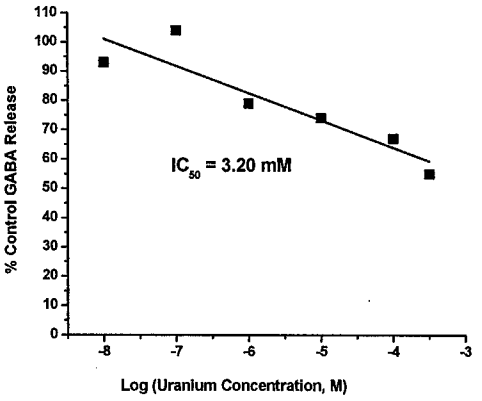

Figure 5. Percentage inhibition of stimulated GABA release from hippocampal synaptosomes as a function of the concentration of uranium in the superfusing solution. GABA release is much less sensitive to acute uranium exposure than that of glutamate. Maximal inhibition is limited by the solubility of uranium in aqueous media at pH 7.4. The data were fitted by linear regression of the types of response inhibition shown in Figure 4.

The basis for the differential potency of uranium on glutamate and GABA release is not known. GABA peaks are smaller, occur later in the chromatogram, and are somewhat susceptible to co-elution with other unknown compounds. But no chromatographic factors have been found to contribute to this differential sensitivity. It is possible that uranium binds glutamate (6) – but not GABA – in a soluble complex, but if this occurs it does not diminish the derivatization efficiency

for glutamate. Each set of samples from an experimental run included triplicate sets of standards derivatized in the presence of the concentrations of uranium used in that assay. No uranium effect on derivatization could be discerned, and thus such a mechanism cannot account for the observed differential potency. Alternatively, it is plausible that uranium could complex glutamate in such a fashion as not to affect derivatization but that might more potently bind to synaptic sites related to transmitter release, e.g., to block voltage-sensitive Ca^{+2} channels. It is worth noting that the diminished neuronal excitability suggested by these observations is consistent with the findings of Pellmar *et al.* (2) who utilized neurophysiological recordings. Elucidation of the mechanism(s) involved will require further investigation.

The uranium IC_{50} for glutamate release of 2.77 μM based on nominal metal concentrations is remarkably similar to the inhibitory potencies of a number of other multivalent metal ions (7). This suggests by inference that the action of uranium is exerted at membrane voltage-sensitive Ca^{+2} channels to interfere with Ca^{+2} influx and diminish exocytosis. Moreover, some of these multivalent metals (e.g., lead) are well known to possess developmental neurotoxicant properties. Thus, it is clear that additional studies are warranted on uranium's neuronal actions in an array of experimental settings.

The acute *in vitro* exposure studies also have assessed the Ca^{+2}-mimetic properties of uranium by measuring synaptosomal depolarization-evoked transmitter release in the absence of Ca^{+2} (replaced by Mg^{+2} to maintain perfusion medium isotonicity) and in the presence of a Ca^{+2} channel antagonist (methoxyverapamil) in the superfusion buffer. Preliminary studies showed that this component of glutamate release constituted only ~25-30% of the total release observed under the conditions described above. If uranium at least partially supports exocytosis – like Pb^{+2} – then this release component should be greater than that found in non-exposed preparations.

Figure 6 displays the time course of K^+-stimulated glutamate release in the absence of Ca^{+2} in control hippocampal synaptosomes, reflecting an approximate 50% increase over baseline values. This compares with a three-fold increase observed in the presence of Ca^{+2} in the superfusion medium as shown in Figure 2 above. However, the data shown in Figure 7 indicate that there is no observable change in the magnitude of this release across a range of nominal superfused uranium concentrations. Similarly, Figure 8 displays the time course of K^+-stimulated GABA release in the absence of Ca^{+2} in non-exposed hippocampal synaptosomes, reflecting a response approximately 60% of that achieved in the presence of Ca^{+2} as shown in Figure 4 above. Again, the data shown in Figure 9 indicate that there is no observable change in the magnitude of this release across a range of nominal superfused uranium concentrations.

In summary, depolarization of superfused hippocampal synaptosomes with high K^+ in Ca^{+2}-free buffer evoked modest increases in glutamate and GABA release, but acute uranium exposure *in vitro* across a range of concentrations did not significantly or systematically alter these response magnitudes. This suggests that UO_2^{+2} does not possess Ca^{+2}-mimetic properties, but it could also be explained if the intrasynaptosomal UO_2^{+2} concentrations did not achieve sufficient levels during the acute exposure to manifest such an effect. This issue can be addressed directly by permeabilizing the synaptosomes with detergent so that the uranium species can enter the cell by diffusion and exert any Ca^{+2}-mimetic actions. These experiments are currently being conducted.

