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14. ABSTRACT

Androgen deprivation therapy is a well-established treatment for prostate cancer, but an important side effect of androgen deprivation therapy is impairment of memory and learning. The goal of this project was to use an animal model to test the hypothesis that impaired adult hippocampal neurogenesis underlies the androgen deprivation therapy-induced impairment of cognitive function. We found that three approaches used to reduce androgenic activity in patients suffering from prostate cancer (i.e., castration, leuprolide and flutamide) all decrease neuronal proliferation and neuronal survival in the dentate gyrus of the hippocampus in mice. Although adult hippocampal neurogenesis is thought to underlie various forms of memory and learning, especially spatial memory, the results of the experiments provided no evidence that androgen deprivation produces deficits in cognitive function. Both the serotonin-selective reuptake inhibitor antidepressant fluoxetine and the uncompetitive N-methyl-D-aspartate receptor antagonist memantine stimulate hippocampal neurogenesis. The results of the experiments show that fluoxetine can reverse castration-induced deficits in neuronal proliferation, but had no effect on neuronal survival. Therefore, the utility of these drugs in preventing or treating cognitive deficits in prostate cancer patients is questionable.

15. SUBJECT TERMS

Neurogenesis, neuron, hippocampus, memory, learning, testosterone, androgen, androgen deprivation, castration, prostate cancer, flutamide, leuprolide, proliferation, survival, immunohistochemistry, Western blot.

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1. INTRODUCTION

Androgen deprivation therapy is a well-established treatment for prostate cancer, but an important side effect of androgen deprivation therapy is impairment of memory and learning. In the hippocampus, a brain region that plays a major role in memory and learning, new nerve cells continue to develop throughout adulthood, a process called neurogenesis. The goal of this project is to use an animal model to test the hypothesis that impaired hippocampal neurogenesis underlies the androgen deprivation therapy-induced impairment of cognitive function. There are four specific aims. Specific Aim 1 tests the hypothesis that androgen deprivation decreases hippocampal neurogenesis. Specific Aim 2 tests the hypothesis that androgen deprivation disrupts cognitive behavior. Specific Aim 3 tests the hypothesis that drugs that increase hippocampal neurogenesis will reduce the effects of androgen deprivation on hippocampal neurogenesis and Specific Aim 4 tests the hypothesis that drugs that increase hippocampal neurogenesis will reduce the effects of androgen deprivation on cognitive behavior. The results of the proposed studies could lead to the development of strategies to optimize the physical and mental health of men with prostate cancer and improve the quality of life and well-being of prostate cancer patients and their families.

2. KEYWORDS

Neurogenesis, neuron, hippocampus, memory, learning, testosterone, androgen, androgen deprivation, castration, prostate cancer, flutamide, leuprolide, proliferation, survival, immunohistochemistry, Western blot.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1: To test the hypothesis that androgen deprivation decreases hippocampal neurogenesis.

Specific Aim 2: To test the hypothesis that androgen deprivation disrupts cognitive behavior.

Specific Aim 3: To test the hypothesis that drugs that increase hippocampal neurogenesis will reduce the effects of androgen deprivation on hippocampal neurogenesis.

Specific Aim 4: To test the hypothesis that drugs that increase hippocampal neurogenesis will reduce the effects of androgen deprivation on cognitive behavior.

What was accomplished under these goals?

The specific aims and the major tasks are as stated in the approved Statement of Work.

<u>Specific Aim 1: To test the hypothesis that androgen deprivation decreases hippocampal neurogenesis.</u>
Major Task 1: Treat Animals

Major Task 2: Sacrifice Animals, Tissue Processing and Data Analysis

Major Task 1 and Major Task 2 were completed.

Following the approved ACURO protocol, six-week-old, male C57/BL6 mice were housed on a 12/12-hour light/dark cycle with food and water available *ad libitum*. One week after arrival, the mice were divided randomly into one of four treatment groups: sham/placebo pellet, castration/placebo pellet, sham/flutamide pellet, and sham/leuprolide pellet. The mice were anesthetized and underwent castration or sham surgery. While still anesthetized, a placebo pellet, a pellet containing flutamide (an androgen receptor antagonist) or a pellet containing leuprolide (a gonadotropin-releasing hormone analog that reduces plasma testosterone levels) were implanted s.c. (i.e., all animals received pellet implants). At the completion of surgery the mice were injected with the analgesic buprenorphine and placed in a clean cage for recovery.

After one week of post-surgery recovery, in order to label proliferating cells the mice were injected with bromodeoxyuridine (BrdU). Twenty-eight days later (i.e., 35 days post castration) the mice were anesthetized and a blood sample withdrawn by cardiac puncture for the measurement of plasma levels of testosterone by

ELISA. The seminal vesicles were removed and weighed as a measure of androgenic activity. The mice were perfused with ice-cold paraformaldehyde and the brains were removed and stored. The left and right-side hemisphere of the brain were separated, cut into 20 µM coronal sections with a vibratome and stored immunohistochemical studies (IHC) to measure neuronal proliferation and neuronal survival.

Separate groups of mice were used for the Western blot (WB) studies (note that IHC and WB procedures cannot be carried out in the same subjects). They were treated as described above, including surgery and pellet implantation, above except they did not receive BrdU injections. On Day 35 post-surgery, mice were anesthetized, blood samples withdrawn by cardiac puncture for the measurement of plasma levels of testosterone and the seminal vesicles were removed and weighed. The brains were removed, the hippocampi (note that this this is the entire hippocampus and not limited to the dentate gyrus) were dissected and snap frozen in dry-ice and stored at -80°C for use in Western blot studies. The hippocampal tissues were processed and the intensity of each protein band was analyzed with software and corrected with the corresponding GAPDH level. The results were expressed as the fold of that of the control. See Figure 1 for a summary of the experimental design.

