AWARD NUMBER: W81XWH-14-1-0523

TITLE: Reducing Disease Activity in Animal Models of MS by Activation of the Protective Arm of the Renin-Angiotensin System

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Fort Detrick, Maryland 21702-5012

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The results we have thus far generated support our hypothesis that ‘skewing’ of the regulatory arm of RAS in the CNS following onset of demyelinating injury in mice is beneficial. We observed in an animal model of multiple sclerosis that the RAS system is pertubated to bias the regulatory arm in the progressive phases of the disease. We demonstrated not just an increase in expression of specific receptors, but also angiotensin metabolites. The regulatory arm of RAS was activated in an effort to stimulate repair once the immunological and pathological destruction of the tissue had reached a “tipping point”. We also demonstrated for the first time that there is differential regulation of the RAS pathway in different regions of the CNS; likely driven by the extent of the pathological insult.

Our data show that a natural response to the damage that occurs in EAE is to skew expression of the RAS system in the brain to the anti-inflammatory, regulatory arm. In our proposal we hypothesized that if we were to administer A1-7 at an earlier stage in the disease we could activate the regulatory arm of RAS at an earlier stage and prevent significant tissue damage. We have shown that administration of an optimal dose of A1-7 at the first sign of clinical disease significantly ameliorated disease course in mice. These data support our hypothesis and would support the development of A1-7 as a potential treatment for multiple sclerosis.
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1. INTRODUCTION:
Multiple Sclerosis (MS) is a chronic autoimmune and neurodegenerative disease of the central nervous system (CNS) with no defined etiology that is characterized by focal infiltration of leukocytes into the CNS white matter, resulting in destruction of myelin, axonal loss, and ultimately clinical symptoms and neurological disability. Although several disease modifying drugs are available to treat relapsing-remitting MS there are presently no treatments that offer neuroprotection, and none targeted for the progressive phase of the disease. Components of the renin-angiotensin-system (RAS) have recently begun to receive attention as novel mediators in the CNS and in neurological disease. RAS exists as a balance between the pro-vasoconstrictor, pro-inflammatory axis of angiotensin converting enzyme 1 (ACE1), angiotensin II (A-II) and angiotensin type 1 receptor (AT1R), and the pro-vasodilatory, anti-inflammatory arm of ACE2, angiotensin 1-7 (A(1-7)) and receptors Mas and AT2R. A(1-7) which is formed from A-II via ACE2 direct effects on immune regulation, wound healing, stem cell mobilization, and counteracts the effects of A-II, decreasing inflammation, oxidative stress and neuronal apoptosis. Thus the ACE2/A(1-7)/Mas axis appears to be critical for protective physiological homeostasis in the CNS. Whereas there is much evidence describing the pathological association of increased expression the ACE1/A-II/AT1R axis in the CNS of MS patients, there is little evidence for a role of ACE2/A(1-7)/Mas axis, only a solitary assessment showing decreased ACE2 levels in the CSF of MS patients. These observations, and our preliminary data, suggest that components of the RAS pathway in the CNS are indeed an integral part of MS pathogenesis. We posit that the ACE2/A(1-7)/Mas axis is impaired in the CNS of MS/EAE to such a degree that it significantly contributes to the disease pathology and we further hypothesize that treatment with A(1-7) will counteract the effects of the pro-vasoconstrictor, pro-inflammatory and attenuate disease severity and neurodegeneration in demyelinating disease of the CNS.

2. KEYWORDS:
Renin-angiotensin system, angiotensin 1-7 (A1-7), experimental autoimmune encephalomyelitis, Multiple Sclerosis, neurodegeneration, neuroprotection.

3. ACCOMPLISHMENTS:
What were the major goals of the project?
Major Goals (Year 1):
1: Measure levels of RAS components in the spinal cord of mice with EAE (animal model of MS) prior to, and at multiple distinct stages of, disease.
2: Assess expression of differentially expressed RAS components in and around CNS lesions at the various stages of disease.
3: Measure expression of protective arm RAS components in MS lesions and NAWM.
4: Determine the efficacy of treatment with increasing doses of A(1-7) at the onset of disease.

