5-14-1991

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AN ANALYSIS OF THE ANATOMICAL PROJECTION FROM THE mZI TO THE dMCG USING HRP HISTOCHEMISTRY: COMPARISON BETWEEN MALE AND FEMALE RATS

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May 14, 1991

Abstract

Anatomical and behavioral studies indicate that the dorsal Midbrain Central Grey (dMCG) and the Ventromedial Nucleus of the hypothalamus (VMH) are the principle brain structures that function in mediating sexual behavior in the female rat. Recently, the medial portion of the Zona Incerta (mZI) has been implicated as an additional brain structure that regulates sexual receptivity (lordosis) in the female rat. When the mZI of the female was destroyed via intracerebral injections with the neurotoxin, ibotenic acid, by Dornan *et al.* (1991), they reported that lordosis behavior was shown to be attenuated. Presently, little is known about the role of the mZI in the expression of male sexual behavior, but several studies indicate that it plays no role. Anatomical studies, however, have revealed that neural connections exist between the mZI and the dMCG in both male and female rats. Collectively this information suggests that the neuronal pathways between the mZI and the dMCG would differ in strength between male and female rats. We addressed this possibility by using the retrograde tract tracing approach with horseradish peroxidase (HRP). A 12% solution of HRP was injected bilaterally (volume, 0.5µl/per side) into the dorsal portion of the Midbrain Central Gray in three animals (2 males and 1 female). After a 3-day survival period, animals were sacrificed and their brains processed for HRP histochemistry using a modified Mesualm method. The results of this analysis revealed more retrogradely labelled cells in the female brain than the male brain with a similiar injection site. These results suggest that in the rat, the neural pathway between the mZI and the dMCG may be a stronger in the female than in the male.

Introduction

Lordosis is a hormone-dependent receptive behavior seen in the female rat (11). This behavior only occurs in late pro-estrus or estrus when levels of circulating estrogen and progesterone are high. Lordosis is initiated when the male mounts the female and places pressure on her posterior rump, tailbase, and perineum (11). The female subsequently arches her back and moves her tail away from her genitalia so copulation may occur (2). The brain regions that have been well documented in playing a role in the expression of lordosis are the dorsal portion of the Midbrain Central Gray (dMCG) and the Ventromedial Nucleus of the hypothalamus (VMH) (11).

Sakuma and Pfaff (16) have demonstrated that lordosis can be facilitated in the female rat by electrical stimulation of the dMCG or the VMH. If an electrode is placed into either of these brain regions and impulses delivered at a rate of 0.5 Hz, the rat will respond by exhibiting lordosis behavior. On the contrary, if either of these regions are lesioned electrically (via an intracerebrally implanted electrode) lordosis behavior is subsequently abolished.

In light of the control the VMH and the dMCG seem to have over the expression of lordosis behavior in the female rat, it is not surprising that they share a strong connection between each other (8). In fact, when Sper *et al.* (13) injected an antereograde tract-tracing material (horseradish peroxidase covalently bound to wheat

germ agglutin (HRP-WGA)) into the VMH, they found many labelled neurons in the dMCG. Morrell *et al.* (1981) used the horseradish peroxidase (HRP) retrograde tract-tracing method to show the connections to the dMCG. When Morrell *et al.* injected the HRP into the dMCG, they found the greatest concentration of neurons that project to the dMCG were located in VMH (8). Both these studies suggest that a strong connection exists from the VMH to the dMCG.

Recently, another brain structure has been implicated in the expression of lordosis behavior. This structure is the medial portion of the Zona Incerta (mZI). In a study by Dornan *et al.* (3), injections of the neurotoxin, ibotenic acid, into the mZI of steroid-primed female rats produced a decrease in lordosis behavior. Since the mZI seems to play a role in female receptivity behavior, the authors suggest that a triad comprising of the VMH, the mZI, and the dMCG is responsible for the expression of sexual behavior observed in the female rat. This conclusion is consistent with the anatomical data collected to date.