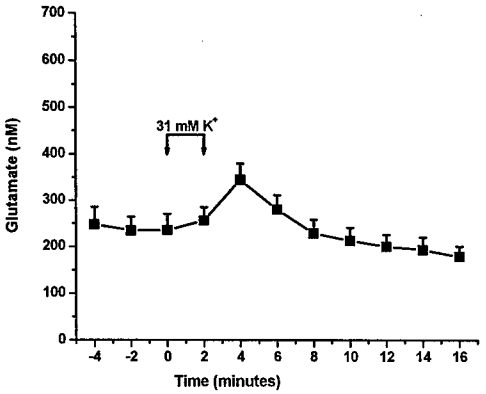

Figure 6. Time course of glutamate concentration in response to superfusion with 31 mM K+ across hippocampal synaptosomes in calcium-free 10 mM HEPES-sucrose buffer (pH 7.4) containing a calcium channel antagonist. Stimulation evoked a peak increase in endogenous glutamate of 44% over baseline concentrations. Values are expressed as mean ± SEM of independent determinations - N = 8 for control conditions and 3-4 for each uranium concentration - conducted in triplicate.

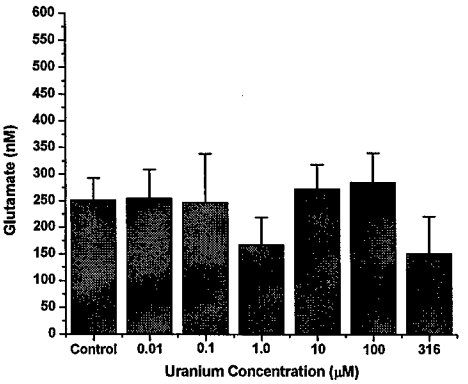

Figure 7. Magnitude of the stimulated calcium-free glutamate responses to 31 mM K+ as in Figure 6 across a range of perfusate uranium concentrations. There were no significant differences in calcium-independent glutamate release from control values at any exposure level. Means are expressed + SEM of independent determinations - N = 6 for control conditions and 3 for each uranium concentration - conducted in triplicate.

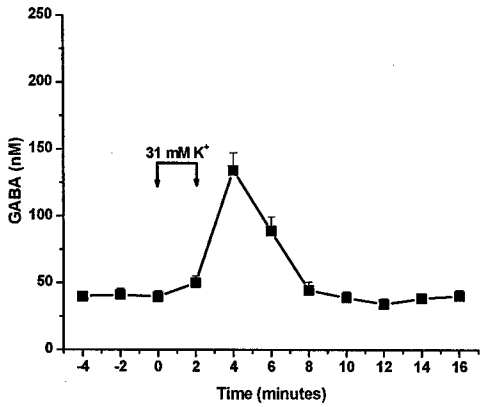

Figure 8. Time course of GABA concentration in response to superfusion with 31 mM K+ across hippocampal synaptosomes in calcium-free 10 mM HEPES-sucrose buffer (pH 7.4) containing a calcium channel antagonist. Stimulation evoked a peak 3.3-fold increase in endogenous GABA over baseline concentrations. Values are expressed as mean + SEM of independent determinations - N = 8 for control conditions and 3-4 for each uranium concentration - conducted in triplicate.

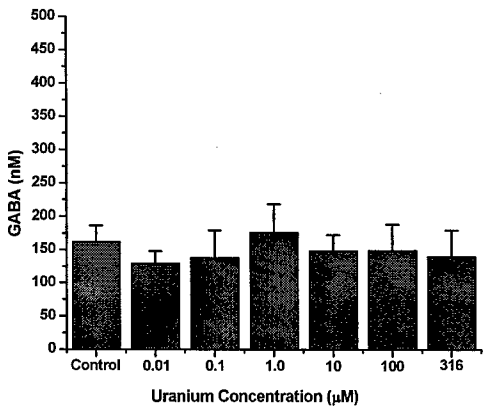

Figure 9. Magnitude of the stimulated calcium-free GABA responses to 31 mM K+ as in Figure 8 across a range of perfusate uranium concentrations. There were no significant differences in calcium-independent GABA release from control values at any exposure level. Means are expressed ± SEM of independent determinations - N = 8 for control conditions and 3-4 for each uranium concentration - conducted in triplicate.

The uranium species involved in the effect on glutamate exocytosis is not known. This study has utilized both uranium (VI) oxide and uranyl nitrate exposures, but a discrimination of the actions of these two uranium species has not been observable. Uranyl ion (UO_2^{+2}) – the most common form produced in the body from all forms of the metal – is converted to diuranate ion ($U_2O_7^{-2}$)

under alkaline conditions (8), so this or another uranium complex (e.g., UO_2OH^+, 9) may be involved.

Additional experiments assessing depolarization-evoked transmitter release after acute uranium exposure are being conducted in terms of free uranium ion concentrations by employing chelating agents (i.e., uranium buffers) to more precisely and reliably identify its potency for glutamate and GABA release. These data would also provide a context within which to interpret the effects of chronic exposure *in vivo*. The accomplishment of Technical Objective 2 is being completed with analogous experiments performed *in vivo* employing intracerebral microdialysis to quantify the changes in hippocampal glutamate/GABA release occurring after a chronic exposure period of 12 months. All animals for this study have been implanted and chronically exposed and the microdialysis sessions with the first cohort of animals have been completed, but the small numbers of animals per group preclude meaningful preliminary data at this time.