Major Goals (Year 2):
5: Correlate improvement in CDS with changes in MRI, histopathology, oxidative stress and immunological bias at the most efficacious dose of A(1-7).

What was accomplished under these goals?
Below is a summary of the statement of work that was submitted with the application which has been updated with achievements in the first year of this award, updates are highlighted in yellow.

<table>
<thead>
<tr>
<th>Specific Aim 1: Assess changes in expression of select components of RAS at distinct stages of autoimmune mediated demyelination.</th>
<th>Timeline</th>
<th>Current STATUS</th>
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Major Task 2: Experiment 2:  

<table>
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<td>Tissues analyzed for RAS</td>
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<td>Analysis of RAS expression and correlates with disease status</td>
<td>8</td>
<td>COMPLETED</td>
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**Milestones:**

- Local IACUC Approval: 3 months COMPLETED
- Tissue collected and stored: 10 months COMPLETED
- Tissues analyzed for RAS: 13 months STILL TO BE COMPLETED
- Analysis of RAS expression and correlates with disease status: 13 months STILL TO BE COMPLETED

Major Task 3: Experiment 3:  

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<tr>
<td>Human tissue collected</td>
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</tr>
<tr>
<td>Tissues analyzed for RAS</td>
<td>12</td>
<td>15 STILL TO BE COMPLETED</td>
</tr>
<tr>
<td>Analysis of RAS expression and correlates with lesion status</td>
<td>12</td>
<td>15 STILL TO BE COMPLETED</td>
</tr>
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</table>

**Milestones:**

- Local IRB Approval: 3 months COMPLETED
- Tissue collected and stored: 9 months COMPLETED
- Tissues analyzed for RAS: 14 months COMPLETED
- Analysis of RAS expression and correlates with lesion status: 14 months COMPLETED

Specific Aim 2: Assess the effect of therapeutic A(1-7) treatment on disease course in progressive EAE and RR-EAE.  

Major Task 4: Experiment 4:  

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<th>Milestone</th>
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<td>14</td>
<td>COMPLETED</td>
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**Milestones:**

- Local IACUC Approval: 3 months COMPLETED
- Clinical measures collected: 12 months COMPLETED
- Analysis of clinical efficacy of varying doses of A(1-7): 14 months COMPLETED

Major Task 5: Experiment 5:  

<table>
<thead>
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<th>Milestone(s) Achieved:</th>
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<tr>
<td>Local IACUC Approval</td>
<td>3</td>
<td>COMPLETED</td>
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</tbody>
</table>
Milestone Achieved - Tissue and images collected and stored  20  NOT YET STARTED

Milestone Achieved - Tissues analyzed for RAS  22  NOT YET STARTED

Milestone Achieved - Analysis of RAS expression and correlates with MRI, disease status 24  NOT YET STARTED

As can be seen from the above table the vast majority of first year milestones have been completed. Due to the tardy receipt of human materials (major task #3) we have also been able to complete all of major task #4 in the first year. Based on the timing thus far we fully expect to complete all of the remaining experiments in the proposed times.

For this report you will find in the following pages a complete summary of all the results thus far obtained, along with some of our early conclusions. At this stage we are in the midst of completing our full analyses of the data obtained in major task #2, and attempting to correlate this with the data obtained in major task #1. These correlations and outcomes will be included in one of the two manuscripts in preparation.