As mentioned before, Morrell's study (8) of the connections between the dMCG and the VMH concluded that a strong pathway exists between these two brain structures. In this same study, Morrell also observed that connections exist between the dMCG and the mZI. This connection is also regarded as another strong pathway projecting to the dMCG since the mZI contained many retrogradely labelled neurons after histological analysis. In addition, Ter Horst and Luiten (1987) also reported the connectivity between the VMH and the mZI. This study used phaseolus vulgaris leuco-agglutin (PHA-L), an antereograde tract-tracing material, and

iontophoretically injected PHA-L into the VMH. Following histological procedures, they found extensive antereograde labelling in the mZI.

In sharp contrast to the female, the male rat depends almost entirely on the medial preoptic area (MPOA) (12), located rostral to the hypothalamus, for the expression of copulatory behavior (15). Copulatory behavior is the sexual behavior that a male exhibits in response to a female in estrus ("in heat") (1). Copulatory behavior entails three essential features: 1) intromission - entry of penis into the female's vagina, 2) pelvic thrusting -rhythmic movement of the hindquarters causing genital friction, and 3) ejaculation - discharge of semen. During copulation, the male will mount the female several times, achieving intromission on most of the mounts. After eight to fifteen intromissions the male will ejaculate. At the time of ejaculation, the male will exhibit a deep pelvic thrust and arch backwards (15).

The MPOA was shown to be contingent in the expression of male copulatory behavior by Sachs and Meisel (1988). When they lesioned the MPOA electrically or via the neurotoxin, ibotenic acid, copulation ceased (15). In addition, Malsbury (1978) made horizontal knife cuts through the primary efferents that extend from the MPOA to the midbrain and further observed copulatory behavior to be attenuated (6).

Although the mZI, the dMCG, and the VMH have not directly been implicated in male copulatory behavior, the anatomical connections between the three brain regions are existent. In 1985, Roger and Codusseau (13) used the HRP-WGA technique to

investigate the afferent projections to the mZI in the male rat. With this anterograde tract-tracing method, they found the most heavily labelled hypothalamic area to be the VMH. Also, the HRP-WGA anterograde study accomplished by Sper *et al.* (18) concluded a pathway exists between the VMH and the dMCG. Upon iontophoretic injection of HRP-WGA into the VMH, many labelled neurons were located in the dMCG. In addition, Morrell *et al.* observed HRP retrogradely labelled cells in the mZI when HRP was iontophoretically injected into the dMCG of the male rat.

Since the connections in the male are present between the major neural structures implicated in lordosis, that are present in the female rat, one might expect that the male rat would have the capacity to express female sexual behavior with proper hormonal therapy. Indeed, male rats do express lordosis when injected systemically with estradiol. The circulating amounts of estradiol present in the male promote lordotic responses (11,14). This response, however, is not nearly as intense as in the female.

In light of the information above, a possible factor involved in the reduced lordotic response displayed by the male may be contingent upon the strength of the pathway between the three brain regions. In other words, the number of the neuronal projections from the mZI to the dMCG, the VMH to the mZI, and the VMH to the dMCG are weaker in the male rat. This may be due to the role these brain structures have in sexual behavior. Since the female implicates these structures in her sexual behavior and the male does not in copulatory behavior, it would be expected that the connections between the brain regions are more numerous in the

female than the male. Therefore, the projections from the mZI to the dMCG would be stronger in the female rats.

The connectivity in both the male and female rat was assessed and compared using a horseradish peroxidase (HRP) retrograde tract-tracing method from a modified Mesulam technique (7). The HRP was injected into the dMCG and taken up endocytotically by the synaptic terminals. The endocytotic vesicles formed in the synaptic button were retrogradely transported, *via* its axon, to the soma located in the mZI. The soma-labelled in the mZI represent the amount of connectivity between the two structures. The greater the number of soma labelled, the greater the connectivity between these two structures assuming all axons endocytotically took up HRP.

