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AWARD NUMBER: W81XWH-07-1-0626

TITLE: The Influence of Neuronal Activity on Breast Tumor Metastasis to the Brain

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REPORT DATE: September 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 September 2009	Final	13 Aug 2007 – 12 Aug 2009

4. TITLE AND SUBTITLE

The Influence of Neuronal Activity on Breast Tumor Metastasis to the Brain

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0626

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Rochester
Rochester, NY 14642

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this grant we aim to use our insights gained in the study of developmental neuroscience to affect breast tumor metastasis to the brain. Specifically, we are focusing on our finding that synaptic plasticity is dependent on the remodeling of the extracellular matrix and that this remodeling is elicited by the altered activity of neurons. Because breast tumor metastasis is sensitive to the extracellular environment we believe that by altering neuronal activity we could also affect breast tumor metastasis to the brain. Significant progress during this reporting period includes the establishment and characterization of a model of breast tumor metastasis to the brain in the mouse and the testing of a neurological stimulant in curtailing breast tumor brain metastasis. Our preliminary data suggests that stimulants may affect the ability of circulating cells to enter and grow within the central nervous system.

15. SUBJECT TERMS
brain, extracellular matrix, stimulant, depressant, neuronal activity, diffusion

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	13	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Most anticancer therapies rely on long standing notions of controlling cell division. There have been few, if any, completely new approaches toward the control of tumors in general and breast cancer in particular. In contrast, we propose to exploit the knowledge gained from recent and ongoing work in the field of neuroscience to both predict and prevent breast tumor metastasis in the brain. Specifically we expect that normal mechanisms used by neurons to alter the extracellular matrix as a result of changes in neuronal firing also affect the ability of breast tumor cells to enter the brain, move about, and grow into a tumor. This innovative insight allows us to immediately test the antimetastatic ability of three drugs *already approved for use in the clinic* and hence offers the possibility of extremely rapid clinical application of our ideas. This is the primary reason we believe that this proposal has extremely high immediate impact and will greatly advance the treatment of breast cancer. We also hope to that our work will lead to a whole new way of thinking about breast cancer metastasis to the brain, and lead ourselves and others to explore other clinically approved therapies targeting neurons to see if they alter the extracellular matrix and impact metastasis. Lastly, we are performing some important basic biology that we think establishes a whole new field in cancer research, specifically the exploration of the molecular and physical mechanisms of extracellular matrix modification by neurons and its inhibition of metastasis.

Body

In order to carry out the goals of our proposal we first needed to establish a mouse model of breast tumor metastasis to the brain in our laboratory. Our initial proposal was to use the MDA-MB231 human metastatic ductal breast carcinoma cells, a well established model of human breast cancer, injected intracardially in immuno-deficient mice. However, we found that our preliminary experiments using these cells did not yield brain tumors when the cells were injected intracardially. Additionally, these cells did not appear to grow when injected intracranially suggesting that this cell line has difficulty growing within the brain environment of the mouse and hence is an inappropriate model of breast tumor metastasis to the brain.