In the first series of experiments in this project we assessed the levels of critical RAS components in the spinal cord of mice with EAE, an experimental model of MS. EAE was induced in 10-wk old, male C57BL/6 mice by subcutaneously (s.c.) inoculation at 2 sites (~50µl each) on their flank with 200µg MOG 35-55 peptide emulsified in 100µl incomplete Freund's adjuvant (IFA) with 4mg/ml M.tub (complete Freund's adjuvant - CFA). Immediately following this immunization, and again 2 days later, mice received, by intra-peritoneal (i.p.) injection, 250ng of pertussis toxin. On day 7 mice received a booster inoculation with 200µg MOG 35-55 emulsified in 100µl IFA only. MOG-EAE shows a chronic course with mice developing an ascending hind-limb paresis 10-12 days after inoculation, followed by progressive accruing disability and paralysis with stabilization at a high level of disability. MOG-EAE is similar in many aspects to progressive MS with extensive neuronal damage both in the brain and spinal cord, and once established treatment intervention can be very limited. Spinal cords were harvested from mice (n≥6) either prior to induction of disease or at various stages thereafter, as shown on the x-axis of figure 1. The times chosen reflect early pre-clinical phases where immune activation may have occurred and later distinct phases of clinical development of the disease. In all experiments we took great care in the harvesting of tissue to ensure the same time of processing for each mice, this is especially important when considering the short half-lives of the metabolites we assess in experiment 3. For this experiment spinal cord samples were homogenized in RIPA buffer containing protease inhibitors, protein concentration determined, and 20µg of protein ran per lane on a 5-20% SDS-PAGE gel: control recombinant proteins were included in a separate lane on each gel. Western blots were probed with antibodies specific for the receptor protein of interest and GAPDH as a loading control. The relative intensity of each band was determined using a Licor Odyssey in a shared facility and the relative amount of each protein determined within each gel by comparing to GAPDH control and across all gels by comparing to control recombinant protein. Figure 1 shows the relative expression of three receptor component of RAS, namely MAS, AT2R and AT1R, and of ACE, one of the major enzymes in the RAS pathway. In our hands the commercially available antibodies specific for ACE2 did not yield reproducible results and unfortunately could not be included in these analyses. Focusing on the regulatory arm of RAS there was a clear and dramatic loss of MAS receptor expression within 4 days of inoculation when innate components of the immune response may become active. This was followed by a dramatic and sustained increased in MAS receptor expression just prior to, and more so after, clinical disease became apparent. This is at a stage when there is significantly tissue damage accruing and as such that this is an expected response of the regulatory arm. The expression of MAS returns to baseline levels as the disease reaches its more severe stages however suggesting either that the increased expression of this receptor is very transient. Interestingly we saw no obvious trends or changes in the expression of AT2R, the other receptor found in the regulatory arm of RAS. In contrast to the regulatory arm of RAS, we saw a significant increase in the expression of both AT1R and ACE within 4 days of inoculation. This expected activation of the pro-inflammatory axis was not surprising at this stage of the disease. However, we did not see any further increases in the expression of components of this arm of RAS, with both of these proteins returning to baseline levels somewhat sooner than anticipated. These data were consistent without hypothesis and showed for the first time that
in an animal model of MS the RAS system is pertubated during disease induction and progression to bias the regulatory arm, at least in the active progressive phases of the disease.

As a follow-up to our encouraging spinal cord data we chose to assess the relative expression of the same proteins in brain tissue where there is much less disease activity and pathology in this model. Using the same experimental design (mice per group ≥6) and timeline as described above for the 1st experiment, the relative changes in these proteins were determined and are shown in figure 2. Similar to the spinal cord there was a dramatic, immediate reduction in the expression of MAS within 4 days of inoculation, but unlike in the spinal cord the levels of MAS did not quickly return to baseline. There was however a dramatic increase in MAS expression at a much later stage of disease (day 20, CDS 3.0-4.0). Similar to the spinal cord there was no there was no discernable change in the expression of AT2R. In contrast to the spinal cord, there was no immediate increase in the expression of AT1R or ACE over baseline, with the only detectable change being reduced ACE expression at very late stages of disease (>38 days after inoculation). This may reflect the different timeline of tissue damage in the brain as compared to the spinal cord in this model, and as far as we are aware this show for the first time in EAE that there is differential regulation of this pathway in different locales of the CNS, namely spinal cord vs. brain.

In the third series of experiments we assessed the relative change in expression of a multitude of metabolites in the RAS. It is important to realize that any hypotheses we make to rationalize the changes in expression of enzymes and specific receptors, as described above, need to be explained in the context of the ligands for these different proteins. Without engagement of the receptor there is no biological significance to having more expressed. Figure 3 on the next page shows a general schematic of the RAS pathway and how the various metabolites are most commonly derived. Assessment of the various components of this, in association with specific receptor and enzyme expression would enable us to absolutely define if there is “skewing” of the RAS from the active to the protective arm during EAE.

