2008

Androgen and Estrogen (α) Receptor Localization on Periaqueductal Gray Neurons Projecting to the Rostral Ventromedial Medulla in the Male and Female Rat

Dayna R. Loyd

Anne Z. Murphy PhD
Georgia State University, amurphy@gsu.edu

Follow this and additional works at: https://scholarworks.gsu.edu/neurosci_facpub

Part of the Neuroscience and Neurobiology Commons

Recommended Citation

This Article is brought to you for free and open access by the Neuroscience Institute at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Neuroscience Institute Faculty Publications by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.
Abstract: The periaqueductal gray (PAG) is involved in many gonadal steroid-sensitive behaviors, including responsiveness to pain. The PAG projects to the rostral ventromedial medulla (RVM), comprising the primary circuit driving pain inhibition. Morphine administered systemically or directly into the PAG produces greater analgesia in male compared to female rats, while manipulation of gonadal hormones alters morphine potency in both sexes. It is unknown if these alterations are due to steroidal actions on PAG neurons projecting to the RVM. The expression of androgen (AR) and estrogen (ERα) receptors in the PAG of female rats and within this descending inhibitory pathway in both sexes is unknown. The present study used immunohistochemical techniques (1) to map the distribution of AR and ERα across the rostrocaudal axis of the PAG; and (2) to determine whether AR and/or ERα were colocalized on PAG neurons projecting to the RVM in male and female rats. AR and ERα immunoreactive neurons (AR-IR, ERα-IR) were densely distributed within the caudal PAG of male rats, with the majority localized in the lateral/ventrolateral PAG.
Females had significantly fewer AR-IR neurons, while the quantity of ERα was comparable between the sexes. In both sexes, approximately 25-50% of AR-IR neurons and 20-50% of ERα-IR neurons were retrogradely labeled. This study provides direct evidence of the expression of steroid receptors in the PAG and the descending pathway driving pain inhibition in both male and female rats and may provide a mechanism whereby gonadal steroids modulate pain and morphine potency.
April 24, 2008

To Whom It May Concern:

This manuscript complies with all contemporary standards of ethical practice and scientific publication regarding such matters study design and ethical approval, data provity and fabrication, authorship, declaration of conflict of interest, plagiarism and redundant publication.

Sincerely,
Anne Z. Murphy
Assoc. Professor
June 26, 2008

Dr. Harry W.M. Steinbusch, Editor-in-Chief
Journal of Chemical Neuroanatomy
European Graduate School of Neuroscience
Department of Psychiatry and Neuropsychology
Maastricht University
Postbus 616, 6200 MD, Maastricht, The Netherlands

Dear Dr. Steinbusch:

We are resubmitting our manuscript [CHENEU-D-00030] entitled:

"Androgen and Estrogen (a) Receptor Localization on Periaqueductal Gray – Rostral Ventromedial Medullary Neurons in the Male and Female Rat",

for publication in the Journal of Chemical Neuroanatomy.

We were very pleased with the positive and thorough reviews of our manuscript, and are submitting a revised version including all major and minor changes as recommended by the three referees. The major changes are underlined in the text. A detailed list of all changes corresponding with each reviewers comments is given below.

Reviewer 1:

1. Sampling. For figure 2, on how many sections were cells counted from each rat for each level? One is not many. Was counting done blind to the treatment group? In cases where significant differences were found, can they be confirmed using repetitive sampling of number of cells per unit area (cell density), which may be more rigorous?

Response: The reviewer is right in that one section per rat per level is not many; we also thought that initially. However, the section we pick is highly representative for that specific level. In the past we have counted 3 sections per level and then averaged the cell counts. However as there is very little variability within a level, we did not consider the additional effort worthwhile. In addition, while the cell counts were conducted on one representative level, our n’s are reasonable (n=6 per sex) and our variability low. The experimenter was blind to the experimental condition and a sentence stating as much has now been added to the Data Analysis and Presentation section of the results. In levels where significant differences were noted, a 2nd observer blind to the condition counted the cells and confirmed our initial findings. A sentence stating this has been added.

2. Statistics: Unpaired t-test is not considered an appropriate post-hoc test for ANOVA. ANOVA is not an appropriate test for fractional data (Fig. 6).

Response: The reviewer is correct about the ANOVA on fractional data & we appreciate this correction. I actually teach stats so this is quite embarrassing. We have converted the
percentile data to standard scores and conducted the ANOVA on these data. Fishers PLSD was used for post-hoc analysis.

3. "PAG-RVM" is jargon and ambiguous. This phrase should be replaced in the title and throughout the manuscript using more precise and descriptive wording.
   
   Response: The term “PAG-RVM” has been replaced throughout the manuscript with either “PAG neurons projecting to the RVM” or “PAG output neurons” where appropriate.

4. How thoroughly were the injection sites in the RVM characterized? Was rostral-caudal, dorsal or lateral extent of the injection variable? How variable? And did this correlate with observations in the PAG?
   
   Response: The RVM injection site and criteria for analysis has been made more clear: “Analysis was limited to injection sites that occurred between the facial nucleus and the olivary complex across approximately 2mm rostrocaudally (Bregma -9.30 to -11.60).” No differences were noted in either the number or distribution of PAG FG+ cells within these constraints. In our studies using anterograde tracing from the PAG to the RVM, we have also noted that this region of RVM contains the highest density of anterogradely labeled fibers and is remarkably consistent throughout this mid-levels of RVM. RVM injections outside of the 2mm window were not analyzed.

5. The first paragraph of the discussion is overly repetitive with the Introduction. Bandler has written several reviews providing a greater synthesis of the function of the PAG that could be employed here.
   
   Response: The discussion has been modified and referenced as suggested.

6. P. 14 "there are numerous behavioral studies that support this hypothesis" is an overstatement. The studies subsequently referenced to do not attest to the specific participation of the PAG.
   
   Response: The statement has been replaced with “numerous behavioral studies indicating a role of gonadal steroids”.

7. The remainder of the discussion is overly long. Paragraphs should focus on specific aspects of the results and put them in context to the literature, rather then digress to reviewing the function of the PAG in general.
   
   Response: The discussion has been reduced.

8. Methods: references supporting the specificity of the antisera used should be added.
   
   Response: Antibody specificity, manufacturers info and references have been added to methods.

9. P. 11, Results. The description of dually labeled cells with respect to FG, AR or ER cells is poorly worded and becomes confusing. The specific denominator in these cases should be described better. "the percentage of AR colocalized with FG+ cells" is ambiguous. Also, this wording is problematic in the abstract "were colocalized with PAG-RVM neurons": phrasing should be more precise.
   