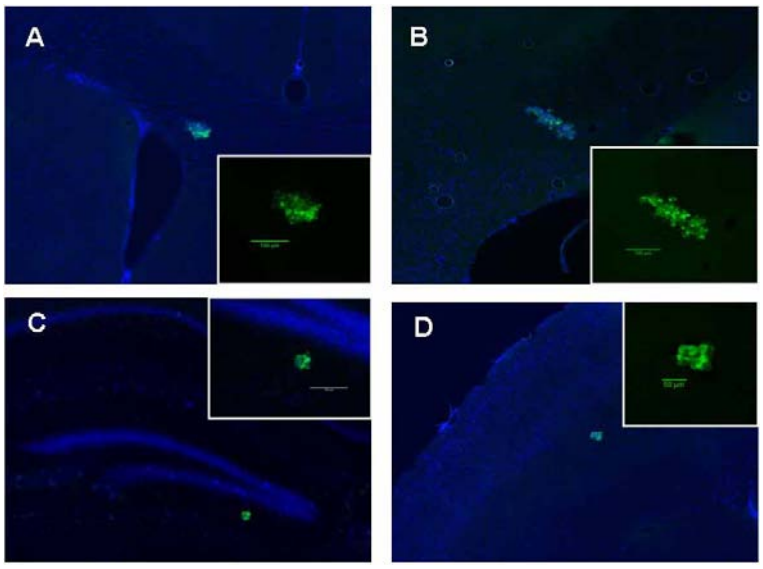

Figure 1. Breast Tumor Metastases to the Brain after Intracardiac Injection

MDA-MB-231BR-YFP cells establish metastases in the Corpus Callosum (A: white matter tumor), thalamus (B), hippocampus (C) and cortex (D). Green is YFP expressed by tumor cells, blue is DAPI nuclear counterstain. Insets are higher magnification images in the YFP channel. Scale bars = 100 μm (A-C); 50 μm (D).

Because of these results we spent the first 6 months of this funding period developing a different mouse model in the lab. As described in the last report, we obtained MDA-MB231BR cells[1] (a generous gift from T. Yoneda) and transfected these cells with the fluorescent protein Venus (a version of YFP) in our laboratory. With this cell line we obtained brain metastases (Figure 1). However, we found an unexpected and unacceptable variability in the number of metastases in the brain following intracardiac injection in SCID mice. After experimenting with a number of immunodeficient mice, we found that nu/nu mice yielded an acceptable level of variability. This exploration of a good cell line and mouse strain for studying breast tumor metastasis to the brain significantly delayed starting the aims of this grant but produced a good model for studying this process.

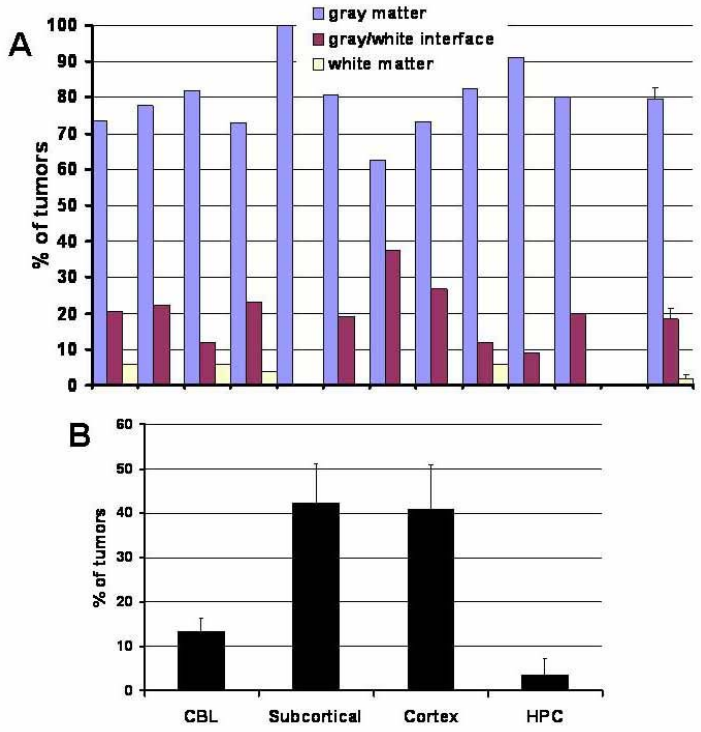

Figure 2. Distribution of tumor metastases within the brain.
A. Percentage of total tumors identified within gray matter, white matter and the gray/white matter interface. The last set of three bars represents the average for the control group. Notice that the majority of tumors are established within the gray matter.
B. Distribution of brain metastases between different brain areas. The majority of tumors were found in cortical and subcortical areas with few being present in the cerebellum (CBL) and hippocampus (HPC).