Using the same mouse model and timeline as described above for the 1st experiment (mice per group ≥5) we harvested spinal cord tissue from mice. For these metabolite assessments we included only those mice which were perfused with saline 5-7 minutes after administering anesthetic and whose spinal cord was homogenized after 14-15 minutes. Spinal cords were homogenized in PBS containing a cocktail of protease inhibitors and enzyme antagonists designed to ensure that there is no degradation of metabolite entities following the harvest due to
contaminating RAS enzymes. This enabled us to get a highly accurate measure of the state of the RAS in the harvested tissue. Figure 4 below shows the changes at various time points following immunization in the expression the major metabolites in this pathway including angiotensin I (A1-10, the parent product of this pathway), the three axes from this, namely angiotensin II (A1-8), A1-9 and A1-7. We also show data on A3-8, another downstream product of A1-8 and A1-5, a direct downstream product of A1-7. In figure 4 it can clearly be seen that all products (except for All) showed increased levels 4 days after immunization and reverted to baseline within the next 7-10 days. The levels of angiotensin I fluctuated mildly after the initial immunization but did not significantly differ from baseline. However, after day 14, the two arms of RAS diverged dramatically. Whereas the end product of the pro-inflammatory arm (A3-8) remained at baseline levels through the remainder of the experiment, the concentrations of A1-7, its precursor A1-9 and its end product A1-5 all increased from day 14 onwards. The concentration of A1-9, A1-7 and A1-5 continued to rise through the last time point (day 30). These data not only support our hypothesis but give us encouragement going forward. These results may suggest that a natural response to the damage that occurs in EAE is to skew expression of the RAS system in the brain to the anti-inflammatory, regulatory arm. Whilst these data provide support for our hypothesis, of concern with our data set is the lack of significant angiotensin II expression. Other groups have described the expression of angiotensin II in normal CNS tissue and altered expression in autoimmune diseases (Manzel et al., 2014, J. Neuroimmune Pharmacol, 9:533). We are currently reassessing the LC-MS data to determine if there were any errors in the analyses and will act accordingly, either re-running samples, or collecting new tissue lysates for a second series. Whilst we would hope to complete this in the expected time frame we may run into some delays, especially if there is need for a second series of mice.

Nevertheless, the results we have thus far generated strongly support our hypothesis of regulatory arm skewing in the CNS following initiation of demyelinating injury in mice. The LC-MS data coupled with the variance in expression of the different receptors strongly support our hypothesis and show activation of the regulatory arm of RAS at a later stage in disease progression. Our hypothesis is that this arm of RAS is activated at this stage in an effort to stimulate repair once the immunological and pathological destruction of the tissue has reached a “tipping point”. In this model, the tissue damage is probably so great that no matter the action of the protective arm this cannot reversed. However, these data lend support to our preliminary observation of efficacy of A(1-7) administration, quite simply based on our observations that A(1-7) and MAS upregulation is somewhat later in the time course. We hypothesised that modulation of the RAS at an early time point in the disease, in favor of the regulatory arm, by administration of A(1-7), would tilt the balance in favor of protection. If we were to administer
A(1-7) at an earlier stage of the disease process (as we did in preliminary experiments), then maybe we could drive the protection and repair processes at an earlier stage prior to significant tissue damage.