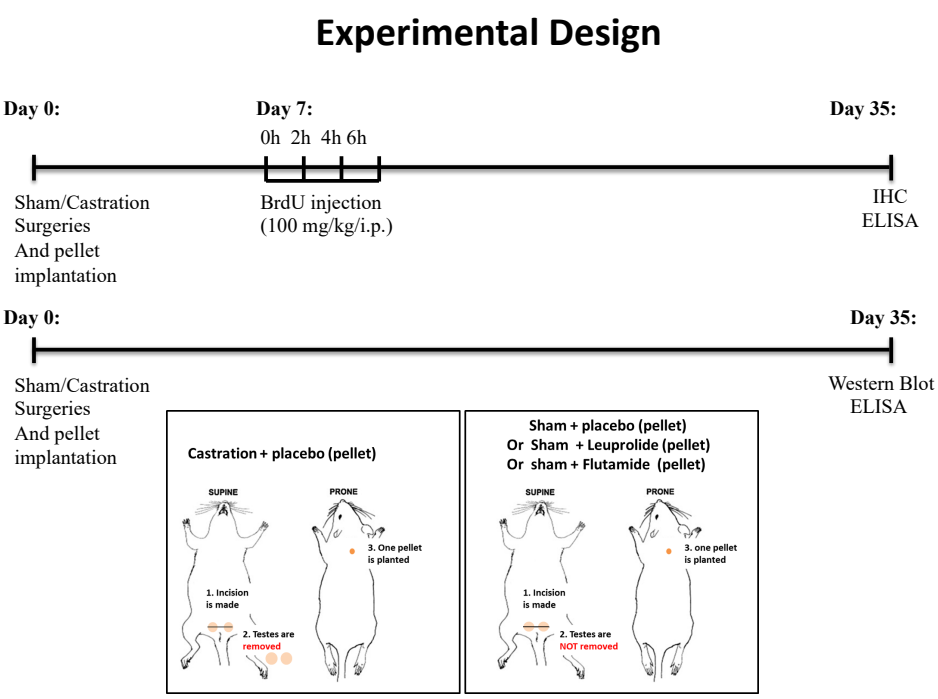

Fig. 1

For the IHC studies, Ki-67, a nuclear protein that is expressed in dividing cells, was used as a marker for neuronal proliferation. BrdU, administered 28 days before sacrifice, is taken up by dividing cells. In order to assess neuronal survival, the number of BrdU-positive cells that co-express NeuN, a marker of mature neurons, was determined. The number of Ki-67-positive and BrdU/ NeuN-positive cells in the subgranular zone of the dentate gyrus of the hippocampus were counted to determine neuronal proliferation and neuronal survival, respectively.

The data were analyzed by ANOVA followed by Bonferroni test to detect differences among treatment groups. Data were presented as means ± SEM. For all analyses the criterion for rejection of the null hypothesis was set at $p<0.05$.

Plasma levels of testosterone at the time of sacrifice were measured by ELISA in the separate IHC and Western blot studies to verify to success of the castration and drug treatment procedures. Analysis of plasma testosterone levels by ANOVA revealed significant difference among treatment groups in both the IHC [$F(3, 28) = 19.18$, $p<0.001$; Fig. 2A] and in the WB studies [$F(3, 28) = 14.50$, $p<0.001$; Fig. 2B]. Both

castration and treatment with leuprolide significantly reduced plasma levels of testosterone compared to controls.

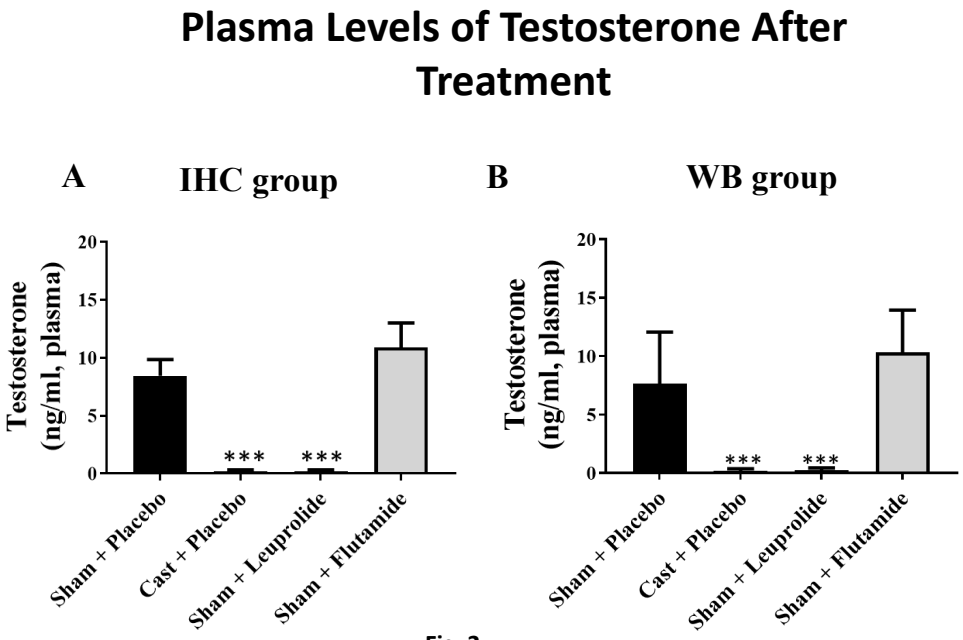

Fig. 2

Flutamide is an androgen receptor antagonist. It does not reduce plasma levels of testosterone, but blocks the androgenic effects of testosterone. The weight of the seminal vesicles can be used as a marker of androgenic activity. Analysis of dry weight of the seminal vesicles by ANOVA revealed significant difference among treatment groups in both the IHC [$F(3, 28) = 16.23$, $p<0.001$; Fig. 3A] and in the WB studies [$F(3, 12) = 26.26$, $p<0.001$; Fig. 3B]. Seminal vesicle weight was significantly reduced in the castrated, leuprolide- and flutamide-treated mice compared to controls. The results of these two experiments measuring plasma testosterone levels and seminal vesicle weight validate the experimental procedures. Note that all three procedures are used in androgen deprivation therapy in patients suffering from prostate cancer.

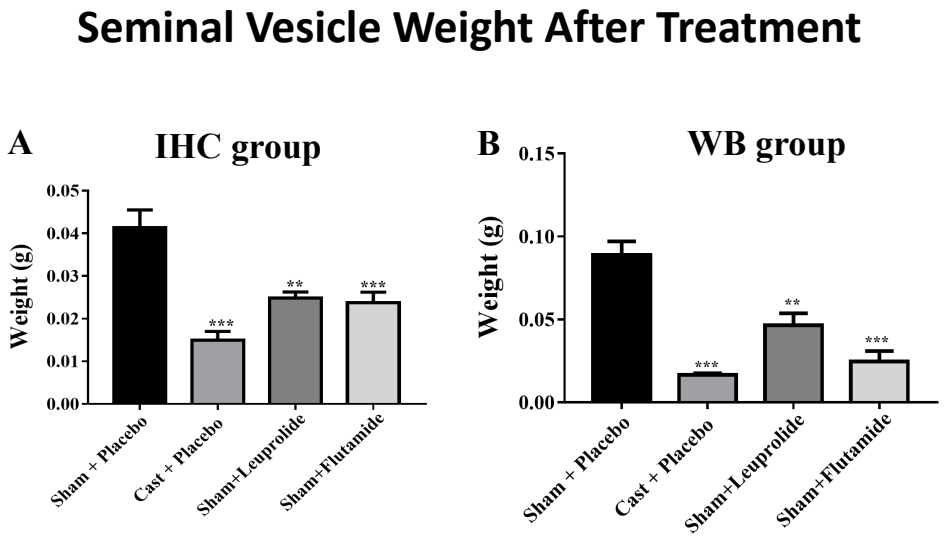

Fig. 3

Analysis of the number of cells expressing Ki-67 as a marker of neuronal proliferation in the subgranular zone of the dentate gyrus of the hippocampus revealed significant differences among treatment groups [$F(3, 28) = 10.94$, $p<0.001$; Fig. 4A, B]. Neuronal proliferation was significantly reduced in the castrated, leuprolide- and flutamide-treated mice compared to controls. The results of this experiment show that all treatments that reduce androgenic activity also decrease neuronal proliferation in the dentate gyrus of the hippocampus in mice.