A retrograde labelling method was chosen over an antereograde method because we wished to analyze the pathway of the mZI to the dMCG. Assessing the neurons in the mZI would give us more information as to the location of the neurons that project to the dMCG. An antereograde method, on the other hand, incorporates the antereograde substance in the soma or dendritic trees by an endocytotic process. The vesicles formed are subsequently transported to the synaptic terminals *via* the axonal passage-way. Although the nerve cell bodies and the axons extending from the mZI would be analyzed, the labelled tissue would not give us any information as to the location and the number of neurons in the mZI that extend to the dMCG. The reason being that all the cells in the mZI would be labelled. These labelled neurons

would included the neurons that extend to the dMCG and those that extend to other brain regions.

HRP was chosen as the tract-tracing method because the substance is not expensive relative to other retrograde labellers, it is well established as a retrograde tracer (7), and a thorough methodology has been established by Mesulam and other neuroanatomists. The other retrograde tract-tracing methods considered were Flourogold and True Blue. These substances were not chosen for this study since our laboratory did not have an ultraviolet microscope available to view the labelled cells, the substances are very expensive, and the labelled cells fade rapidly (within one month after labelling occurs) (7).

Mesulam's HRP methodology for the light microscope, in particular, was implicated as the HRP technique of choice for several reasons. A light microscope was available to us for histological analysis. It is a well established methodology in HRP technology. The procedure is easily understood and well explained for people with no previous experience in the HRP technique. It also incorporates tetramethylbenzidine, a non-carcinogen, as the substrate of choice.

Tetramethylbenzidine is recommended for use by Mesulam due to the sensitivity the molecule exhibited in the enzymatic reaction with HRP. In the reaction, hydrogen peroxide combines to the ferric atom located on the HRP enzyme. This enzyme-substrate complex then binds to the TMB molecule and oxidizes it. When oxidized, the TMB is released from the enzyme as a blue precipitate. The reaction with TMB is more sensitive when compared to other

chromogens often used in HRP histochemistry due to its ability to cross the plasma membrane of the soma. TMB is more readily soluble in the lipid membrane than any other HRP chromogen so it easily passes into the soma where it is incorporated to the HRP-hydrogen peroxide complex.

Methods and Materials

Subjects

Six adult male and six adult female Long-Evans rats obtained from the IWU breeding colony were used in this study. The animals were approximately 1.5 years old. The males ranged in weight between 500-600 grams while the females were between 300-350 grams. The rats were housed individually in stainless steel wire-mesh cages and maintained in a temperature-controlled room (21^0C) with a 15:9 light-dark cycle. Food and water were available *ad lib.* All animals were sexually inexperienced at the time of surgery. Although 12 animals were processed through part or all of the surgical and histological procedures (a modified form of Mesulam (7)), only three animal (2 males and 1 female) were adequate for analysis. Each of these 9 animals not available for analysis either died before the brains could be processed, or the histological procedure failed to adequately prepare the brain tissue for analysis. From the three animals that survived, only 1 male and 1 female were used to report the results. Both of these animals had injection sites in the same portion of the dMCG. The second male, not used in the analysis, had an injection site that was assessed to be more anterior than that of the male or female rat used in this study.

Surgical and Histological Procedure

1) Anesthesia - Each animal was injected with sodium pentobarbital (50ml/kg) and placed into a Kopf stereotaxic apparatus. One animal died in this first step due to respiratory failure (induced by the sodium pentobarbital).

2) HRP injections - A 0.5 µl pressure injection of a 12% solution of horseradish peroxidase (Sigma VI) in 0.9M NaCl were made with a 1.0 µl Hamilton syringe into the dorsal portions of the Midbrain Central Gray. The injection sites were based on the coordinates derived by Dornan *et al.* (1990). For the males the coordinates were AB= -7.5, ML= .6, DV= 4.5, and the females were AB= -7.5, ML= .5, DV= 4.7. This was the site found to be most receptive for expression of lordosis behavior (11) All coordinates were determined from bregma and the atlas of Paximos and Watson (1982) (9) was used as a stereotaxic guide. Bregma is the intersection of the medial lateral and the antereoposterior sutures.