Our preliminary data had already suggested that this hypothesis would be proven correct and our next series of experiments was aimed not only at testing the above hypothesis and confirming our preliminary findings, but also to determine the most efficacious dose of A(1-7). Our preliminary data showed that twice daily administration of A(1-7) for a total daily dose of 0.5mg/kg significantly reduced disease activity in the MOG-EAE even more so than a single daily dose of the same amount. Using the same mouse model as described above for the 1st experiment we assessed the efficacy of twice daily administration of A(1-7) for total daily doses of 0.5, 1.0, 2.0 and 4.0mg/kg as compared to twice daily administration of saline only (n≥8 mice per group). In this experiment mice were immunized as described earlier and A(1-7) or saline treatment was started when clinical disease was already established (i.e. mice had hind limb or tail weakness). Clinical disease was scored using a common scale that is well-established. This clinical disease score (CDS) varies from 0-5 and is graded as follows: 0 = no symptoms as compared with non-immunized mice; 0.5 = weight loss and/or subtle weakness in tail or gripping of hind limbs; 1 = loss of muscle tone in tail and/or mild weakness in gripping of hind limbs; 1.5 = tail paralysis or very limp tail, hind limbs have very weak grip; 2 = hind limb weakness, resulting in ataxia; 2.5 = more severe ataxia and hind limb weakness where mouse may drag one limb occasionally but can still move joints; 3 = mild paresis or paralysis of one (3.0) or both (3.5) hind limbs and possible incontinence; 4 = complete paralysis of both hind limbs, 4.5 = paresis or paralysis of forelimbs, 5 = loss of temperature control and inactivity. A(1-7) or saline were administered s.c. twice daily (~10-12hrs apart). Mice were evaluated daily for clinical disease in a blinded manner in consultation with Dr. Leslie Weiner. Figure 5 below shows that when administrated in a therapeutic manner A(1-7) caused significant improvement in the clinical disease course. Mice receiving 0.5mg/kg/day (red plot) showed clinical improvement early on, but over time showed progression similar to that of saline-treated mice (black plot). In contrast, mice receiving either 1.0 (green), 2.0 (purple) or 4.0 (blue) mg/kg/day showed a significantly better disease course: disease onset was slowed and the severity of disease was significantly less over time with the average mouse presenting with a clinical disease score of 2.0 (mild ataxia) compared to untreated mice which all showed severe paresis (≥3.0). These data supported our original hypothesis and suggested that there was no increased benefit from doses greater than 1mg/kg/day.

Figure 5: Clinical efficacy of twice daily administration of A1-7.

Given that the half-life of A(1-7), when given sub-cutaneously, is approximately 30 minutes we next sought to determine if the efficacy of A(1-7) would be increased if given as a continuous infusion. Using the same experimental outline as in the previous experiment and the same total daily doses we assessed clinical disease course over time in mice receiving A(1-7) or saline via an implantable minipump. Figure 6 below shows that
similar to what was seen with the twice-daily dosing, all doses significantly slowed disease at the early stages with little variances between the doses. Again, over time little efficacy was observed with a total daily dose of 0.5mg/kg/day (red plot). 1mg/kg/day (green plot) was the most efficacious dose when given as a continuous infusion; mice presenting with a clinical disease score of 2.0-2.5 (ataxia). In contrast, mice receiving either 2 (purple) or 4 (blue) mg/kg/day deteriorated rapidly after day 16 and were not significantly different to saline-treated mice by day 20. However, quite surprisingly, both of these groups of mice recovered over time and showed significant clinical improvement as compared to the saline-treated group over the remainder of the study. By the end of the study (day 40) there was little difference between the 1.0, 2.0 and 4.0mg/kg/day groups. Based on this data, continuous subcutaneous treatment with A(1-7) did not appear to show any significantly greater benefit that twice-daily treatment, the 1mg/kg/day group in both experiments showed similar trends.

We had expected at this stage to have carried out all the histological and IHC assessments of the human tissue looking at the various components of RAS that we have assessed in the murine model. Unfortunately, it took longer than expected to obtain the human tissue for these experiments. At the time of writing this report, all the human MS tissue has been received and we have characteristics for each of the lesions defining them as either active or chronic. We also have adjacent normal appearing white matter (NAWM) and are in the process of processing these tissues for the characterization. We expect these data to be compiled within the next 3 months.

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest?
1 - University of British Columbia, Vancouver Grand Rounds presentation. Drs. Lund, Rodgers and Kelland attended meeting with research and clinical faculty at the UBC Medical Center and Djavad Mowafaghian Centre for Brain Health and presented preliminary pre-clinical data of efficacy of A(1-7).