   Response: We now use 3 terms to more precisely describe the data: (1) IR neurons that were retrogradely labeled; (2) the percentage of receptor that was localized in retrogradely labeled cells; and (3) the percentage of retrogradely labeled cells that expressed receptor.
Abstract: First line, "behaviors, including pain and antinociception" is poorly worded. 'Pain' is a perception or sensation, rather than an action in response to a stimulus (a behavior).

Response: The first line of the abstract was altered to "behavior, including responsiveness to pain".

Reviewer 2:
1. Throughout the paper the authors use the generic term "estrogen" in place of estradiol. It is preferable (more accurate) to name the particular estrogen being referred to (usually estradiol in most rat studies), since "estrogens" are a class of hormone, not a specific hormone - see the Greenspan et al., Pain 2007 consensus paper (on which Dr. Murphy is a co-author!).

Response: We were using the term ‘estrogen’ to refer to all potential ligands that could bind to the estrogen receptor. However, we did note several occasions where we incorrectly used the term “estrogen” instead of “estradiol”. This has been corrected.

2. The authors should add a justification to the introduction that explains why they limited examination of ER to the alpha subtype rather than -beta (or both). Given that ER-beta has also been implicated in pain modulation, this should be acknowledged as a limitation of the present study, in the discussion.

Response: Unfortunately, as there were no reliable antibodies available commercially for identifying ERß at the time these studies were conducted, we were unable to include it in our analysis. We now address this in the introduction and discussion. To my knowledge, this is still true, which is why most people look at mRNA rather than protein.

3. The authors chose to use weight- rather than age-matched males and females, and provide an acceptable rationale for this choice; however, presumably AR and ER change with age, so it would be best to state what the ages of the males vs. females were, and indicate whether such an age difference might contribute to the observed sex differences.

Response: We now indicate the ages of the animals used. Age-related changes in steroid receptor expression are generally limited to aged animals (12-18 months of age). In this study, we used animals that were between 70-100 days old; therefore it is unlikely that there were any age-related changes in ERα or AR expression.

4. The use of multiple unpaired t-tests as post-hoc tests without any apparent adjustment of alpha is problematic, as it increases the chance of a Type I error (false significance). Instead, the authors should use a post-hoc test that is appropriate for multiple tests (e.g., Tukey or SNK), or adjust alpha via Bonferroni (though this method is likely far too conservative, so the former tests are preferable). Note also that on p. 9, the sentence describing unpaired t-tests is incorrect (should read "... used to determine specific group differences when a main effect or interaction was observed" or something like that). Finally, please provide precise p values instead of "p<0.05" - these values, along with the F values and df, provide the reader with a sense of the reliability/strength of each effect.

Response: The problem with our statistics has been addressed (see #2 above). Precise p values are now stated for significant results.

5. In each section of the results, the authors claim that there were "no qualitative or quantitative differences" between AR or ERα in females in different estrous stages. All of these sentences should be removed, as there is no way estrous stage could be reasonably examined in this
study (n was far too small/stage, and all females fell into only two stages). The inability to formally examine this variable given the present design should simply be acknowledged in the discussion.

Response: This section has been deleted and are now acknowledged in the discussion.

6. The discussion is FAR too long and should be condensed. The section on p. 15-16 could easily be deleted entirely - it is very speculative and far beyond the data. Moreover, the section following it ("Other Functional Considerations") needs to be cut in half at the very least. This section is dissertation- (or review-) appropriate only.

Response: The discussion has been reduced as recommended.

7. The references need adjustment: On p. 4 (and p.14), Stoffel et al., 2005 is cited as a study demonstrating gonadal hormone modulation of morphine antinociception, but morphine was not tested in that study (other opioids were). Additionally, the last sentence in the 1st paragraph should include Stoffel et al., 2003, as this study included hormone replacement manipulations.

Response: The references have been corrected to be accurate.

8. p. 5: Pittsburgh is misspelled; p. 7: mater is misspelled

Response: The misspellings have been corrected.

9. On the figures, it would be better to use open and closed symbols instead of (or in addition to) the different colors - many readers will print out your paper on a printer that does not allow for green and purple, in which case they won't be able to distinguish among the groups.

Response: The color figures 1, 2 and 4 have been changed to red and black. The different groups were identifiable when printed in black/white.

Reviewer 3:

1. It is well-known that AR and ER expression are, in part, responsive to circulating levels of gonadal steroid hormones. This is of particular importance in female rats, which normally exhibit dramatic changes in estrogen levels during the estrus cycle. The general accepted experimental design to account for the fluctuations is to compare OVX females-treated with vehicle or estrogen. Why didn't the authors choose this procedure?

Response: Previous studies examining hormonal changes in ERα expression used an antibody (H222) that was sensitive to the occupied versus unoccupied receptor. The antibody we used in the present study targets the C terminus, and therefore is not sensitive to changes in estradiol levels. Both my laboratory as well as Gloria Hoffman's laboratory has examined whether there are changes in ERα expression across the estrus cycle within several brain regions and found none.

2. The authors report that they examined vaginal cytology and state the female animals were in estrus (n=4) or proestrus (n=2), and conclude that AR and ER expression do not change across the estrous cycle. First, these low numbers are insufficient to warrant any conclusion about AR and ER protein expression during the estrous cycle. Second, there are no observation points during metestrus or diestrus phase of the estrous cycle.

Response: This section has been deleted and is now acknowledged in the discussion.
3. Why were unpaired t-tests used instead of a post-hoc test such as the Tukey’s test after main effects were found using the three-way ANOVA, which is much more an accepted procedure for statistical analysis?

Response: This has been corrected.

4. The retrograde data from the RVM to the PAG is very interesting, however, the authors have to specifically state how precise the injections were (i.e., number of misses versus number of hits). Of course the most animals receiving the retrograde injections must be hits.

Response: We have included more details about how we judged ‘hits’ versus ‘misses’.

5. The authors provide very specific data about the distribution of AR and ER at different anatomical levels in the PAG. However, how does the data look when it is expressed for the entire length of the PAG. Do the sex differences that are reported by the authors persist?

Response: Yes, the sex differences in AR expression were consistent across the rostrocaudal axis of PAG. We have now made this clearer in the Results.

6. The authors results showed that the presence of a sex difference in AR, but not in ER. However, the authors do not, but should, discuss the implication of the AR sex difference for the sex difference as related to the pain circuitry. Especially, the possible role of the androgenic metabolites of testosterone in the circulation.

Response: The implication for our observed sex differences in AR expression as it relates to the pain circuitry is now included in the Discussion.

7. Figure 3 is not required as long as the success rate of the RVM injections are stated.

Response: Figure 3 has been deleted as recommended.

In summary, we would like to thank the reviewers for taking the time and effort to review our manuscript. The reviews and comments were highly constructive, and we feel that the manuscript is much stronger with these revisions. We hope that you find our revised manuscript suitable for publication in the Journal of Chemical Neuroanatomy.