As reported in the last report, within the first year we were able to characterize our breast tumor brain metastasis model. We carried out control experiments to characterize the metastasis pattern of MDA-MB231BR cells. Nude mice were intracardially injected with a 100 µl volume containing 200,000 cells. After 3 weeks the animals were deeply anesthetized and perfused with saline followed by fixative. Their brains were removed, dehydrated and sectioned to a thickness of 50 µm. Sections were scanned under epifluorescence and tumors were identified (Figure 1). The number of cells comprising a tumor as well as tumor location within the brain was noted. Brain metastases were identified in all the mice although the number of tumors varied (normalized to the number of sections obtained from individual mouse brains – 73±2 sections were analyzed per mouse). On average 0.37±0.07 tumors/section were identified. The range was 0.11-0.88 tumors/section. Tumors ranged in size from 3 cells to 270 cells. The average tumor size was 31±7 cells. The majority (80±3%) of the tumors were found in the gray matter, 18±3% were located at the gray/white matter interface, and very few tumors (2±1%) were located in the large white matter tracts (Figure 2). 13±3% of tumors were located in the cerebellum, 42±8%

were found in subcortical areas, 41±10% in the cerebral cortex, and 4±4% in the hippocampus (Figure 2).

Aim 1: Drugs known to increase neural activity (and hence reduce spine motility) inhibit breast tumor metastasis to the brain.

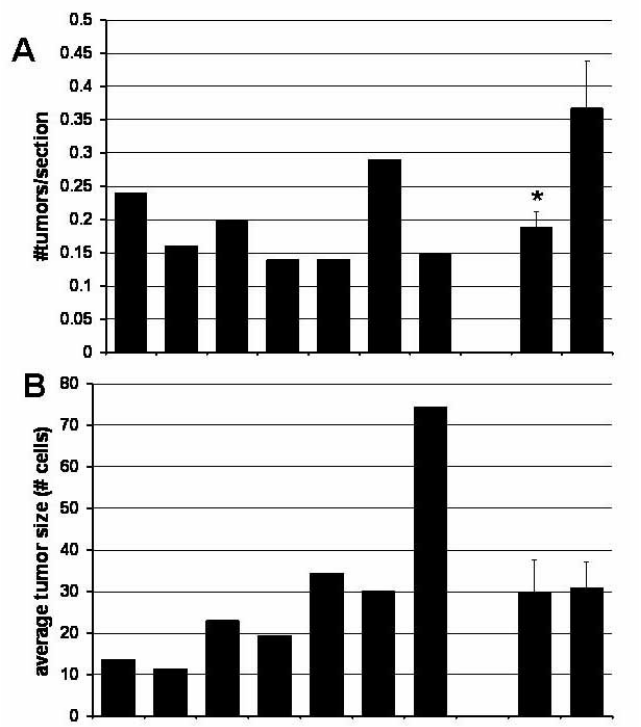

Figure 4. Quantification of tumor metastasis in caffeine-treated animals.

A. Number of tumors/section analyzed in 7 injected animals. The last bars in the graph represent the average number of tumors/section in caffeine-treated (left) and control (right) mice, while the 7 previous bars represent the number of tumors/section in each of the 7 caffeine treated mice, to provide a visual demonstration of the typical variability in these groups. Notice that caffeine-treated mice have significantly fewer tumors in the brain. * indicates $p<0.05$ t-test. B. Average tumor size (number of cells counted per tumor). The last bars in the graph represent the average number of cells/tumor in caffeine-treated (left) and control (right) mice, while the 7 previous bars represent the tumor size in each of the 7 caffeine treated mice, to provide a visual demonstration of the typical variability in these groups. Notice the lack of difference between tumor size in the two groups. Error bars show the standard error.

After establishing the baseline of tumor growth within the brain after intercardiac injection of breast cancer cells, we turned to exploring whether stimulants which increase neuronal activity affect this metastasis. We proposed to use three different drugs to alter brain activity: caffeine, methylphenidate and modafinil. While the smallest effect may be expected from caffeine which is not as potent as the other drugs, we decided to begin our experiments with caffeine because it can be orally delivered and thus is easier to work with than the other injectable drugs. We pretreated nude mice for 5 days with caffeine by providing the animals with 0.5g/L of caffeine in their drinking water. After 5 days of treatment MDA-MB231BR-YFP cells were injected intracardially and caffeine treatment was continued throughout the 3 week survival period. Interestingly, animals treated with caffeine had fewer tumors (0.19±0.02 tumors/section (76±3 sections were examined); 7 animals; $p<0.05$ t-test when compared to controls; Figure 4a). The average number of cells per tumor was not statistically significantly different than in control conditions (29±8 cells; $p>0.05$; Figure 4b), suggesting that caffeine affected the ability of breast tumor cells to establish tumors within the brain but not to grow once established. Further supporting the idea that caffeine causes differences in "seeding", caffeine treated animals had significantly larger proportion of white matter tumors (Figure 5).