3 – University of Southern California, School of Medicine, Department of Neurology, Multiple Sclerosis division meetings. Research progress has been presented to colleagues within our research group.
4 – University of Southern California, School of Pharmacy. Research progress has been presented to collaborators in the school of Pharmacy and at research progress meetings therein.

What do you plan to do during the next reporting period to accomplish the goals?
As discussed above, based on the timing thus far we will conduct all of the remaining experiments in the coming year and fully expect to complete all of this in the proposed times.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
We had expected at this stage to have carried out all the histological and IHC assessments of the human tissue. However, it took longer than expected to obtain the tissue for these experiments. As such we have encountered a 3-4 month delay. All of the tissue is now in place at USC and will be assessed and analyzed in the coming 3 months. This is a minor delay of no significance the whole project.

Changes that had a significant impact on expenditures
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects
Nothing to Report.

Significant changes in use or care of vertebrate animals.
Nothing to Report.

Significant changes in use of biohazards and/or select agents
Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.
Nothing to Report.

Please note that two manuscripts are in preparation just now describing: (i) our observations of changes in the RAs system components during disease onset and progression, and (ii) our dose response findings in the treatment of MOG-EAE. We expect these manuscripts to be submitted early next year.

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.

Conference poster:

Presentations:
University of British Columbia, Vancouver Grand Rounds presentation. Drs. Lund, Rodgers and Kelland attended meeting with research and clinical faculty at the UBC Medical Center and Djavad Mowafaghian Centre for Brain Health and presented preliminary pre-clinical data of efficacy of A(1-7).

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Brett T. Lund, Ph.D.
Project Role: Principal Investigator
Researcher Identifier:
Nearest person month worked: 7
Contribution to Project: Dr. Lund has overseen the whole progress of the project. He wrote the IACUC protocol and obtained approval for all of the outlined experiments, carried out with the help of others on the project all of the animal experiments and has worked hand-in-hand with colleagues in the histological analyses and assessments of expression of components of the RAS system throughout disease. Dr. Lund has held monthly meetings with the research team to go over the data and the research progress.

Name: Eve E. Kelland, Ph.D.
Project Role: Co-investigator
Researcher Identifier:
Nearest person month worked: 4
Contribution to Project: Dr. Kelland has worked closely with Dr. Lund on all aspects of the project but most specifically focusing on the histological analyses and assessments of expression of components of the RAS system throughout disease. She aided in all the animal experiments thus far and has taken an important role in ensuring the successful completion of each experiment.

Name: Kathleen Rodgers, Ph.D.
Project Role: Co-investigator
Researcher Identifier:
Nearest person month worked: 1
Contribution to Project: Dr. Rodgers has sat in on many of the research group meetings and has aided in the interpretation of the RAS data and clinical data. She has advised on our pre-clinical approaches and how best to utilize the drug in our animal models.

Name: Stan Louie, Pharm. D.
Project Role: Co-investigator
Researcher Identifier:
Nearest person month worked: 1
Contribution to Project: Dr. Louie has analyzed all of the serum and brain samples thus far collected for the levels of the various soluble components of RAS. He has also sat in on the research group meetings and has aided in the interpretation of these data.

Name: Roslynn Stone, Pharm. D.
Project Role: Post-graduate student
Researcher Identifier:
Nearest person month worked: 6
Contribution to Project: Dr. Stone has worked on most aspects of the project but most specifically focusing on the western blot analyses of expression of the various receptors of the RAS. She also aided in all the animal experiments thus far. Dr. Stone sits in on research group meetings and has critically analyzed the hitocnehmichall and RAS metabolite data collected thus far.
Funding Support: USC School of Pharmacy, Ph.D. program.

Name: Christopher Meek, B.S., M.S.
Project Role: Senior research technician
Researcher Identifier:
Nearest person month worked: 4
Contribution to Project: Mr. Meeks has provided expert technical support for the conduct of the animal experiments completed thus far.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

SPECIAL REPORTING REQUIREMENTS
Not applicable.

APPENDICES
None.