Sincerely,

Anne Z. Murphy, Ph.D
Associate Professor, Biology
Detailed Response to Reviewers:

Reviewer 1:

1. Sampling. For figure 2, on how many sections were cells counted from each rat for each level? One is not many. Was counting done blind to the treatment group? In cases where significant differences were found, can they be confirmed using repetitive sampling of number of cells per unit area (cell density), which may be more rigorous?

Response: The reviewer is right in that one section per rat per level is not many; we also thought that initially. However, the section we pick is highly representative for that specific level. In the past we have counted 3 sections per level and then averaged the cell counts. However as there is very little variability within a level, we did not consider the additional effort worthwhile. In addition, while the cell counts were conducted on one representative level, our n’s are reasonable (n=6 per sex) and our variability low. The experimenter was blind to the experimental condition and a sentence stating as much has now been added to the Data Analysis and Presentation section of the results. In levels where significant differences were noted, a 2nd observer blind to the condition counted the cells and confirmed our initial findings. A sentence stating this has been added.

2. Statistics: Unpaired t-test is not considered an appropriate post-hoc test for ANOVA. ANOVA is not an appropriate test for fractional data (Fig. 6).

Response: The reviewer is correct about the ANOVA on fractional data & we appreciate this correction. I actually teach stats so this is quite embarrassing. We have converted the percentile data to standard scores and conducted the ANOVA on these data. Fishers PLSD was used for post-hoc analysis.

3. "PAG-RVM" is jargon and ambiguous. This phrase should be replaced in the title and throughout the manuscript using more precise and descriptive wording.

Response: The term “PAG-RVM” has been replaced throughout the manuscript with either “PAG neurons projecting to the RVM” or “PAG output neurons” where appropriate.

4. How thoroughly were the injection sites in the RVM characterized? Was rostral-caudal, dorsal or lateral extent of the injection variable? How variable? And did this correlate with observations in the PAG?

Response: The RVM injection site and criteria for analysis has been made more clear: “Analysis was limited to injection sites that occurred between the facial nucleus and the olivary complex across approximately 2mm rostrocaudally (Bregma -9.30 to -11.60).” No differences were noted in either the number or distribution of PAG FG+ cells within these constraints. In our studies using anterograde tracing from the PAG to the RVM, we have also noted that this region of RVM contains the highest density of anterogradely labeled fibers and is remarkably consistent throughout this mid-levels of RVM. RVM injections outside of the 2mm window were not analyzed.

5. The first paragraph of the discussion is overly repetitive with the Introduction. Bandler has written several reviews providing a greater synthesis of the function of the PAG that could be employed here.

Response: The discussion has been modified and referenced as suggested.
6. P. 14 "there are numerous behavioral studies that support this hypothesis" is an overstatement. The studies subsequently referenced to do not attest to the specific participation of the PAG.

Response: The statement has been replaced with "numerous behavioral studies indicating a role of gonadal steroids".

7. The remainder of the discussion is overly long. Paragraphs should focus on specific aspects of the results and put them in context to the literature, rather than digress to reviewing the function of the PAG in general.

Response: The discussion has been reduced.

8. Methods: references supporting the specificity of the antisera used should be added.

Response: Antibody specificity, manufacturers info and references have been added to methods.

9. P. 11, Results. The description of dually labeled cells with respect to FG, AR or ER cells is poorly worded and becomes confusing. The specific denominator in these cases should be described better. "the percentage of AR colocalized with FG+ cells" is ambiguous. Also, this wording is problematic in the abstract "were colocalized with PAG-RVM neurons": phrasing should be more precise.

Response: We now use 3 terms to more precisely describe the data: (1) -IR neurons that were retrogradely labeled; (2) the percentage of receptor that was localized in retrogradely labeled cells; and (3) the percentage of retrogradely labeled cells that expressed receptor.

10. Abstract: First line, "behaviors, including pain and antinociception" is poorly worded. 'Pain' is a perception or sensation, rather than an action in response to a stimulus (a behavior).

Response: The first line of the abstract was altered to "behavior, including responsiveness to pain".

Reviewer 2:

1. Throughout the paper the authors use the generic term "estrogen" in place of estradiol. It is preferable (more accurate) to name the particular estrogen being referred to (usually estradiol in most rat studies), since "estrogens" are a class of hormone, not a specific hormone - see the Greenspan et al., Pain 2007 consensus paper (on which Dr. Murphy is a co-author!).

Response: We were using the term 'estrogen' to refer to all potential ligands that could bind to the estrogen receptor. However, we did note several occasions where we incorrectly used the term "estrogen" instead of "estradiol". This has been corrected.

2. The authors should add a justification to the introduction that explains why they limited examination of ER to the alpha subtype rather than -beta (or both). Given that ER-beta has also been implicated in pain modulation, this should be acknowledged as a limitation of the present study, in the discussion.

Response: Unfortunately, as there were no reliable antibodies available commercially for identifying ERβ at the time these studies were conducted, we were unable to include it in our analysis. We now address this in the introduction and discussion. To my knowledge, this is still true, which is why most people look at mRNA rather than protein.
3. The authors chose to use weight- rather than age-matched males and females, and provide an acceptable rationale for this choice; however, presumably AR and ER change with age, so it would be best to state what the ages of the males vs. females were, and indicate whether such an age difference might contribute to the observed sex differences.

Response: We now indicate the ages of the animals used. Age-related changes in steroid receptor expression are generally limited to aged animals (12-18 months of age). In this study, we used animals that were between 70-100 days old; therefore it is unlikely that there were any age-related changes in ER\textsubscript{\(\alpha\)} or AR expression.

4. The use of multiple unpaired t-tests as post-hoc tests without any apparent adjustment of alpha is problematic, as it increases the chance of a Type I error (false significance). Instead, the authors should use a post-hoc test that is appropriate for multiple tests (e.g., Tukey or SNK), or adjust alpha via Bonferroni (though this method is likely far too conservative, so the former tests are preferable). Note also that on p. 9, the sentence describing unpaired t-tests is incorrect (should read "used to determine specific group differences when a main effect or interaction was observed" or something like that). Finally, please provide precise p values instead of "p<0.05" - these values, along with the F values and df, provide the reader with a sense of the reliability/strength of each effect.

Response: The problem with our statistics has been addressed (see #2 above). Precise p values are now stated for significant results.

5. In each section of the results, the authors claim that there were "no qualitative or quantitative differences" between AR or ER\textsubscript{\(\alpha\)} in females in different estrous stages. All of these sentences should be removed, as there is no way estrous stage could be reasonably examined in this study (n was far too small/stage, and all females fell into only two stages). The inability to formally examine this variable given the present design should simply be acknowledged in the discussion.

Response: This section has been deleted and are now acknowledged in the discussion.