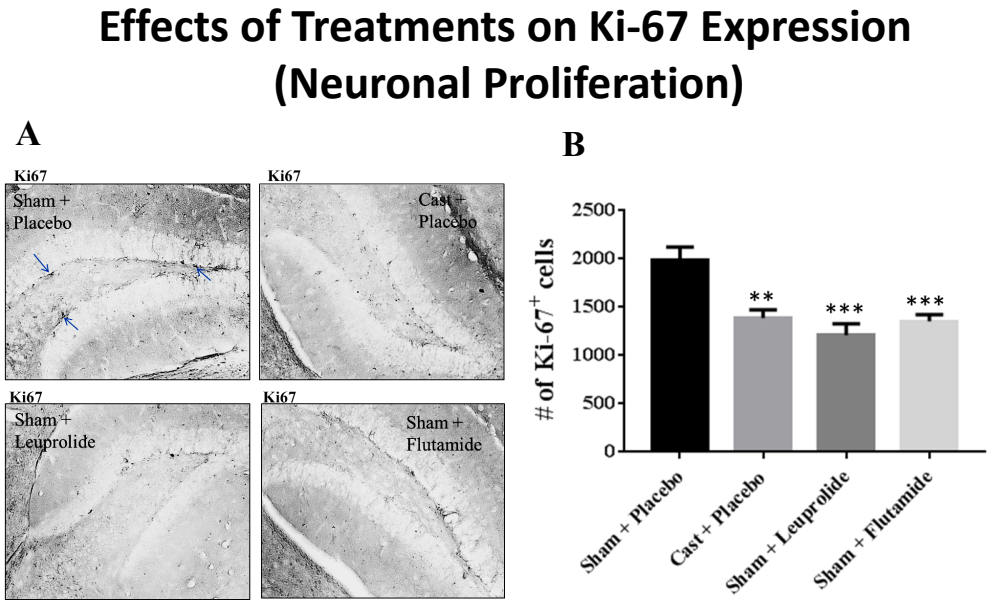

Fig. 4

Cells that co-express both BrdU and NeuN (BrdU/NeuN positive) reflect cells that proliferated and took up BrdU and matured (i.e., survived) into adult neurons. Analysis of the number of BrdU/NeuN positive cells in the subgranular zone of the dentate gyrus of the hippocampus revealed significant differences among treatment groups [$F(3, 28) = 14.79$, $p<0.001$; Fig. 5A, B]. Neuronal survival was significantly reduced in the castrated, leuprolide- and flutamide-treated mice compared to controls. The results of this experiment show that all treatments that reduce androgenic activity decrease neuronal survival in the dentate gyrus of the hippocampus in mice.

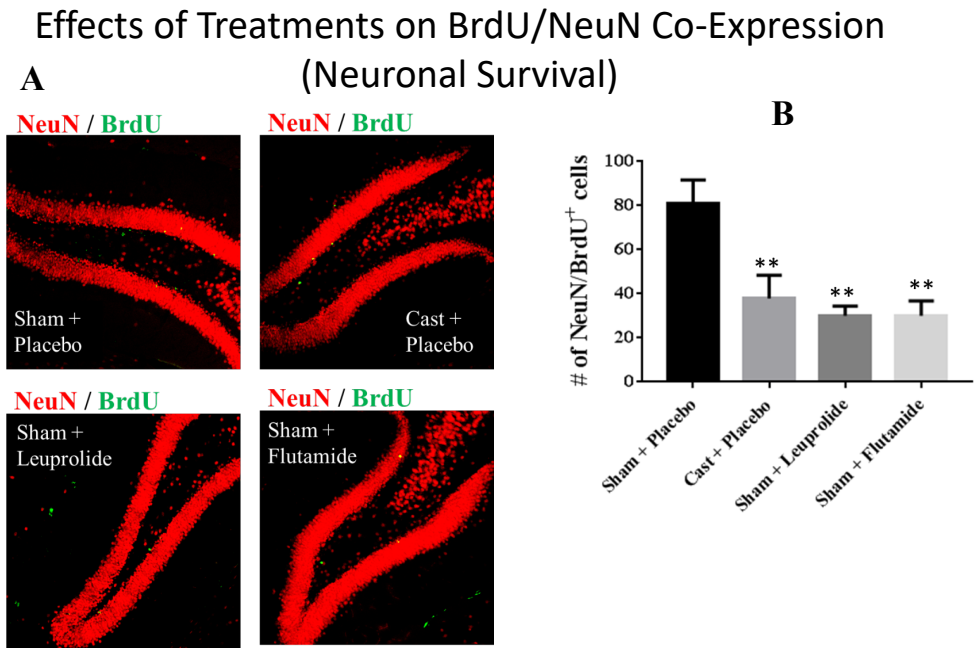

Fig. 5

In WB analysis, the levels of Ki-67 protein expression in the hippocampus (note that this is the entire hippocampus and not limited to the dentate gyrus) were significantly different among groups [$F(3, 40) = 7.292$, $p<0.001$; Fig. 6A]. Ki-67 protein expression was significantly reduced in the castrated, leuprolide- and flutamide-treated mice compared to controls. There was no difference in the protein expression of NeuN in the hippocampus among groups [$F(3, 40) = 0.04647$, $p>0.05$; Fig. 6B]. We believe that this is because the Western Blot analyses use the entire hippocampus, whereas the immunohistochemistry studies focus only on the subgranular zone of the dentate gyrus of the hippocampus. The changes in the dentate gyrus might be diluted out when the whole hippocampus is evaluated.

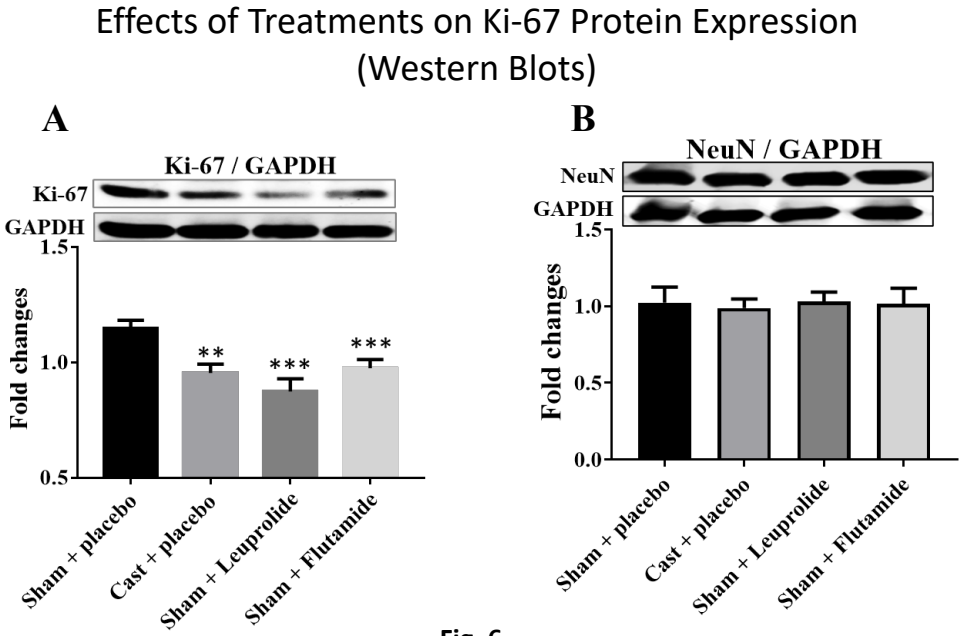

Fig. 6

Specific Aim 2: To test the hypothesis that androgen deprivation disrupts cognitive behavior.
Major Task 3: Treat Animals
Major Task 4: Behavioral Testing, Data Analysis and Manuscript Preparation

Major Task 3 was completed. With the exception of manuscript preparation, Major Task 4 was completed.