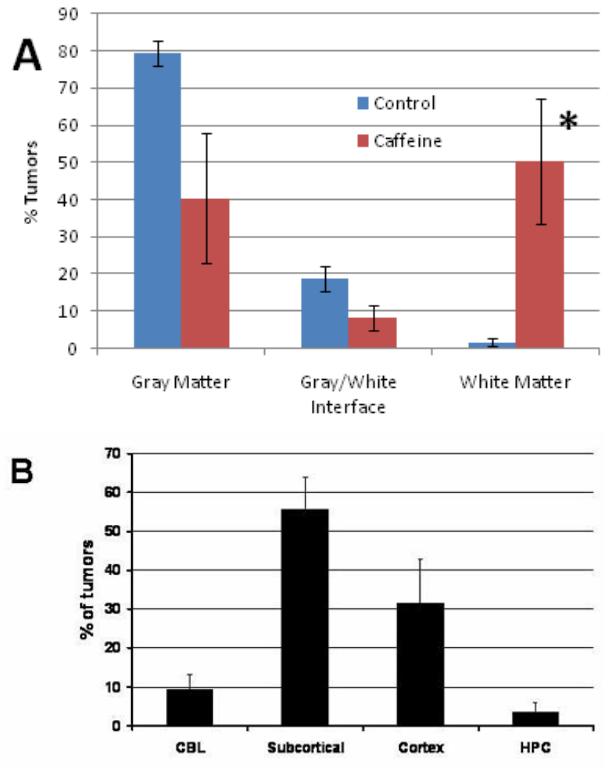

Figure 5. Distribution of tumor metastases within the brain of caffeine-treated animals.

A. Percentage of total tumors identified within gray matter, white matter and the gray/white matter interface for the caffeine-treated group (left) and the control group (right). Notice the increase in the proportion of tumors established in the white matter in caffeine-treated animals.
B. Distribution of brain metastases between different brain areas in caffeine-treated animals. The majority of tumors were found in cortical and subcortical areas with few being present in the cerebellum (CBL) and hippocampus (HPC) similarly to control animals.

After this success with caffeine, we have initiated experiments to test the effects of modafinil on breast tumor metastasis to the brain in our model. Unfortunately, we have had additional unexpected set backs when we found that many of our mice (control and treated) ceased to yield brain metastases. Because we noticed that our nu/nu mice had a significantly different appearance than when we carried out our caffeine experiments, we consulted with our immunology colleagues and determined that there may have been a drift in the immune system function of the mice we were using. We expended significant effort and time to repeat the intracardiac injections in different kids of immunodeficient mice but did not see improved metastatic burden. Therefore we tested our cells by directly injecting into the brains of immunodeficient mice (Figure 6). We obtained no growth of these intracranially implanted cells suggesting to us that our cells had lost the ability to grow within the brain. We consulted with Dr. Yoneda who informed us that this does occasionally happen over time and we recently obtained new cells from him. We have transfected these cells with Venus and are testing their efficacy at metastasizing to the brain after intracardiac injection in nu/nu mice. We have also obtained a different version of these cells from Dr. Steeg at NCI and are testing these as well. At 21 days post-implantation of $1x10^6$ MB-231BR cells from our old batch, 45% of SCID mice implanted in the mammary fat pad had measurable tumors (14 out of 31). The average tumor volume was 37.6 mm^3 (standard error of 8.2 mm^3). At that same time point, new MB-231BR cells from the Yoneda lab grew tumors in 75% of mice (6 out of 8) with an average tumor volume was 33.6 mm^3 (standard error of 13.0 mm^3). MB-231BR from the Steeg lab grew in 100% of mice (6 out of 6) with an average tumor volume was 42.6

mm^3 (standard error of 9.5 mm^3). Therefore we believe that we can now carry out experiments using the new cells we obtained.

Thus while we were only able to characterize one of the stimulants named in the original aim, we hope to continue this project with new cells even though the grant period is over.