6. The discussion is FAR too long and should be condensed. The section on p. 15-16 could easily be deleted entirely - it is very speculative and far beyond the data. Moreover, the section following it ("Other Functional Considerations") needs to be cut in half at the very least. This section is dissertation- (or review-) appropriate only.

Response: The discussion has been reduced as recommended.

7. The references need adjustment: On p. 4 (and p.14), Stoffel et al., 2005 is cited as a study demonstrating gonadal hormone modulation of morphine antinociception, but morphine was not tested in that study (other opioids were). Additionally, the last sentence in the 1st paragraph should include Stoffel et al., 2003, as this study included hormone replacement manipulations.

Response: The references have been corrected to be accurate.

8. p. 5: Pittsburgh is misspelled; p. 7: mater is misspelled

Response: The misspellings have been corrected.

9. On the figures, it would be better to use open and closed symbols instead of (or in addition to) the different colors - many readers will print out your paper on a printer that does not allow for green and purple, in which case they won't be able to distinguish among the groups.
Response: The color figures 1, 2 and 4 have been changed to red and black. The different groups were identifiable when printed in black/white.

Reviewer 3:

1. It is well-known that AR and ER expression are, in part, responsive to circulating levels of gonadal steroid hormones. This is of particular importance in female rats, which normally exhibit dramatic changes in estrogen levels during the estrus cycle. The general accepted experimental design to account for the fluctuations is to compare OVX females-treated with vehicle or estrogen. Why didn't the authors choose this procedure?
Response: Previous studies examining hormonal changes in ER$\alpha$ expression used an antibody (H222) that was sensitive to the occupied versus unoccupied receptor. The antibody we used in the present study targets the C terminus, and therefore is not sensitive to changes in estradiol levels. Both my laboratory as well as Gloria Hoffman's laboratory has examined whether there are changes in ER$\alpha$ expression across the estrus cycle within several brain regions and found none.

2. The authors report that they examined vaginal cytology and state the female animals were in estrus (n=4) or proestrus (n=2), and conclude that AR and ER expression do not change across the estrous cycle. First, these low numbers are insufficient to warrant any conclusion about AR and ER protein expression during the estrous cycle. Second, there are no observation points during metestrus or diestrus phase of the estrous cycle.
Response: This section has been deleted and is now acknowledged in the discussion.

3. Why were unpaired t-tests used instead of a post-hoc test such as the Tukey's test after main effects were found using the three-way ANOVA, which is much more an accepted procedure for statistical analysis?
Response: This has been corrected.

4. The retrograde data from the RVM to the PAG is very interesting, however, the authors have to specifically state how precise the injections were (i.e., number of misses versus number of hits). Of course the most animals receiving the retrograde injections must be hits.
Response: We have included more details about how we judged 'hits' versus 'misses'.

5. The authors provide very specific data about the distribution of AR and ER at different anatomical levels in the PAG. However, how does the data look when it is expressed for the entire length of the PAG. Do the sex differences that are reported by the authors persist?
Response: Yes, the sex differences in AR expression were consistent across the rostrocaudal axis of PAG. We have now made this clearer in the Results.

6. The authors results showed that the presence of a sex difference in AR, but not in ER. However, the authors do not, but should, discuss the implication of the AR sex difference for the sex difference as related to the pain circuitry. Especially, the possible role of the androgenic metabolites of testosterone in the circulation.
Response: The implication for our observed sex differences in AR expression as it relates to the pain circuitry is now included in the Discussion.

7. Figure 3 is not required as long as the success rate of the RVM injections are stated.
Response: Figure 3 has been deleted as recommended.
* Potential Reviewers

Possible Reviewers

Rebecca M Craft, PhD  
Washington State Univ  
Dept Psychol  
PO BOX 644820  
Pullman WA 99164-4820  
Work Phone: 509-335-5040  
Fax:  509-335-5043  
E-mail Address:  craft@wsunix.wsu.edu

Richard J Bodnar, PhD  
Queens Col CUNY  
Dept Psychology  
65-30 Kissena Blvd  
Flushing NY 11367  
Work Phone:  718-997-3543  
Fax:  718-997-3257  
E-mail Address:  Richard_Bodnar@qc.edu

Peggy Mason, PhD  
Univ Chicago  
Dept Neurobiol, Pharmacol & Physiol  
947 E 58th St, MC 0926  
Chicago IL 60637  
Work Phone: 773-702-3144  
Fax:  773-702-1216  
E-mail Address:  pmason@midway.uchicago.edu

Kathryn G Commons, PhD  
Children' Hosp, Harvard MS  
Anesthesiology  
300 Longwood Ave Enders 1070  
Boston MA 02115  
Work Phone: 617-919-2220  
Fax:  617-730-0199  
E-mail Address:  kathryn.commons@childrens.harvard.edu
Androgen and Estrogen (α) Receptor Localization on Periaqueductal Gray Neurons Projecting to the Rostral Ventromedial Medulla in the Male and Female Rat

Dayna R. Loyd and Anne Z. Murphy

Department of Biology, Center for Behavioral Neuroscience
Georgia State University, PO Box 4010, Atlanta, Georgia 30302-4010

Running Title: AR and ERα Localization on PAG-RVM Pathway

Correspondence to: Anne Z. Murphy, Ph.D.
Dept. Biology,
Center for Behavioral Neuroscience
Georgia State University
PO Box 4010
Atlanta, GA 30303-4010
amurphy@gsu.edu

Phone: 404.413.5351
Fax: 404.413.2509

Key Words: gonadal steroid receptor, hormone, immunohistochemistry, sex differences, descending modulatory pathway, pain, antinociception

Grant Support: NIH grants DA16272 and AR49555 to AZM
ABSTRACT

The periaqueductal gray (PAG) is involved in many gonadal steroid-sensitive behaviors, including responsiveness to pain. The PAG projects to the rostral ventromedial medulla (RVM), comprising the primary circuit driving pain inhibition. Morphine administered systemically or directly into the PAG produces greater analgesia in male compared to female rats, while manipulation of gonadal hormones alters morphine potency in both sexes. It is unknown if these alterations are due to steroidal actions on PAG neurons projecting to the RVM. The expression of androgen (AR) and estrogen (ERα) receptors in the PAG of female rats and within this descending inhibitory pathway in both sexes is unknown. The present study used immunohistochemical techniques (1) to map the distribution of AR and ERα across the rostrocaudal axis of the PAG; and (2) to determine whether AR and/or ERα were colocalized on PAG neurons projecting to the RVM in male and female rats. AR and ERα immunoreactive neurons (AR-IR, ERα-IR) were densely distributed within the caudal PAG of male rats, with the majority localized in the lateral/ventrolateral PAG. Females had significantly fewer AR-IR neurons, while the quantity of ERα was comparable between the sexes. In both sexes, approximately 25-50% of AR-IR neurons and 20-50% of ERα-IR neurons were retrogradely labeled. This study provides direct evidence of the expression of steroid receptors in the PAG and the descending pathway driving pain inhibition in both male and female rats and may provide a mechanism whereby gonadal steroids modulate pain and morphine potency.
INTRODUCTION