As described above, six-week-old, male C57/BL6 mice were housed on a 12/12-hour light/dark cycle with food and water available *ad libitum*. One week after arrival, the mice were divided randomly into one of four treatment groups: sham/placebo pellet, castration/placebo pellet, sham/flutamide pellet, and sham/leuprolide pellet. The mice were anesthetized and underwent castration or sham surgery. While still anesthetized, a placebo pellet, a pellet containing flutamide (an androgen receptor antagonist) or a pellet containing leuprolide (a gonadotropin-releasing hormone analog that reduces plasma testosterone levels) were implanted s.c. (i.e., all animals received pellet implants). At the completion of surgery the mice were injected with the analgesic buprenorphine and placed in a clean cage for recovery.

Five weeks later (i.e., 35 days post castration) the mice began behavioral testing. On the first (Day 1), the mice were evaluated using the Mouse Neurological Screen, a test provides a basic neurological assessment by evaluating sensory and neuromuscular function. On the following day (Day 2) they underwent the Open Field Test. The Open Field Test measures locomotor activity, and together with the Mouse Neurological Screen helps rule out nonspecific sensory/motor impairment that could affect performance on the other behavioral tests and confound the subsequent interpretation of the data. The next (Day 3) the mice were tested for spontaneous alternation using the Y-maze, a test that indicates deficits in spatial working memory. The Barnes maze was the final test and began the following day (Day 4). The Barnes maze assesses hippocampal-

dependent spatial memory and testing takes 9 days. An additional test, Novel Object Recognition, was added to the behavioral testing battery (note that the addition was approved by ACURO). This test also evaluates spatial memory and will be used in conjunction with the results obtained from the Y- and Barnes mazes. In the Novel Object recognition test the mice are first habituated to an object and then later evaluated in terms of time spent investigating the familiar object versus a novel objective.

After the completion of Barnes maze testing the mice were anesthetized, sacrificed by rapid decapitation, trunk blood was collected for the measurement of plasma levels of testosterone and seminal vesicles were removed and weighed. Because behavioral testing per se can affect neurogenesis, brain tissue was not collected.

Evaluation of plasma testosterone levels and/or seminal vesicle weights indicated that compared to controls androgenic activity was significantly reduced in all subjects who were castrated or received leuprolide or flutamide. The Mouse Neurological Screen revealed no gross differences in sensory and motor function among the treatment groups. In an Open Field Test of there was no differences among the treatment groups in locomotor activity in the periphery [F (3, 44) = 0.2518, P=0.8596; Fig. 7A] or the center of the apparatus [F (3, 44) = 0.1549, P=0.9260; Fig. 7B). Reduced activity in the center of the apparatus can be taken as an indicator of anxiety-like behavior, a finding which was not present. Taken together, the lack of significant differences among the treatment groups in the Mouse Neurological Screen and the Open Field Test suggest that the experimental treatments did not produce nonspecific sensory/motor impairment that could affect performance on the other behavioral tests and confound the subsequent interpretation of the data.

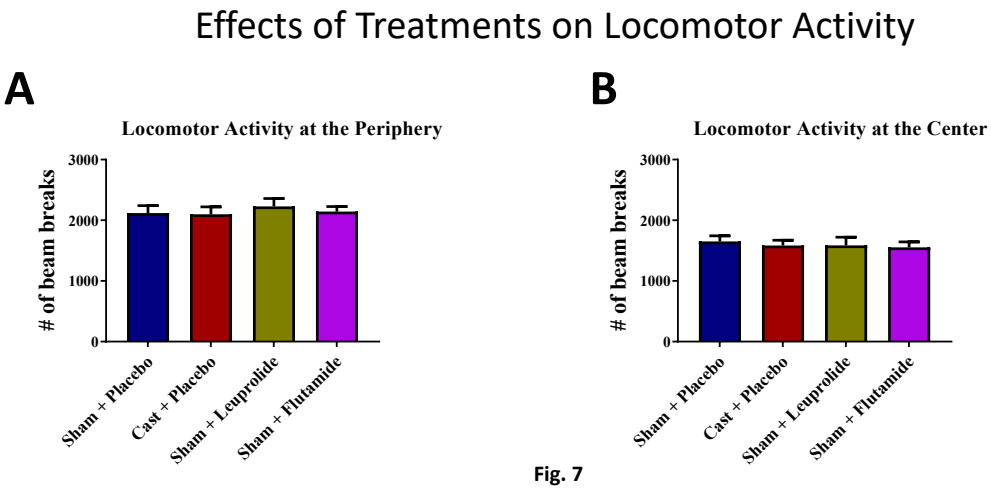

Fig. 7

In the Y-maze there were no significant differences in spontaneous alternation among the treatment groups (3, 44) = 0.7170, P=0.5471; Fig. 8A). In Novel Object Recognition test there were no significant differences in objective recognition among the treatment groups [F (3, 44) = 1.521, P=0.2224; Fig. 8B).

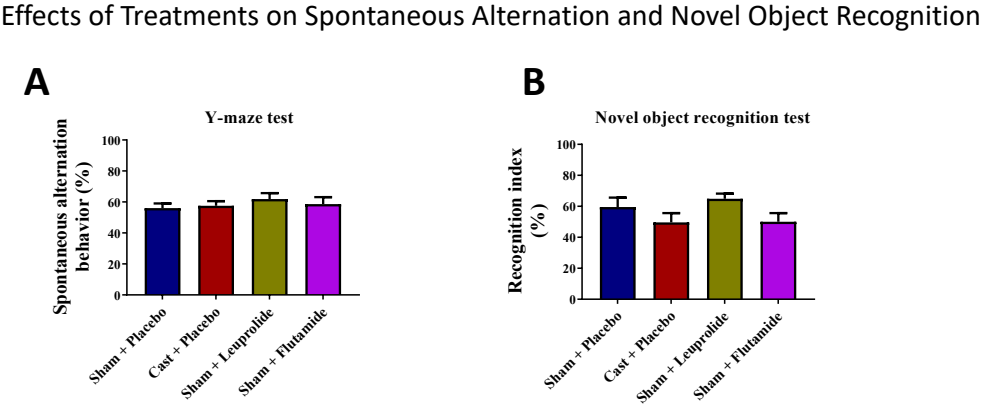

Fig. 8

The Barnes maze, there is a training phase of 4 days during which mice became progressively more efficient at finding the escape box in terms of committing fewer errors. The mice showed that they learned their performance improved [F (3, 176) = 45.70, p<0.001], but there were no significant differences among treatment groups (Fig. 9A). After a pause of 2 days (i.e., Day 7), the mice were assessed for memory retention. There were no significant differences among treatment groups in term of memory retention [F (1, 88) = 0.07544, P=0.7842; Fig. 9B]. This was followed on Days 8 and 9 by the reversal phase, in which the position of the escape box was altered to test the ability of the mice to re-learn a new location. There were no significant differences among treatment groups in the reversal phase [F (1, 88) = 6.547, P=0.0; Fig. 9B]. Taken together, the results of the behavioral experiments provide no evidence that androgen deprivation produces deficits in cognitive function.