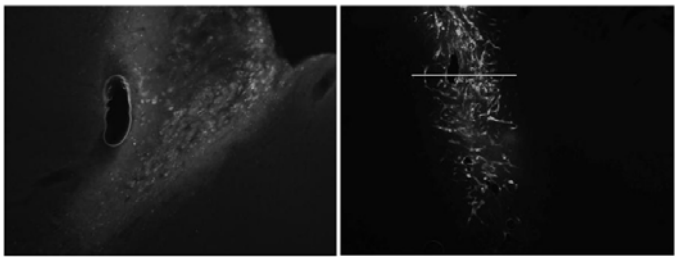

Figure 6. Brain tumors established after intracranial injection of cells.
Right. Intracranial injection of CNS-1 cells, a rat glioma cell line, results in large, diffuse tumors 7 days after cell implantation. Left. Intracranial injection of our MDA-MB231BR cell line resulted in no tumor growth but visible cellular debris 7 days after implantation.

Aim 2: Test the involvement of the tPA-plasmin axis in the effects that activity-altering drugs have on the brain extracellular matrix (ECM).

To determine whether tPA was involved in the effects of caffeine on breast tumor metastasis to the brain in our model, we carried out immunostaining experiments on fixed brain sections using tPA antibodies. Unfortunately we found that commercially available antibodies did not yield reliable results in our hands despite the description of their use in the literature. Staining was non-specific and was also present in tPA KO mice. We tried the following antibodies: Molecular Innovations ASMTPA-GF. Rabbit anti-Mouse. Oxford Biomedical Rabbit anti-human. PA 57.

Therefore we continued our work using zymographical approaches to examine tPA activity in the neuropil rather than tPA protein expression. We spent considerable time developing in situ methods for investigating tPA activity in the hope that we could see localized tPA elevations around areas where tumors are likely to form. First, we tried a modified in situ technique on acute brain slices. We prepared 400 µm thick slices of brain from animals that were treated with caffeine for 5 days or control animals. These were allowed to recover at room temperature in artificial cerebral spinal fluid before being incubated at 37°C with a fluorescent quenched gelatinase subtrate. The activity of MMPs was blocked with specific inhibitors. Slices were then fixed and photographed on a fluorescent microscope. Our analysis using this technique revealed that caffeine increases the levels of tPA activity in the brain (Figure 7). While contrary to our original hypothesis, this is interesting as it suggests that tPA is upregulated following caffeine treatment and this should result in a more labile ECM that is permissive for tumor growth. However, tumor size does not increase in caffeine-treated animals. This implies that breast tumors in the brain parenchyma may be less sensitive to the brain ECM than other more invasive resident cancers such as gliomas. However, it is also possible that changes in the blood brain barrier (which are believed to be mediated by proteases) could result in a blood brain barrier that is less permissive for tumor entrance to the brain after caffeine-treatment.

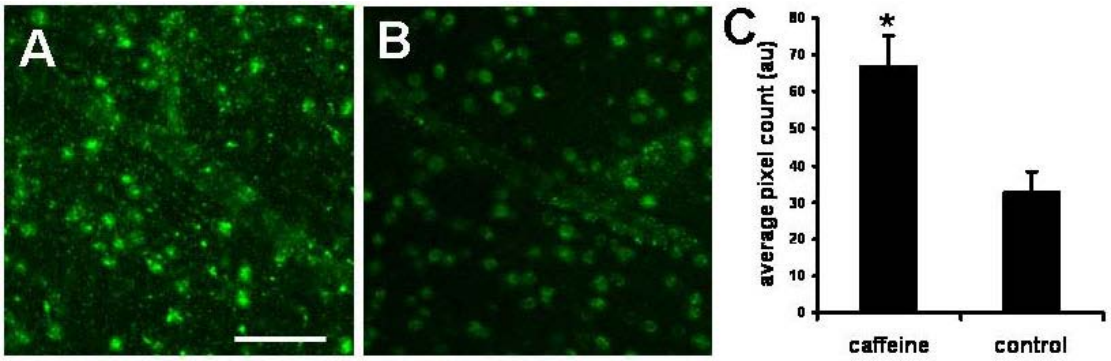

Figure 7. Caffeine induces tPA activity.
A. tPA in situ zymography from a mouse treated with caffeine for 5 days. Notice tPA activity in neuronal somata, neuropil and blood vessels. Scale bar = 50 μm. B. In a control mouse, tPA activity staining is dim and stained cell bodies/neuropil puncta are less frequent. C. Quantification showing that caffeine elevates tPA activity in the brain (67±8 a.u. caffeine, n=10; 33±6 a.u. control, n=6; p=0.016 Mann-Whitney test).