Anatomical and physiological studies have shown that the midbrain periaqueductal gray (PAG) plays a modulatory role in a variety of behaviors including antinociception (Reynolds, 1969; Behbehani & Fields, 1979; Heinricher et al., 1987; Behbehani, 1995; Budai et al., 1998), reproduction (McCarthy et al., 1991; Ogawa et al., 1991; Murphy & Hoffman, 1998; Daniels et al., 1999), fear and anxiety (Kim et al., 1993), aggression (Bandler et al., 1985; Bandler & Carrive, 1988; Depaulis et al., 1992; Scordalakes & Rissman, 2004) and vocalization (Davis et al., 1993; Zhang et al., 1994). While these behaviors have been shown to be modulated by gonadal steroids, our knowledge of the qualitative and quantitative aspects of gonadal steroid receptors in the PAG is incomplete. The PAG has been shown to contain a large number of both androgen receptor (AR) and estrogen receptor (ER$\alpha$) immunoreactive neurons (Murphy & Hoffman, 1999; Murphy & Hoffman, 2001), however these studies were conducted exclusively in male rats. While the distribution of ER$\alpha$ in the female PAG has been reported in a few species, including the cat (VanderHorst et al., 1998), the golden hamster (Boers et al., 1999), the guinea pig (Turcotte & Blaustein, 1993) and the rhesus monkey (Vanderhorst et al., 2002; VanderHorst et al., 2004), the quantity and distribution of AR and ER$\alpha$ in the female rat is currently unknown.

The PAG projects heavily to the rostral ventromedial medulla (RVM), which in turn projects to the dorsal horn of the spinal cord. This PAG-RVM-spinal cord circuit is the primary neural pathway that elicits the antinociceptive effects of opiates. Previous studies have reported sex differences in the anatomical organization of the projections from the PAG to the RVM and activation of these neurons by inflammatory pain (Loyd &
Murphy, 2006). In addition, there are significant sex differences in the activation of the this pathway by systemic morphine, both in the presence and absence of inflammatory pain (Loyd & Murphy, 2006; Loyd et al., 2007; Loyd et al., 2008). To date, it is not known whether ERα and AR are expressed on PAG neurons projecting to the RVM. Numerous behavioral studies have shown that sex differences in opioid analgesia are modulated by both the organizational and activational effects of gonadal steroids (Kepler et al., 1989; Islam et al., 1993; Krzanowska & Bodnar, 1999; 2000; Stoffel et al., 2003; Cataldo et al., 2005; Stoffel et al., 2005). Male rats castrated at birth experience decreased morphine potency in adulthood, while female rats masculinized at birth experience greater morphine potency in adulthood whether morphine is administered systemically (Cicero et al., 2002) or directly into the PAG (Krzanowska et al., 2002). Similarly, both systemic and central administration of morphine is less effective in gonadectomized adult males and more effective in ovariectomized adult females (Kepler et al., 1989; Ratka & Simpkins, 1991; Krzanowska & Bodnar, 1999; Terner et al., 2002; Stoffel et al., 2003; Stoffel et al., 2005; Terner et al., 2005); effects are reversed with hormone replacement (Ratka & Simpkins, 1991; Kiefel & Bodnar, 1992); Stoffel et al., 2003; (Ji et al., 2007).

While the organizational and activational effects of gonadal steroids are likely to contribute to the sexually dimorphic actions of morphine, it is currently unknown whether gonadal steroid receptors are expressed on PAG neurons projecting to the RVM. In addition, the qualitative and quantitative aspects of AR and ERα expression in the PAG of the female rat are not known. The present studies utilized immunohistochemistry to map (1) the quantity and distribution of AR and ERα immunoreactive neurons across
the rostrocaudal axis of the PAG; and (2) to determine if the PAG neurons projecting to
the RVM express AR and ERα immunoreactivity. Due to a lack of commercially
available antibodies at the time these studies were conducted, ERβ was not analyzed in
this study. This study is the first to report AR and ERα immunoreactivity in the PAG and
its descending projections to the RVM in both male and female rats.

MATERIALS AND METHODS

Subjects

Six adult male and six weight-matched (250-350g; approximately 70-100 days of
age) cycling female Sprague-Dawley rats were used in these experiments (Zivic-Miller;
Pittsburgh, PA). Rats were housed in same-sex pairs on a 12:12 hour light:dark cycle.
Access to food and water was ad libitum throughout the experiment except during
surgery. These studies were performed in compliance with the Institutional Animal Care
and Use Committee at Georgia State University. All efforts were made to reduce the
number of animals used in these experiments and to minimize any possible suffering by
the animal.

Vaginal Cytology

Vaginal lavages were performed daily beginning two weeks prior to experimental
manipulations to confirm that the female rats were cycling normally and to keep daily
records on the stages of their cycle up to the day of sacrifice. Proestrus was identified
as a predominance of nucleated epithelial cells and estrus was identified as a
predominance of cornified epithelial cells. Diestrus 1 was differentiated from Diestrus 2
by the presence of leukocytes. Rats that appeared between phases were noted as being in the more advanced stage.

*Retrograde Tracer Injections*

Animals were deeply anesthetized with a cocktail of ketamine/xylazine/acepromazine (50 mg/kg / 3.3 mg/kg / 3.3 mg/kg; i.p.; Henry Schein, Melville, NY). When a surgical plane of anesthesia was reached each animal was placed in a stereotaxic frame and the skull was adjusted so bregma and lambda were at the same dorsal-ventral plane. Glass micropipettes (10-20 µM) filled with the retrograde tracer Fluorogold (FG; 2% soln. w/v in saline; Fluorochrome LLC; Denver, CO) were lowered into the RVM using the following coordinates (in mm): AP: -2.0 Lambda; ML: 0.0; DV: -8.5). FG was iontophoresed (50/50 duty cycle, 7.5 µA current) into the RVM for 25 minutes to facilitate neuronal uptake. The current was then turned off and the pipettes remained in place for an additional 5 minutes prior to removal to minimize backflow of the tracer along the pipette track. Following tracer injections, wounds were sutured closed, the antibiotic Neosporin was applied to the wound, and the animals were placed in clean cages to recover under a heat lamp. Upon complete recovery from the anesthetic, animals were returned to their original housing facilities.