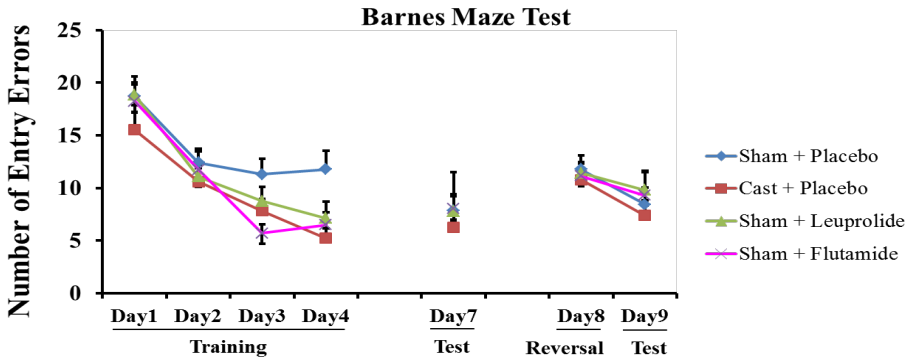

Fig. 9

Disruption of adult hippocampal neurogenesis is thought to be linked to deficits in spatial memory, and patients with cognitive impairment after androgen-deprivation therapy have problems with memory. Our results were not expected and there could be several explanations. First, the effects on behavior could be too subtle to detect using these tests of spatial memory. Other more sensitive tests could be used. It is important to point out that the effects in human are rather subtle in nature. Second, patients who have prostate cancer and undergo androgen deprivation therapy tend to be older, whereas our experimental animals were young. Therefore, the animal model using young mice might not have been appropriate. Normal age-related deficits in prostate cancer patients could have additive or synergistic effects with the effects of androgen deprivation on memory. Studies could be carried out in old mice to test whether this could be true. Third, it is possible that androgen deprivation-induced decreases in neurogenesis might not occur in and/or such decreases might be linked to memory deficits in humans.

Specific Aim 3: To test the hypothesis that drugs that increase hippocampal neurogenesis will reduce the effects of androgen deprivation on hippocampal neurogenesis.
Major Task 5: Treat Animals
Major Task 6: Sacrifice Animals, Tissue Processing and Data Analysis

Major Task 5 and Major Task 6 were completed.

Both the serotonin-selective reuptake inhibitor antidepressant fluoxetine and the uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist memantine stimulate hippocampal neurogenesis. The purpose of this experiment is to determine whether these drugs will reduce or block androgen deprivation-induced decreases in hippocampal neurogenesis.

One week after arrival the mice were divided randomly into one of six treatment groups: sham/vehicle, sham/fluoxetine, sham/memantine, castration/vehicle, castration/fluoxetine and castration/memantine. Mice that were castrated or undergo sham surgery were treated as described above except pellets (i.e., placebo, leuprolide or flutamide) were not implanted. Beginning immediately after castration (or sham) surgery the mice began to receive fluoxetine (18 mg/kg/day) or memantine (7.5 mg/kg/day) in their drinking water and this

continued for the duration of the study. One week after recovery from surgery (and after initiating drug administration) the mice were injected with BrdU as described in Specific Aim 1. Four weeks after BrdU administration the mice were anesthetized, a blood sample was taken for the measurement of plasma levels of testosterone, and in this experiment plasma levels of fluoxetine or memantine also were measured using gas chromatography/mass spectrometry (GC/MS). The seminal vesicles were removed and weighed and the mice sacrificed by cardiac perfusion with paraformaldehyde. The brains were removed and sections processed using standard IHC procedures as described in Specific Aim 1.

A second set of mice were used for Western blot analyses. They were treated as described above, including surgery and drug administration, above except they did not receive BrdU injections. Five weeks after surgery the mice were sacrificed, trunk blood was collected for the measurement of plasma levels of testosterone and fluoxetine or memantine and the seminal vesicles weighed. The brains were removed and rapidly cooled in ice-cold saline. The hippocampi were dissected out and processed for Western blot analyses of proteins as described in Specific Aim 1.

Evaluation of plasma testosterone levels and/or seminal vesicle weights indicated that compared to controls androgenic activity was significantly reduced in all subjects who were castrated. Means plasma levels of fluoxetine in the experimental subjects were 285.7 ± 29.59 ng/ml and mean plasma levels of memantine were 78.57 ± 29.59 ng/ml. The therapeutic levels of fluoxetine in humans are within the range of 91 – 302 ng/ml, whereas the therapeutic levels of memantine are within the range of 16 – 264 ng/ml. Therefore, the plasma levels of the drugs that were achieved in the mice were within the range found in humans.

In WB analysis, the levels of Ki-67 protein expression in the hippocampus were significantly different among treatment groups [$F(5, 42) = 22.54$, $p<0.001$; Fig. 10]. Ki-67 protein expression in the castration plus vehicle group was significantly reduced compared to that of sham plus vehicle group mice. In the castrated mice, treatment with fluoxetine reversed the reduction in Ki-67 found in the vehicle-treated group. Treatment with memantine had no effect on castration-induced decreases in Ki-67 protein expression.

Effects of Fluoxetine and Memantine on Castration-Induced Decreases in Ki-67 Protein Expression

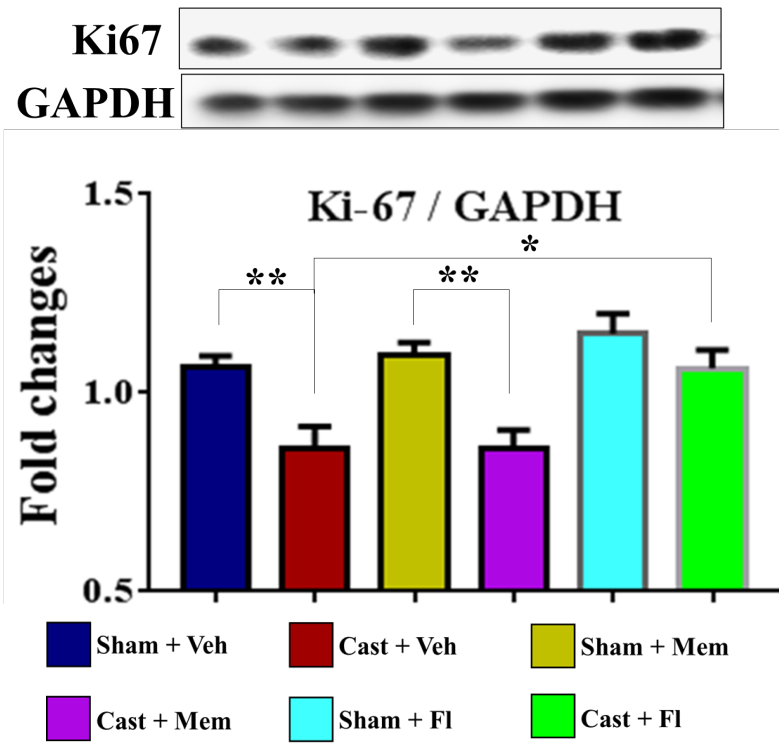

Fig. 10

Analysis of the number of BrdU/NeuN positive cells in the subgranular zone of the dentate gyrus of the hippocampus revealed significant differences among treatment groups [F (5, 42) = 34.36, p<0.001; Fig. 11]. Neuronal survival in the castration plus vehicle group was significantly reduced compared to that of sham plus vehicle group. Neither treatment with fluoxetine nor memantine increased neuronal survival. The results of this experiment show that memantine has no effect on neuronal proliferation or survival after androgen deprivation (i.e., castration). Although fluoxetine reversed the androgen deprivation-induced decrease in neuronal proliferation, it had no effects on neuronal survival.