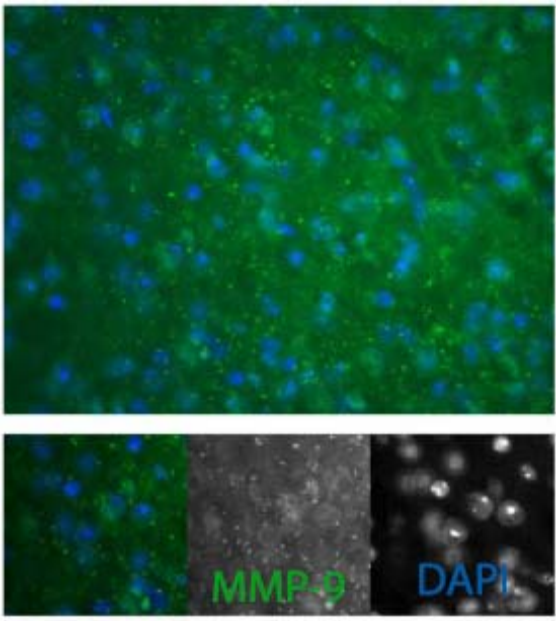

Figure 8. Alcohol fixation method of in situ zymography for matrix metalloproteinases (MMPs)

Top: Brain section from a control mouse after alcohol fixation, wax embedding and in situ zymography with a fluorescent gelatin substrate. Green indicates MMP activity while blue denotes DAPI staining of nuclei demonstrating that this method can easily be combined with immunocytochemistry.
Bottom: merged images, MMP zymography and DAPI stain shown individually from left to right.
Notice the many puncta of MMP activity in the neuropil and light staining of cell bodies indicative of reduced staining artifact.

Interestingly, we also noticed that blood vessels in caffeine-treated animals showed more uniform tPA activity along their length, while vessels from control animals had variable staining at different points along the blood vessel. In light of this we wanted to determine whether there were changes in tPA activity specifically around blood vessels following caffeine-treatement. However, while this zymography technique yielded staining indicative of protease activity, it also contained many artifactual stained areas due to damage sustained by the tissue during the cutting process and incubation period. These were particularly prominent around blood vessels. Therefore we have adapted a different protocol, recently developed in the Wilczynski lab at the Nencki institute (Gawlak et al., 2009) that maintains protease activity by alcohol fixation and allows relatively artifact-free assay of protease activity. While we are still in the process of setting up this technique routinely in the laboratory, we have begun to get very good,

high resolution images of protease activity. The other advantage of this technique is that is allows immunostaining of zymographical sections allowing the co-registration of tPA activity with endothelial cell markers to delineate blood vessels. Additionally, this technique can also be easily adapted to determine the changes in the activity of other proteases (such as the MMPs) after caffeine treatment (Figure 8) and we are currently quantifying these changes to see if other proteases may be important for the effects of caffeine on brain tumor metastasis to the brain.

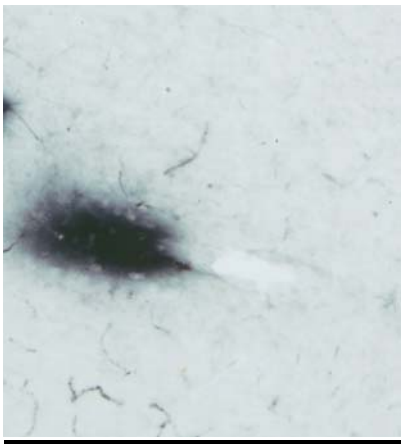

Figure 9. Assaying blood brain barrier breakdown.

Brain section from a mouse injected with MDA-231BR cells intracardially reacted with biotinylated anti-mouse IgG (H+L). The dark area is mouse IgG (normally confined to the circulatory system) which has entered the brain through a break in the BBB.