A 12% HRP solution was determined to be an adequate concentration of HRP according to the guidelines of Mesulam (1982). Mesulam determined that a HRP solution above 20% would be too high a concentration of HRP therefore toxic to the animal. The toxicity would damage the synaptic terminals in the dMCG and retrograde transport would not be able to occur. A solution below 6%, however, would be too aqueous. Therefore, the injection site could become too large by the primary diffusion that occurs from the injection in the first 10 minutes of administration of HRP into the dMCG. Also, the HRP could be

at such a low concentration that adequate amounts of HRP would not be available for determination of retrograde transport. The retrograde tracer must be at a concentration where reaction with hydrogen peroxide and tetramethylbenzidine will form a precipitate that can be examined in light microscopy (7).

The Sigma VI HRP was specifically used because it contains much of the isoenzyme C. HRP has many isoenzymes that are characterized by their carbohydrate and amino acid concentrations. Isoenzyme C has an active site that is more prone to substrate than any of the other isoenzymes. The active site is less sterically hindered so the substrate (Hydrogen peroxide and TMB) binds more readily (7).

A 0.9M NaCl solvent was used to make the 12% HRP solution since it is a very ionic solvent. The ions orientate themselves with each other and the molecules of HRP in order to keep the injection site more localized. The electrostatic interactions between the ions and HRP promote this localization (7).

3) Survival period - After surgery, the animals were placed back into their cages where they survived for approximately 72 hours (+/- 2 hours) before being deeply anesthetized with sodium pentobarbital. The survival period determined for the rats was in accordance to the guidelines suggested by Mesulam (7). Mesulam suggests that any time period less than 1 day would not allow for a sufficient amount of HRP to move retrogradely to the soma and be detected after the enzymatic reaction. After 5 days, however, degradation of the HRP begins to occur and enzymatic activity is lost.

4) Perfusion - The perfusion was performed transcardially.

a.)The transcardial perfusion was initiated by a rapid bolus of 100-150ml of a 0.9% saline solution (pH 7.4) at 21^{0}C. The perfusion of the physiological saline (0.9%) lasted approximately 10 minutes. The perfusion was performed long enough to remove all the blood (mainly the erythrocytes and leukocytes) from the circulatory system. The erythrocytes and leukocytes need to be removed since they tend to react enzymatically with Hydrogen peroxide and TMB to form artifacts in the brain tissue (7). This makes it difficult to distinguish neurons from these artifacts. The pH of the solution was 7.4 in order to save the HRP's enzymatic activity. HRP is most reactive at this pH (7). The saline was 0.9% (physiological saline) so reaction with the blood will not readily occur. Physiological saline is less likely to disrupt the osmotic potentials of the molecules in the blood so clotting does not occur. The temperature of the saline was 21^{0}C to allow for convenient storage and to allow for better penetration of the tissue (7).

b.) Saline was immediately followed by 500ml of fixative. The 1.00% paraformaldehyde and 1.25% glutaraldehyde fixative solution in a 0.2M phosphate buffer (pH 7.4) at 21^{0}C was delivered over 30 minutes. Half the volume was delivered at a fast rate and the other half at a slow rate. The rates were determined experimentally in order for the perfusion with the fixative to extend the full 30 minutes. The fixative is needed to denature the autolytic and catalytic enzymes, present in the brain tissue, because they destroy the neuronal and HRP integrity very quickly after death of the animal. The

aldehydes bind to the autolytic and catalytic enzymes and denature them. In order for the fixative to be effective in rendering the autolytic and catalytic enzymes ineffective, the aldehyde concentration must be at least 2.25% (7).