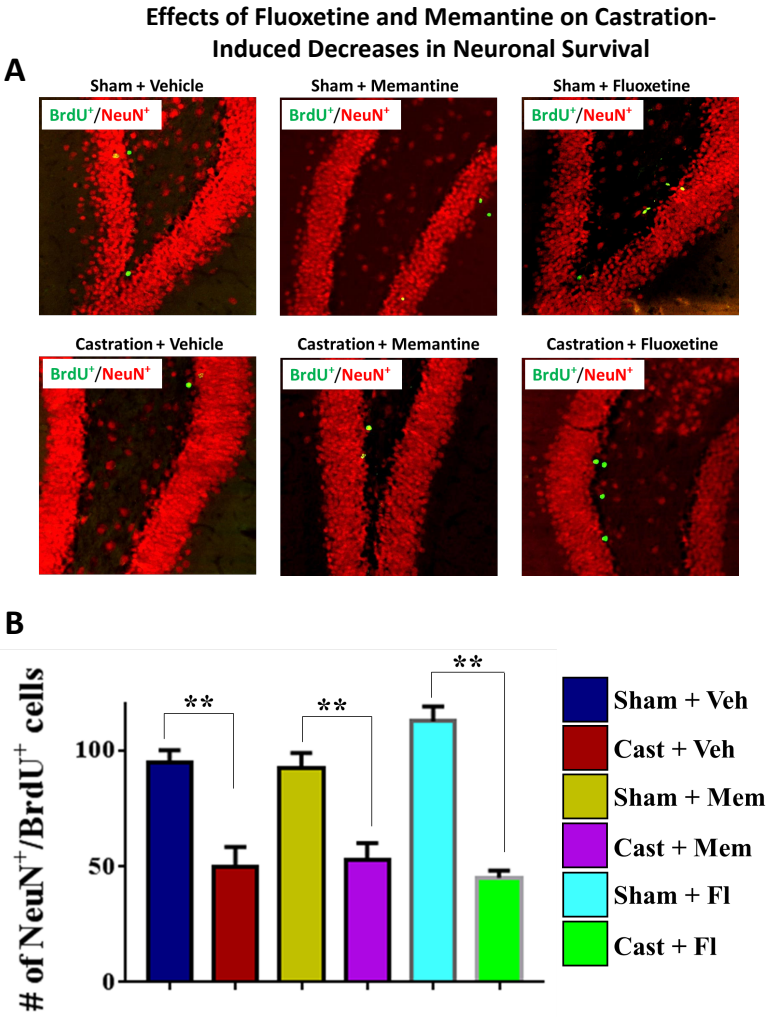

Fig. 11

There are a number of critical steps involved in adult neurogenesis in the subgranular zone of the dentate gyrus of the hippocampus. In this region neuronal stem cells first proliferate and then differentiate into immature neurons. Approximately 60% of the newborn cells that don't terminally differentiate do not survive and die within one week of their generation (46). Neurons that survive migrate into the granular cell layer of the dentate gyrus, develop the morphological and functional properties of granule cell neurons and become integrated into the existing neuronal circuitry. It is these mature neurons that express NeuN. The data from this experiment] indicate that fluoxetine can reverse the effects of androgen deprivation, but has no effect on neuronal survival This finding suggests that androgen deprivation must affect another critical step or steps in adult hippocampal neurogenesis besides impairing neuronal proliferation.

Specific Aim 4: To test the hypothesis that drugs that increase hippocampal neurogenesis will reduce the effects of androgen deprivation on cognitive behavior.

Major Task 7: Treat Animals

Major Task 8: Behavioral Testing, Data Analysis and Manuscript Preparation

Because of the clear-cut lack of effect of the androgen deprivation on behavior found in Specific Aim 2, we chose not to pursue the behavioral experiments in Specific Aim 4 because it would not be a productive use of time and effort.

What opportunities for training and professional development has the project provided?
Although the project was not specifically intended to provide training and professional development, having medical and undergraduate students assist with the work provided an excellent training opportunity for learning about experimental design, carrying out Western blot and immunohistochemistry procedures and data analysis. The students were mentored by the Principal Investigator and the Research Scientist. The following medical students contributed to the research effort by working on the project as volunteers: Ryan Shota, Derek Oh and Austin Alquisola. Mr. Oh was awarded a competitive summer research scholarship from Western University to support his work on the project. In addition, these students presented some of the research findings at a student research forum that was part of the Western Medical Research Conference (see abstract below). The following undergraduate students also volunteered to work on the project: Jonathon Jo, Ekatarina Smith, Mary Van Schaick, Morgan McCoy, Adrienne Jo, Lily Harris and Joshua Chan.

How were the results disseminated to communities of interest?
The results were disseminated by presentations at the following meetings (see Appendix):

1. "The Effects of Androgen Deprivation Therapy on the Adult Hippocampal Neurogenesis and Cognition in Mice." Alkam, T., Atkinson, K., Jo, J., Chan, J., Smith, E. and Pechnick, R.N. Presented at the annual meeting of the Society for Neuroscience, San Diego, CA, November, 2018.

2. "Androgen Deprivation Disrupts Adult Hippocampal Neurogenesis". Alkam, T., Jo, J. and Pechnick, R.N. Presented at the 7th Mediterranean Neuroscience Conference, Marrakech, Morocco. June 2019.

3. "Disruption of Adult Hippocampal Neurogenesis Following Androgen Deprivation". Alkam, T., Jo, J., Shota, R., Oh, D., Alquisola, A. and Pechnick, R.N. Presented at the annual meeting of the American College of Neuropsychopharmacology, Orlando, FL, December, 2019.

4. "The Selective Serotonin Reuptake Inhibitor Fluoxetine Reverses Androgen Therapy-Induced Disruption of Hippocampal Neurogenesis". Shota, R., Oh, D., Alquisola, A., Jo, J., Alkam T. and Pechnick, R.N. Presented at the Western Medical Research Conference, Carmel, CA, January, 2020. Published in J. Investigative Medicine 68 (Suppl 1):A60, 2020.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to report.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Our finding that all three treatments tested (castration, leuprolide and flutamide) reduce both neuronal proliferation and survival in the subgranular zone of the dentate gyrus of the hippocampus is significant because all three methods are used in the treatment of prostate cancer in humans. This suggests that patients might show similar deficits in hippocampal neurogenesis. This provides a proof-of concept that could drive the field forward in a number of ways. Lack of evidence of cognitive deficits in the experiments conducted might be due to the limitations of the animal model and suggests that other models should be utilized to study this phenomenon. Furthermore, it does not rule out the possibility that impaired hippocampal neurogenesis might cause or contribute to the androgen deprivation therapy-induced impairment of cognitive function in humans. Currently little is known regarding the fundamental mechanism(s) underlying androgen deprivation therapy-induced impairment of adult hippocampal neurogenesis, and this represents a gap in our knowledge base. The finding that fluoxetine can reverse the effects of androgen deprivation, but has no effect on neuronal survival suggests that androgen deprivation must affect another critical step or steps in adult hippocampal neurogenesis besides impairing neuronal proliferation. These exact mechanisms by which androgen deprivation impair adult hippocampal neurogenesis need to be defined.