Additionally, we are developing methods to look at the blood brain barrier in the hopes of determining whether caffeine treatment decreases its permeability and hinders the entrance of breast tumor cells into the brain. We are currently using immunostaining for IgG on fixed brain sections of caffeine-treated and control animals to determine how frequently the blood brain barrier is compromised and allows the entrance of blood elements into the brain in these mice.

Thus this aim was completed for the drug caffeine although with opposite results to those expected. This has prompted us to examine other proteases as well as to concentrate on the blood brain barrier as a possible substrate for caffeine action on the brain.

Aim 3: Investigate the effects of activity-altering drugs on the diffusive hindrance of the ECM.

The purpose of aim 3 was to determine whether the effects of stimulants on breast tumor metastasis to the brain were mediated by changes in the brain extracellular matrix structure. To assay this we proposed to use fluorescence recovery after photobleaching (FRAP) in acute slices of brain made from animals treated with stimulants and control animals. Our working hypothesis was that caffeine treatment would increase the diffusive hindrance of the brain ECM (via a downregulation of protease expression). However, as described above, we unexpectedly found that caffeine increased the expression of proteases in the brain parenchyma without changing the growth of tumors within the brain (as assayed by average tumor size). Therefore, we were still interested in understanding whether caffeine altered the structure of the brain ECM and whether breast tumor metastases were not sensitive to this change or whether brain tumor growth was not altered because the ECM was similarly stable. We are currently carrying out these experiments, however, we are assaying diffusive hindrance in the intact brain using in vivo two-photon FRAP rather than generating brain slices. While this technique limits our analysis to superficial areas of the cerebral cortex, breast tumor metastases were

observed in these areas. Additionally, this technique avoids the artifactual release of proteases that we observed using our original in situ zymography method during brain slice preparation. Such protease release, which may be linked to cell death during de-enervation, may alter the ECM structure and complicate the in vitro FRAP experiments. We have recently developed a method to deliver dye to the superficial cortex without the need for intracranial injection by topical application of dye to the cortex after durotomy. This method precludes cortical damage that may alter ECM structure and using a FITC solution we can label the extracellular space without labeling cells (Figure 10), therefore allowing the determination of ECM hindrance using FRAP.

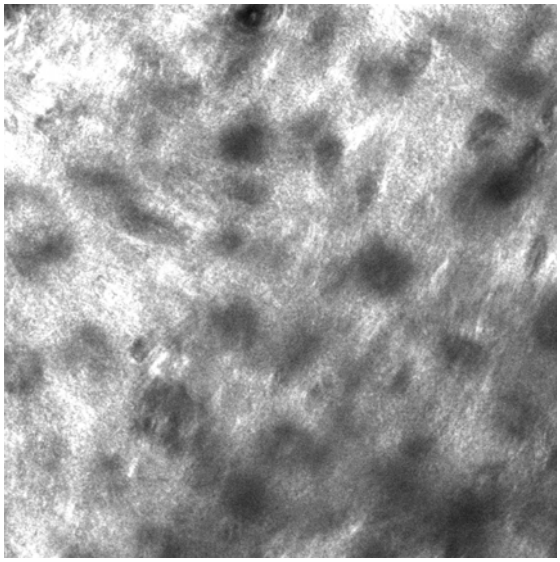

Figure 10. Labeling the extracellular space with fluorescent dye in the brian in vivo.

Two-photon image taken 50 µm below the level of the pia. The brain was exposed and the dura was removed. The cortex was bathed in solution of FITC for 30 minutes and then washed with saline. Notice that FITC accumulates in the extracellular space without entering cell bodies which appear dim.

In summary, while we encountered some unforeseen delays in establishing our mouse model of breast tumor metastasis to the brain, and spent a significant amount of time during this funding period establishing and troubleshooting this model, we have completed the analysis of the effects of one of the drugs (caffeine) on breast tumor metastasis to the brain (aim 1), and discovered that caffeine administration significantly alters the frequency of brain metastasis and their locations within the brain. We have also explored the effects of caffeine on the expression of proteases in the brain (aim 2), and discovered that caffeine administration significantly alters tPA activity in the brain parenchyma. Due to the effects of caffeine on the frequency of brain metastases, we are also currently exploring changes in the blood brain barrier during caffeine treatment. Finally, we have made progress towards developing methods for assaying the extracellular matrix diffusive hindrance in vivo, to determine whether caffeine alters the brain extracellular matrix structure (aim 3).