Work has also been initiated on Technical Objective 3 to identify changes in glutamate receptor subtypes induced by uranium exposure. Studies on the effects of acute exposure to free UO_2^{+2} ion concentrations on access to the NMDA receptor channel (10) have begun. In addition, a cohort of animals have undergone pellet implantation and are nearing the end of their 12-month chronic exposure period at which time binding to the NMDA receptor channel will be quantified. Similarly, the effects of acute and chronic uranium exposure on the AMPA subtype of glutamate receptors will be assessed in an analogous manner beginning in the next few months. A separate cohort of rats for these latter experiments also has received DU pellet implants. These binding studies will provide an evaluation of the postsynaptic actions of uranium exposure and elucidate potential toxic mechanisms, while also complementing the work on Technical Objective 2 on transmitter release *in vitro* and *in vivo*. By the end of year 4 of the project these observations will have resulted in a thorough and integrated picture of the actions of DU on hippocampal synaptic function.

The dearth of information in the literature on uranium chemistry and the metal's solubility in physiological buffers has posed occasional challenges. Uranium is poorly soluble at alkaline pH, precipitates phosphate, and forms complexes with carbonate ion. Thus, the composition of superfusion buffers has had to be modified. Of greater importance has been the small number of research reports on the neurotoxic actions of uranium – this has limited the ability to create a context for the findings from this project and thus provide more specific interpretation of the results.

Key Research Accomplishments

The key research accomplishments on this project to date are:

- Standardization of DU/tantalum pellet preparation and implantation procedures
- Establishment of chronic DU exposure protocol with blood and brain uranium concentrations
- Validation of an effect of chronic DU exposure on rate of somatic growth

- Demonstration that acute uranium exposure *in vitro* diminishes endogenous depolarization-evoked synaptosomal glutamate and GABA release

- Establishing a differential potency for acute uranium exposure *in vitro* to diminish stimulated glutamate and GABA release

- Identifying the potency of nominal uranium concentrations to diminish stimulated glutamate release as similar to that of other multivalent metal ions, suggesting the presence of developmental neurotoxicant properties and thereby opening other avenues of investigation

- Demonstrating the apparent absence of Ca^{+2}-mimetic properties for acute exposure to uranium *in vitro*

Reportable Outcomes

Lasley, S.M. and Vietti, K.R. Acute exposure to uranium decreases potassium-stimulated hippocampal glutamate release. The Toxicologist 78, 1140, 2004.

Lasley, S.M. and Vietti, K.R.N. Acute exposure to uranium *in vitro* decreases potassium-stimulated hippocampal glutamate release. Military Health Research Forum, San Juan, Puerto Rico, April 25-28, 2004.

Lasley, S.M. and Vietti, K.R.N. Acute exposure to uranyl ion (UO_2^{+2}) *in vitro* diminishes K^+-stimulated glutamate/GABA release independent of extracellular calcium. The Toxicologist 90, 628, 2005.

Lasley, S.M. and Vietti, K.R.N. Model of long-term exposure to depleted uranium via intramuscular implants in rats: Accumulation in blood and brain tissue. Submitted.

Lasley, S.M. and Vietti, K.R.N. Acute exposure to uranium *in vitro* differentially diminishes K^+-stimulated hippocampal glutamate and GABA release. Submitted.

Conclusions

Blood and hippocampal uranium concentrations in chronically exposed animals increase monotonically up to 12 months exposure duration at the exposure levels utilized in this work. In addition, a small but significant impairment in the rate of somatic growth is present in the exposed groups compared to controls. Notably, the hippocampal uranium levels measured by ICP-MS are substantially lower than those reported by Pellmar *et al.* (4,11) even though the observed decrease in body weight (4) is remarkably similar to that reported in Figure 1.

Acute uranium exposure *in vitro* clearly produces a differential inhibitory effect on potassium-stimulated Ca^{+2}-dependent glutamate and GABA release in hippocampal synaptosomes, exhibiting a substantially more potent effect on the glutamatergic process. The resulting

imbalance in neuronal excitation and inhibition may account for the decreased excitability reported by Pellmar *et al.* (2) in chronically DU-exposed rats. The IC_{50} (2.77 uM) for glutamate release based on nominal uranium concentrations is remarkably similar to those determined for other multivalent metal ion effects on transmitter release, and suggests that uranium also possesses developmental neurotoxicant properties. On the other hand, acute exposure to uranium *in vitro* does not appear to result in Ca^{+2}-mimetic actions on K^{+}-stimulated glutamate and GABA release.

The value of this knowledge as a scientific product resides in the establishment of the DU chronic exposure protocol as a shrapnel wound model based on blood and brain levels of the metal and altered rates of growth. These measures provide benchmark values for future studies in this project and for correlation of results from this project to those obtained in other laboratories. In addition, given the similarity of the effects of uranium on transmitter release to those of other multivalent metals (e.g., lead) and the fact that exposure in military scenarios is continuing, it is clear that additional studies are warranted on uranium actions in an array of experimental settings. It is particularly noteworthy that some of the multivalent metals exhibiting effects on transmitter release (e.g., lead) possess well-known developmental neurotoxicant properties.

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