What was the impact on other disciplines?
It is hoped that the findings will stimulate further research in the area by other researchers. The experiments were designed to increase their translational impact. First, we chose to compare and contrast the effects of three approaches to androgen deprivation therapy that are used in prostate cancer patients. Second, we tested two drugs the stimulate hippocampal neurogenesis, fluoxetine and memantine, and both these drugs are approved for the treatment of other clinical conditions. Thus there is no need for new drug development and safety testing. The finding that fluoxetine reversed castration-induced decreases in neuronal proliferation is provocative. A clinical trial could easily be conducted to test whether fluoxetine blocks or reverses cognitive impairment caused by androgen deprivation therapy in prostate cancer patients.

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

What was the impact on society beyond science and technology?
The present study should increase public awareness of the possible negative consequences of the use of androgen deprivation therapy in the treatment of prostate cancer and the potential impact on the quality of life.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
None

Actual or anticipated problems or delays and actions or plans to resolve them
We had two problems while carrying out the studies that negatively impacted the rate of progress of the work. The first involved the mice used in the studies. All mice that are received from the vendor are subject to in-house quarantine for seven days. With one of our shipments several of the mice died within two days of arrival. We immediately contacted our in-house Veterinarian and he strongly suggested not to use the other animals in the shipment because they might be diseased and/or had undergone problems during shipping (e.g., over-heating and/or dehydration). A necropsy of the dead mice yielded no definitive findings. Subsequently, we ordered another shipment of mice, but again several mice died within two days of arrival and our Veterinarian suggested that we not use the other mice in the shipment. We contacted the Veterinarian at the vendor, Charles River Laboratories, and he requested that we send them the dead mice for necropsy and histological analyses. After several weeks the reports came in and there was no definitive finding of disease or other reasons for the unexpected loss of the mice. Although we considered switching to another vendor, this would cause problems because mice from different vendors can show subtle differences that could affect or confound the interpretation of the data. We requested that the vendor ship fewer mice per shipping container and this alleviated the situation. We also had problems with the male mice fighting after arrival. In some cases the wounds were severe enough that the subjects had to be deleted from the experiment and required ordering replacement animals. After further research, consultation with our Veterinarian and the supplier, we began to order litter mates for each experiment. This reduced the problem, however; litter mates take longer for delivery, i.e., 5-6 weeks. We also found that some mice in the leuprolide-treated group had a tendency to fight. In consultation with our Veterinarian, we concluded that this might be due to a surge in testosterone and associated aggressiveness that can occur after the initiation of treatment with leuprolide. We closely monitored the mice on a more regular basis and this solved the problem. These issues were all documented in the annual reports.

Second, due to COVID Western University and the laboratory were closed down effective 3/15/2020. This had a negative impact on the work that was to occur from this date until the end of the no cost extension (7/30/2020).

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
Nothing to report.

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

6. PRODUCTS

Publications, conference papers, and presentations

1. "The Effects of Androgen Deprivation Therapy on the Adult Hippocampal Neurogenesis and Cognition in Mice." Alkam, T., Atkinson, K., Jo, J., Chan, J., Smith, E. and Pechnick, R.N. Presented at the annual meeting of the Society for Neuroscience, San Diego, CA, November, 2018.

2. "Androgen Deprivation Disrupts Adult Hippocampal Neurogenesis". Alkam, T., Jo, J. and Pechnick, R.N. Presented at the 7th Mediterranean Neuroscience Conference, Marrakech, Morocco. June 2019.

3. "Disruption of Adult Hippocampal Neurogenesis Following Androgen Deprivation". Alkam, T., Jo, J., Shota, R., Oh, D., Alquisola, A. and Pechnick, R.N. Presented at the annual meeting of the American College of Neuropsychopharmacology, Orlando, FL, December, 2019.

4. "The Selective Serotonin Reuptake Inhibitor Fluoxetine Reverses Androgen Therapy-Induced Disruption of Hippocampal Neurogenesis". Shota, R., Oh, D., Alquisola, A., Jo, J., Alkam, T and Pechnick, R.N. Presented at the Western Medical Research Conference, Carmel, CA, January, 2020. Published in J. Investigative Medicine 68 (Suppl 1):A60, 2020.

Journal publications.
Nothing to report. Manuscript preparation currently is under way.

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

Other publications, conference papers and presentations.
Nothing to report.

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?

Robert N. Pechnick, Ph.D. Principal Investigator – no change

Tuerxun Ailikemu, M.D., Ph.D. Research Scientist – no change

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.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
N/A

QUAD CHARTS:
N/A

9. APPENDICES

Copies of Meeting Presentations

1. "The Effects of Androgen Deprivation Therapy on the Adult Hippocampal Neurogenesis and Cognition in Mice." Alkam, T., Atkinson, K., Jo, J., Chan, J., Smith, E. and Pechnick, R.N. Presented at the annual meeting of the Society for Neuroscience, San Diego, CA, November, 2018.

2. "Androgen Deprivation Disrupts Adult Hippocampal Neurogenesis". Alkam, T., Jo, J. and Pechnick, R.N. Presented at the 7th Mediterranean Neuroscience Conference, Marrakech, Morocco. June 2019.

3. "Disruption of Adult Hippocampal Neurogenesis Following Androgen Deprivation". Alkam, T., Jo, J., Shota, R., Oh, D., Alquisola, A. and Pechnick, R.N. Presented at the annual meeting of the American College of Neuropsychopharmacology, Orlando, FL, December, 2019.

4. "The Selective Serotonin Reuptake Inhibitor Fluoxetine Reverses Androgen Therapy-Induced Disruption of Hippocampal Neurogenesis". Shota, R., Oh, D., Alquisola, A., Jo, J., Alkam T. and Pechnick, R.N. Presented at the Western Medical Research Conference, Carmel, CA, January, 2020. Published in J. Investigative Medicine 68 (Suppl 1):A60, 2020.

Presented at the annual meeting of the Society for Neuroscience, San Diego, CA, November, 2018.

The Effects of Androgen Deprivation Therapy on the Adult Hippocampal Neurogenesis and Cognition in Mice

Tursun Alkam, Kelley Atkinson, Jonathan Jo, Joshua Chan, Ekaterina Smith, Robert N. Pechnick

College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, Pomona, CA

Among the various therapeutic approaches, androgen deprivation therapy (ADT) is a well-established treatment for prostate cancer. Its goal is to lower levels of testosterone, the main factor driving the progression of prostate cancer. Although this treatment strategy can slow disease progression, patients receiving ADT show significant declines in executive functioning, spatial reasoning, spatial abilities and working memory. At the present time, however, little is known regarding the fundamental mechanisms underlying ADT-induced impairment of cognition. We hypothesize that the cognitive impairment observed in prostate cancer patients following ADT is due to treatment-induced reduction in hippocampal neurogenesis. In this study, in order to assess neuronal survival, the number of BrdU-positive cells and percentage of BrdU-positive cells that co-express NeuN, a marker of mature neurons was determined in the dentate gyrus of mice using immunofluorescent double-staining and confocal microscopy. Proliferation was assessed by counting the number of Ki-67-positive cells and percentage of Ki-67-positive cells that co-express nestin (a marker of proliferating neural progenitors) and doublecortin (DCX), a protein expressed in immature neurons (i.e., neuroblasts). We found that ADT, using either surgical or pharmacological castration (i.e., the androgen receptor antagonist flutamide or down-regulating the secretion of gonadotropins using leuprolide), affected both neuronal proliferation and survival. In addition, the effects of ADT on learning and memory were determined using open field activity, Y-maze, Barnes maze, and novel object recognition tests. The results of the present study might lead to the development of an animal model to help understand the pathophysiological processes underlying ADT in humans. (Supported by USAMRMC PC150494).