*Perfusion fixation*

Ten days following surgery, animals were given a lethal dose of Nembutal (160 mg/kg; i.p.) and transcardially perfused with 200-250 ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator to remove blood from the brain. Immediately following removal of blood, 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein (Polyscience; Niles, IL) was perfused
through the brain as a fixative. A final rinse with 200-250 ml of the sodium chloride/sodium nitrate solution was perfused through the brain to remove any residual acrolein. Immediately following perfusion, the brains were carefully removed, placed in a 30% sucrose solution and stored at 4°C for at least one week prior to sectioning. Sucrose solutions were changed daily to optimize saturation of sucrose into the tissue. To section the brain, the dura and pia mater were carefully removed and the brains were cut into six series of 25 µm coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution (Watson et al., 1986) at –20°C until immunocytochemical processing. The tissue was sectioned at 25 µm so that 125 µm separates each analyzed level of the PAG thus eliminating any possible bias from counting the same cell twice during data collection.

**Immunocytochemistry**

A 1:6 series through the rostrocaudal axis of each brain was processed for FG immunoreactivity and AR (n=5 males; n=6 females) or ERα (n=6 males; n=5 females) immunoreactivity as previously described (Murphy & Hoffman, 2001). Briefly, sections were rinsed extensively in potassium phosphate-buffered saline (KPBS) to remove cryoprotectant solution, immediately followed by a 20-minute incubation in 1% sodium borohydride to remove excess aldehydes. The tissue was then incubated in either primary antibody solution rabbit anti-AR (Santa Cruz Biotechnology; Santa Cruz, CA, lot no. L0407; 1:10,000) or rabbit anti-ERα (Santa Cruz Biotechnology; Santa Cruz, CA, lot no. I2607; 1:20,000) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. The rabbit anti-AR antiserum was prepared against a peptide mapping at the N-terminus of AR of human origin.
(MEVQLGLGRVYPRPPSKTYRG) corresponding to amino acids 2-21 (manufacturer’s technical information) and specificity has been confirmed (Creutz & Kritzer, 2004). The rabbit anti-ERα antiserum was prepared against a peptide mapping at the C-terminus of ERα of mouse origin (HSLQTYYIPPEAEGFPNTI) corresponding to amino acids 580-559 (manufacturer’s technical information) and specificity has been confirmed (Quesada et al., 2007).

After rinsing out the primary antibody with KPBS, the tissue was incubated for one hour in biotinylated goat anti-rabbit IgG (Jackson Immunoresearch; West Grove, PA, 1:600), rinsed with KPBS, followed by a one hour incubation in an avidin-biotin peroxidase complex (1:10; ABC Elite Kit, Vector Labs). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), AR or ERα immunoreactivity was visualized as a black reaction product using nickel sulfate intensified 3,3’-diaminobenzidine solution containing 0.08% hydrogen peroxide in sodium acetate buffer. After rinsing, AR or ERα labeled sections were then placed in primary antibody solution rabbit anti-FG (Chemicon; Billerica, MA, lot no. 25060005; 1:10,000) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. FG was visualized as a brown reaction product using 3,3’-diaminobenzidine containing 0.08% hydrogen peroxide in Trizma buffer (pH 7.2). After 15-30 minutes, three rinses in sodium acetate buffer terminated the reaction and tissue was given a final rinse in KPBS. Sections were then mounted out of saline onto gelatin-subbed slides, air-dried and dehydrated in a series of graded alcohols. Tissue-mounted slides were then cleared in xylene and glass cover-slipped using Permount.
Data Analysis and Presentation

Data were analyzed across six representative levels through the rostrocaudal axis of the PAG (Bregma -6.72, -7.04, -7.74, -8.00, -8.30, -8.80). The number of AR immunoreactive neurons (AR-IR), ERα immunoreactive neurons (ERα-IR), and the number of AR-IR and ERα-IR neurons that were retrogradely labeled (AR/FG+, ERα/FG+) were quantified. The experimenter was blind to the experimental condition. In levels where significant differences were found, a second blinded observer confirmed results. Cell counts were conducted unilaterally as there are no differences in the number of FG+ cells (Loyd & Murphy, 2006) or the number of AR-IR and ERα-IR neurons (Murphy & Hoffman, 2001) for the left versus right side of PAG. Additionally, previous data have shown that there are no sex differences in total area (mm²) of the PAG between weight-matched male and female Sprague-Dawley rats (Loyd & Murphy, 2006).

Data are reported as the mean ± standard error of the mean (SEM) from which percentages were calculated and reported as the percentage of receptor that was localized in retrogradely labeled cells (%AR/FG+; %ERα/FG+) or as the percentage of retrogradely labeled cells that were colocalized with receptor (%FG/AR; %FG/ERα). A three-way analysis of variance (ANOVA) was used to test for significant main effects of sex (male, female), PAG level (Bregma -6.72 through -8.80), and PAG subdivision (dorsomedial, lateral/ventrolateral). For percentile date, percentages were transformed to standard scores. Fishers’s post hoc tests were used to determine specific group differences when a main effect or interaction was observed. P ≤ .05 was considered significant for all analyses. For data presentation, a representative animal from each
experimental group was selected and the distribution of (1) AR-IR neurons, (2) ERα-IR neurons, (3) FG+ neurons, (4) AR/FG+ neurons and (5) ERα/FG+ neurons within the PAG were plotted using a Nikon Drawing Tube attached to a Nikon Optiphot microscope. Plots were then scanned onto the computer and adjusted to figure format using Adobe Illustrator 10. Photomicrographs were generated using a Synsys digital camera attached to a Nikon Eclipse E800 microscope. Images were captured with IP Spectrum software and adjusted to figure format by alterations in brightness and contrast levels using Adobe Photoshop 7.0.

RESULTS

Androgen Receptor Distribution in the PAG

AR-IR neurons were distributed across the rostrocaudal axis of the PAG in both male and female rats (Figure 1; red circles). AR-IR neurons were confined to the dorsomedial and lateral/ventrolateral subdivisions of the PAG, with the dorsolateral subdivision of the PAG lacking AR-IR neurons. These results are consistent with previous studies showing AR localization in the PAG of male rats (Murphy & Hoffman, 1999; Murphy & Hoffman, 2001). While the qualitative distribution of AR-IR neurons was similar in both males and females, quantitatively males had a significantly greater number of AR-IR neurons compared to females \(F(1, 54)=22.7, p<.00001\) (Figure 2; red bars) with a significantly greater number of AR-IR neurons localized in the lateral/ventrolateral PAG compared to the dorsomedial subdivision \(F(1, 108)=22.1, p<.0001\). This sex difference was evident across the rostrocaudal axis of the PAG. There was no main effect of level of PAG \(F(5,54)=1.2;\) n.s.] and no significant sex by
level interaction \[F(5,54)=0.3; \text{n.s.}\], indicating that the number of AR-IR neurons remained consistent across the rostrocaudal axis of the PAG of both male and female rats.