Key outcomes

1. We have established and characterized a murine model of breast tumor metastasis in our laboratory
2. We have shown that caffeine treatment alters the rate of breast tumor metastasis to the brain and the location of brain metastases but not their size.

3. We have shown the caffeine affects the activity of tPA although tPA activity increases after caffeine treatment, reversing our original working hypothesis.
4. We have established a novel method for assaying the brain extracellular matrix in vivo.

Reportable Outcomes

Presentations:

Zettel. M., Cash, S.S., Brown, E.B., Majewska, A. (2008) Intracardiac injection of MDA-MB-231BR cells in Nu/Nu mice as a model of breast tumor metastasis to the brain. *Soc. Neurosci. Abstr.*

Zettel. M., Cash, S.S., Brown, E.B., Majewska, A. (2008) The influence of neuronal activity on breast tumor metastasis to the brain. *DoD Era of Hope meeting*

Grants secured:

"Brain plasticity and its effects on breast tumor metastasis to the brain"
Principal Investigator: Anna Majewska *Effort:* 10%
Agency: DoD/BCRP *Period:* 09/01/09-08/31/10
Amount:

"Exploiting collagen organization to predict and prevent tumor metastasis"
Principal Investigator: Edward Brown *Effort:* 25%
Agency: NIH *Period:* 10/1/09-9/1/14
Amount

"Understanding collagen organization in breast tumors to predict and prevent metastasis."
Principal Investigator: Edward Brown *Effort:* 25%
Agency: DoD/BCRP *Period:* 9/1/09-8/1/14
Amount:

"The role of caffeine in breast tumor metastasis to the brain"
Principal Investigator: Anna Majewska *Effort:* 10%
Agency: American Institute for Cancer Research *Period:* 01/01/10-12/31/11
Amount:

Cell lines generated:

MDA-MB231BR-YFP

List of personnel:

Anna Majewska
Edward Brown
Sydney Cash (MGH)
Martha Zettel
Marie-Eve Tremblay
Yu Zhang

Conclusion

Our starting hypothesis was that neuronal activity within the brain could affect breast tumor metastasis to this organ. This hypothesis came out of our experiments with synapses (the structures that allow neurons to communicate with one another) that showed that decreasing neuronal activity led to release of tissue plasminogen activator (tPA), degradation of the extracellular matrix and increased synaptic structural changes. We postulated that since breast tumor metastasis is sensitive to the structure of the extracellular matrix such changes (along with the possible effects of tPA on the blood brain barrier) could affect breast tumor metastasis to the brain. Thus we sought to limit this metastasis by increasing brain activity and limiting the release of tPA. This approach of affecting metastasis indirectly by acting on the host tissue rather than the tumor cells themselves is novel and could provide a radical improvement in the treatment of tumors that target the brain, as the brain is largely protected from conventional chemotherapy but not from drugs which are already approved for use to alter brain activity in neurological diseases. The results reported here using caffeine, a known mild brain stimulant, validate this approach to brain tumor treatment and provide great hope in using this approach in the future. We showed that pre-treating animals with caffeine reduced the brain tumor burden twofold after introduction of human breast tumor cells to the mouse circulatory system. This finding is very exciting and we are in the process of testing other stimulants currently used in the clinic which might provide a more potent reduction of breast tumor metastasis to the brain. Additionally, we determined that caffeine increased the expression of tPA suggesting that our initial working hypothesis that increased brain activity would reduce breast tumor metastasis through reduced tPA expression is incorrect. We are currently looking at other proteases which are also sensitive to neuronal activity and could implement the changes we observe. Additionally, based upon the increase in metastasis seeding but not in tumor growth, we believe caffeine may affect the integrity of the blood brain barrier and we are currently testing this idea. These experiments may provide additional targets for therapeutics in the treatment of breast tumor metastasis.

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