Presented at the 7th Mediterranean Neuroscience Conference, Marrakech, Morocco. June 2019.

Androgen Deprivation Disrupts Adult Hippocampal Neurogenesis

Tursun Alkam, Jonathan Jo and Robert N. Pechnick

Department of Basic Medical Sciences, College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, Pomona, CA, USA

Androgen deprivation therapy is a well-established treatment for prostate cancer. Almost half of all prostate cancer patients receive this therapy. An important side effect is impairment of cognitive function, with many patients showing significant declines in executive functioning, spatial abilities and working memory. In the dentate gyrus of the hippocampus, neural stem cells and progenitors proliferate and differentiate into new neurons throughout adulthood; a phenomenon known as adult neurogenesis. Adult hippocampal neurogenesis is thought to be involved in some aspects of cognition. The central hypothesis of this study is that reduced hippocampal neurogenesis underlies the androgen deprivation therapy-induced impairment of cognitive function. Androgen deprivation was produced in male mice by castration, the administration of the androgen receptor antagonist flutamide, or leuprolide, a gonadotropin-releasing hormone analog that reduces plasma testosterone levels. The mice were sacrificed 35 days after castration or the beginning of drug treatment. Using immunofluorescent double-staining and confocal microscopy, the number of BrdU-positive cells and percentage of BrdU-positive cells that co-express NeuN were used to determine neuronal survival, whereas proliferation was assessed by counting the number of Ki-67-positive cells and percentage of Ki-67-positive cells that co-express nestin and doublecortin. All three treatments decreased both neuronal proliferation and survival. In contrast, neurogenesis was not affected in the subventricular zone, another region where adult neurogenesis occurs. These results support the hypothesis that disruption of adult hippocampal neurogenesis might underlie some of the cognitive impairment found in prostate cancer patients undergoing androgen deprivation therapy.
Supported by DOD grant W81XWH-16-1-0429.

Presented at the annual meeting of the American College of Neuropsychopharmacology, Orlando, FL, December, 2019.

Disruption of Adult Hippocampal Neurogenesis Following Androgen Deprivation

Tursun Alkam, Jonathan Jo, Ryan Shota, Derek Oh, Austin Alquisola
and Robert N. Pechnick

Department of Basic Medical Sciences, College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, Pomona, CA, USA

At some point almost half of all prostate cancer patients receive androgen deprivation therapy. This involves surgical castration, the administration of an androgen receptor antagonist or a gonadotropin-releasing hormone analog that reduces plasma testosterone levels. However, an important side effect experienced in many patients is impairment of cognitive function. This is manifested by deficits in executive functioning, spatial abilities and working memory. In the dentate gyrus of the hippocampus, throughout adulthood neural stem cells and progenitors proliferate and differentiate into new neurons. This phenomenon is known as adult neurogenesis and is involved in some aspects of memory and learning. The goal of this study was to test the hypothesis that androgen deprivation disrupts adult hippocampal neurogenesis and to compare and contrast three approaches to androgen deprivation that are analogous to how human prostate cancer patients are treated. Androgen deprivation was produced in male mice by surgical castration, the administration of the androgen receptor antagonist flutamide, or leuprolide, a gonadotropin-releasing hormone analog (n=8/group). Sham surgery and saline-treated controls were included in the experimental design. The mice were sacrificed 35 days after castration or the beginning of drug treatment. Using immunofluorescent double-staining and confocal microscopy, the number of BrdU-positive cells and percentage of BrdU-positive cells that co-express NeuN were used to determine neuronal survival, whereas proliferation was assessed by counting the number of Ki-67-positive cells and percentage of Ki-67-positive cells that co-express nestin and doublecortin. The data will be analyzed by ANOVA, followed where appropriate by Newman-Keuls tests to detect differences among treatment groups. The criterion for rejection of the null hypothesis will be set at $p<0.05$. All three treatments disrupted hippocampal neurogenesis as both neuronal proliferation and survival were decreased in the dentate gyrus of the hippocampus. In contrast, neurogenesis was not affected in the subventricular zone, another region where adult neurogenesis occurs. As adult hippocampal neurogenesis is involved in memory and learning, these results support the conjecture that disruption of adult hippocampal neurogenesis might underlie some of the cognitive impairment found in prostate cancer patients undergoing androgen deprivation therapy. Supported by DOD grant W81XWH-16-1-0429.

Presented at the Western Medical Research Conference, Carmel, CA, January, 2020. Published in J. Investigative Medicine 68 (Suppl 1):A60, 2020.

The Selective Serotonin Reuptake Inhibitor Fluoxetime Reverses Androgen Therapy-Induced Disruption of Hippocampal Neurogenesis

Ryan Shota, Derek Oh, Austin Alquisola, Jonathan Jo, Tursun Alkam, Robert N. Pechnick

Purpose of Study:
Cognitive impairment (CI) is an important side effect of androgen deprivation therapy (ADT), a widely used treatment for prostate cancer. Previous studies show androgen deprivation reduces adult hippocampal neurogenesis (AHN), whereas selective serotonin reuptake inhibitors (SSRIs) stimulate it. Therefore, SSRIs may be a potential therapeutic approach to preventing and/or treating disruption of AHN and the subsequent CI. This investigation sought to test the hypothesis that the SSRI, fluoxetine, can reduce or block androgen deprivation-induced reduction of AHN.

Methods Used:
Mice were randomly divided into four groups: sham/vehicle, sham/fluoxetine, castration/vehicle, and castration/fluoxetine. They underwent castration or sham surgery and were then given fluoxetine in their drinking water or plain drinking water. The mice were sacrificed five weeks post-surgery and their brains harvested for Western blot analyses of Ki-67 (marker for neuron proliferation), doublecortin (DCX, protein expressed in immature neurons and neuron precursors), and NeuN (protein expressed in mature neurons). GAPDH (a housekeeping protein) was used as a loading control.

Summary of Results:
Our investigation showed decreased Ki-67 protein levels in castration/vehicle compared to the sham/vehicle group. The decrease in Ki-67 in the castration/vehicle groups was prevented in the castration/fluoxetine group. Furthermore, there were no differences between sham/vehicle and castration/fluoxetine groups. There were no differences in NeuN and DCX levels among the four treatment groups.

Conclusions:
The results suggest that treatment with fluoxetine reduced the effects of ADT on AHN. It will be important to test whether other drugs that stimulate AHN also are effective in reducing the effects of ADT and whether they can reverse the effects once they develop. This approach may be useful in preventing and/or treating ADT-induced CI in patients with prostate cancer.

Disclosures page:
Funding for this study was provided by DOD grant W81XWH-16-1-042.