**Estrogen (α) Receptor Distribution in the PAG**

ERα-IR neurons were densely distributed throughout the rostrocaudal axis of the PAG in both male and female rats (Figure 1; black circles). Similar to the distribution of AR-IR neurons, ERα-IR neurons were confined to the dorsomedial and lateral/ventrolateral subdivisions of the PAG, with the majority of ERα-IR neurons localized in the lateral/ventrolateral PAG \[F(1, 108)=105.8, p<.0001\]. Overall, there was no sex difference in the number of ERα-IR neurons in the PAG \[F(1, 54)=1.2, \text{n.s.}\] (Figure 2; black bars). A significant increase in the number of ERα-IR neurons \[F(5, 54)=9.02, p<.0001\] was noted along the rostrocaudal axis of the PAG.

**Androgen Receptor Distribution in PAG Neurons Projecting to the RVM**

All iontophoretic injections of the retrograde tracer Fluorogold (FG) into the RVM were located on the midline and dorsal to the pyramidal tract, at the level of the caudal pole of the facial nucleus (lambda –2.0mm). Analysis was limited to injection sites that occurred between the facial nucleus and the olivary complex across approximately 2mm rostrocaudally (Bregma -9.30 to -11.60). In our studies using anterograde tracing from the PAG to the RVM, we have noted that this region of RVM contains the highest density of anterogradely labeled fibers and is remarkably consistent throughout this 2mm window of RVM (unpublished observations). Injections outside of the RVM were not included for analysis. Only male and female rats with comparable injection sites were used for analysis. Injection of FG into the RVM produced dense retrograde
labeling throughout the rostrocaudal axis of the PAG consistent with our previous studies (Loyd et al., 2006, Loyd et al., 2007; Loyd et al., 2008). Females had a significantly greater number of PAG cells retrogradely labeled from the RVM compared to males [F(1,70)=14.4, p<.0003].

Figure 3 shows an example of AR and FG immunoreactivity within the lateral PAG of a representative male (A-B) and female (C-D) rat. AR-IR neurons there were retrogradely labeled were densely localized throughout the rostrocaudal axis of the PAG in both male and female rats (Figure 4; red stars), with males expressing more dual labeled cells [F(1,54)=19.5; p<.0001]. The percentage of retrogradely labeled cells that expressed AR was comparable between the sexes [F(1,54)=13.51; n.s.] (Figure 5, %FG/AR), and significantly increased moving caudally through the PAG [F(5,54)=7.29; p<.0001]. Since female rats had a greater number of PAG neurons projecting to the RVM compared to males, the percentage of AR that was localized in retrogradely labeled cells was also determined (Figure 5; %AR/FG+) and was found to be comparable between the sexes [F(1, 54)=1.4; n.s.].

*Estrogen (α) Receptor Distribution in PAG Neurons Projecting to the RVM*

An example of ERα and FG immunoreactivity within the lateral PAG of a representative male (A-B) and female (C-D) rat is shown in Figure 6. ERα-IR neurons that were retrogradely labeled were densely localized throughout the rostrocaudal axis of the PAG in both male and female rats (Figure 4; open stars) [F(1,54)=1.1; n.s.], with the majority of localized in the lateral/ventrolateral subdivision [F(1, 54)=7.5; p<.0001]. Across all levels and regions of the PAG, the percentage of retrogradely labeled cells that expressed ERα was comparable between the sexes [F(1,54)=0.176; n.s.] (Figure 5,
Similarly, the percentage of ER\(\alpha\) that was localized in retrogradely labeled cells was comparable between the sexes \([F(1, 54)=0.292;\ n.s.]\) (Figure 5; %ER\(\alpha\)/FG+). Additionally, a significantly greater percentage of retrogradely labeled cells that expressed ER\(\alpha\) were observed in the caudal PAG \([F(5,54)=15.1;\ p<.0001]\).

**DISCUSSION**

The PAG has been implicated in a variety of hormone-sensitive behaviors (Bandler & Shipley, 1994; Keay & Bandler, 2001; 2002); however, gonadal steroid receptor expression in the PAG had not been reported in both male and female rats. Here we report that the expression of AR is sexually dimorphic along the entire rostrocaudal axis of the PAG, with males having a significantly greater number of immunoreactive neurons. No sex differences were noted in the qualitative or quantitative aspects of ER\(\alpha\) expression in the PAG. In the present study, ER\(\beta\) was not examined due to a lack of a reliable antibody at the time these studies were conducted; therefore, the possibility remains that the expression of ER\(\beta\) in the PAG is sexually dimorphic. Similarly, in this study we were unable to determine the effects of estrous on ER\(\alpha\) expression in the PAG due to low number of animals. On the day of sacrifice, ten days following tracer injections, all female rats were in either the estrus \((n=4)\) or proestrus \((n=2)\) phase of their cycle; no animals were in the diestrus phase.

The sex difference in the expression of AR in the PAG may play a role in sex differences in pain and analgesia. There are numerous behavioral studies indicating a role of gonadal steroids in modulating morphine potency. Gonadectomy reduces morphine potency in male rats (Kepler et al., 1989) and increases morphine potency in
females (Terner et al., 2002; Terner et al., 2005), while hormone replacement reverses these effects (Stoffel et al., 2003; Stoffel et al., 2005). Masculinizing female rat pups with testosterone increases morphine potency to male-like levels (Cicero et al., 2002). In addition, testosterone has been shown to oppose the effects of estradiol on neuronal excitability (Edwards et al., 1999) and decrease pain sensitivity in both male and female rats (Aloisi et al., 2004). A greater expression of AR in the PAG of males may provide an anatomical substrate for the sexually dimorphic modulation of pain by gonadal steroids.

The distribution of AR-IR and ERα-IR neurons was remarkably similar; both receptor types were preferentially localized within the dorsomedial and lateral/ventrolateral subdivisions of the PAG and both increased in density along the rostrocaudal axis of the PAG. These results are similar to the distribution of AR-IR and ERα-IR neurons previously reported in the PAG of the male rat (Murphy & Hoffman, 1999; Murphy & Hoffman, 2001). In addition, the distribution of ERα-IR neurons in the female rat PAG is similar to that previously reported in the cat (VanderHorst et al., 1998), the golden hamster (Boers et al., 1999), the guinea pig (Turcotte & Blaustein, 1993) and the rhesus monkey (Vanderhorst et al., 2002; VanderHorst et al., 2004).

Steroid Receptor Colocalization within the Endogenous Descending Pathway Driving Pain Inhibition

The dense projections from the PAG to the RVM provide an essential neural circuit for the antinociceptive effects of opiates. Many behavioral studies have reported an effect of steroid hormones on morphine potency; however, this study is the first to report the expression of AR and ERα within the endogenous descending pathway.
driving pain inhibition. Using immunohistochemical analysis, we report that AR and ERα were expressed on PAG neurons projecting to the RVM in both the dorsomedial and lateral/ventrolateral subdivisions of the PAG. Male rats had a greater number AR-IR neurons that were retrogradely labeled, however, there was so sex difference in either the percentage of retrogradely labeled cells that expressed AR or the percentage of AR that was located within retrogradely labeled cells. Similarly, the percentage of ERα that was localized in retrogradely labeled cells was comparable between the sexes and was significantly greater in the caudal PAG with the majority localized in the lateral/ventrolateral subdivision.

*Role in Pain and Analgesia*

The present results report a dense colocalization of gonadal steroid receptors on PAG neurons projecting to the RVM, which may provide the anatomical substrate for the reported sex differences in morphine potency. Between 27-50% of PAG neurons projecting to the RVM contain mu opioid receptor (MOR); these MOR+ cells are localized primarily within the caudal lateral/ventrolateral PAG (Commons *et al*., 2000; Wang & Wessendorf, 2002), in the same subdivision of the PAG that we report a dense distribution of both steroid hormone receptors. Estradiol has been shown to both uncouple MORs from G protein-gated inwardly rectifying potassium channels causing a reduction in hyperpolarization by MOR agonists (Kelly *et al*., 2003) and induce mu opioid receptor (MOR) internalization (Eckersell *et al*., 1998). Furthermore, MOR internalization requires the presence of ERα (Micevych *et al*., 2003) suggesting that colocalization of MOR and ERα in the descending inhibitory circuit may provide a
mechanism through which gonadal hormones differentially affect morphine potency in male and female rats.

Although not determined in the present study, it is possible that both AR and ERα are colocalized within the same PAG cells, as is the case in other brain areas (Wood & Newman, 1995); (Greco et al., 1998). A population of neurons expressing both AR and ERα in the PAG may provide a potential mechanism for the diverse effects of gonadal steroid hormones. For example, there are numerous reports of sex differences in pain sensitivity; however, there is no clear consensus on the direction of the sex difference (Mogil et al., 2000; Gaumont et al., 2002; Aloisi et al., 2004; LaCroix-Fralish et al., 2005). In addition, pain sensitivity varies across the rat estrous cycle (Gintzler, 1980) and the human menstrual cycle (Cogan & Spinnato, 1986; Hellstrom & Anderberg, 2003). Sex differences in circulating gonadal steroids acting via a differential expression of AR and ERα within the same PAG neuron may provide a mechanism for the diverse effects of gonadal steroids on pain sensitivity.

Other Functional Considerations

The PAG has also been implicated in the regulation of the autonomic system controlling blood pressure, heart rate, and regional blood flow, all of which have been shown to be modulated by gonadal hormones (Alper & Schmitz, 1996); (Morgan & Pfaff, 2001). In parallel, the PAG initiates defensive and aggressive behaviors, such as the ‘fight or flight’ response (Bandler et al., 1985; Bandler & Carrive, 1988; Depaulis et al., 1992; Scordalakes & Rissman, 2004), and evidence suggests that gonadal hormones increase these behaviors in both male and female rats (Albert et al., 1990; Albert et al., 1991; Johansson et al., 2000). Additionally, the PAG has also been
implicated in initiating sex behavior, in that stimulation of the PAG facilitates lordosis in female rats (Sakuma & Pfaff, 1979a; 1979b; McCarthy et al., 1991), while lesions of the PAG suppress this behavior (Sakuma & Pfaff, 1979b; Lonstein & Stern, 1998). Here we report that the PAG, an anatomical substrate essential for the integration of sensory input and autonomic output, contains a large population of gonadal steroid receptor-expressing neurons, which appear to be involved in modulating both autonomic and sensory responses involved in producing steroid-sensitive behaviors.

Summary

The present study demonstrates that there are sex differences in the qualitative and quantitative aspects of the gonadal steroid receptors in the PAG. These reported differences in AR and ERα immunoreactivity in the PAG have an important impact on steroid-sensitive behaviors modulated by the PAG, such as reproduction, aggression, and autonomic regulation. We additionally report that the primary neural circuit for the antinociceptive effects of opioids expresses steroid receptors and may provide a direct mechanism for sex differences in morphine analgesia.
ACKNOWLEDGMENTS

The authors would like to acknowledge the technical assistance of Leslie Bush on data collection. This work was supported by NIH grants DA16272 and P50 AR49555 awarded to Anne Z. Murphy, Ph.D.
REFERENCES


TITLES AND LEGENDS TO FIGURES

**Figure 1.** Distribution of cells in the PAG immunoreactive for AR (red circles) and ER\(\alpha\) (black circles) in male (left side of plots) and female rats (right side of plots) at six rostrocaudal levels (A-F) of the PAG.

**Figure 2.** Bar graphs display the mean number (± S.E.M.) of AR immunoreactive cells (green bars) and ER\(\alpha\) immunoreactive cells (purple bars) across six rostrocaudal levels of the PAG. Cell counts were combined for the dorsomedial and lateral/ventrolateral subdivisions of PAG. *# denotes a significant sex difference in mean # of steroid receptors.***

**Figure 3.** Color photomicrograph showing a low (A,C) and high (B,D) power example of single- and double-labeled AR and FG immunoreactive cells in the lateral PAG (bregma -8.00) of a male (A-B) and female rat (C-D). Scale bar = 100 \(\mu\)m for low power images; scale bar = 50 \(\mu\)m for high power images.

**Figure 4.** Distribution of cells in the PAG retrogradely labeled from the rostral ventromedial medulla (black circles) and immunoreactive for AR (red stars) and ER\(\alpha\) (open stars) in male (left side of plots) and female rats (right side of plots) at six rostrocaudal levels of the PAG.

**Figure 5.** Bar graphs display the mean (± S.E.M.) %AR/FG+, %FG/AR+, %ER\(\alpha\)/FG+, and %FG/ER\(\alpha\)+ immunoreactive neurons for the dorsomedial combined with lateral/ventrolateral regions of PAG across six rostrocaudal levels of the PAG.

**Figure 6.** Color photomicrograph showing a low (A,C) and high (B,D) power example of single- and double-labeled ER\(\alpha\) and FG immunoreactive cells in the lateral PAG
(bregma -8.00) of a male (A-B) and female rat (C-D). Scale bar = 100 µm for low power images; scale bar = 50 µm for high power images.
Figure(s)

A Bregma -6.72

B Bregma -7.04

C Bregma -7.64

D Bregma -8.00

E Bregma -8.30

F Bregma -8.80

AR-IR Cells
ERα-IR Cells
Figure(s)
Figure(s)

A Bregma -6.72

B Bregma -7.04

C Bregma -7.64

D Bregma -8.00

E Bregma -8.30

F Bregma -8.80

- FG+
- AR/FG+
- ERα/FG+