A combination of two different aldehydes was used in the fixative because each is, defined by Mesulam, to have positive and negative effects on the sensitivity of the reaction. The paraformaldehyde is more effective in maintaining the resiliency of the tissue in the subsequent reactions, but it tends to bind to the HRP enzyme in such a way to decrease its enzymatic activity. The aldehyde sterically hinders the active site when it binds. Glutaraldehyde, on the other hand, does not affect the active site of the enzyme at all, but it does not maintain the resiliency of the brain tissue as well as paraformaldehyde either (7). Tissue penetration by the fixative is enhanced at 21^0C (room temperature) and also is convenient temperature for storage. The 0.2M phosphate buffer was at a high enough molarity to sustain rather large changes in pH. Also, phosphate molecules do not interfer with the TMB reaction as some other buffers tend to do.

c.) Fixation was immediately followed by perfusion with 500ml of a 10% sucrose-0.2M phosphate buffer solution at 4^0C. The solution was delivered over a 30 minute time period in the same fashion as the fixative. The sucrose-buffer solution is needed to clear the fixative from the tissue and protect the cells. Fixative must be flushed from the tissue so further reaction does not occur with the HRP. Also, the sucrose acts

like a cryoprotectant to sustain the tissue integrity. The solution was administered into the animal at 4^0C in order to slow down the reactivity of the HRP (7).

5) Brain storage - The brain was removed from the skull after the perfusion was completed and placed into the 10% sucrose-buffer solution previously mentioned. The brains were stored in this solution at 4^0C for 2-3 days before sectioning occurred. The extended period of time in the sucrose-buffer is necessary to further remove the aldehydes that still may be present in the tissue and to allow for more cryoprotection of the tissue by the sucrose (7).

6) Sectioning - The brains were removed from the 10% buffer -sucrose solution and frozen quickly in liquid nitrogen. Subsequently, the frozen brains were left in the cryostat to equilibriate for at least one hour. Equilibration was performed in order to reduce the temperature of the brain tissue. If sectioning the brain tissue was attempted immediately after submersion into the liquid nitrogen, the brains would have cracked. The tissue would have been too brittle to section at this time. Once equilibrated, the brains were sectioned at 40 μm and collected in a 0.1m phosphate buffer solution (pH 7.4) at 4^0C. Serial sections were made beginning at the injection site and in the area of the mZI. It is necessary to freeze the brains quickly in order to protect the cells from being destroyed. The slow freezing method normally, accomplished via the cryostat, tends to lessen the neurons integrity (7). The process of freezing the brains in liquid nitrogen, however, does entail problems of its own. If the brains are dipped into the liquid nitrogen too quickly, the tissue tends to crack. Infact, we lost two of the rat brains via this procedure because they did indeed crack.

Embedding the tissue in a 10% gelatin block and subsequently placing it in a 4% glutaraldehyde and 30% sucrose solution for 12 hours will protect the brains from cracking while further enhancing the fixation of the tissue (5). The gelatin allows for better resiliency of the tissue when frozen in liquid nitrogen while the glutaraldehyde fixes the tissue further.

Unfortunately, we also lost two other brains when we placed the brain sections into the 0.1M phosphate buffer. The sections rolled up in a tube-link form when introduced to the buffer. We believe this occurred due to a malfunctioning cryostat blade. It seemed to cut the sections so that tissue damage and subsequent "rolling up" occurred.

7) Rinse - The collected sections were rinsed in distilled water at 21^{0}C for 5 minutes. This was necessary to remove the buffer and bring the reaction temperature back up to room temperature so the enzyme would be more reactive (7).

8) Incubation solution - Next, the sections were immersed in the incubation solution (21^{0}C) and shaken gently. This solution consisted of 92.5ml distilled water, 5ml of a pH 3.3 acetate buffer, 100mg of sodium nitroferricyanide, 5mg of tetramethylbenzidine (TMB), and 2.5ml of absolute ethanol. The sections remained in the solution for 20 minutes. This step was necessary to introduce TMB (the chromogen) into the cells (7). The buffer added to the solution needs to be at pH 3.3 in order to maintain the sensitivity of the HRP for the TMB reaction. If the pH of the solution is above 4.0, the sensitivity of the reaction with TMB is suppressed. When the pH is below 2.0, the tissue is subsequently hard to handle (7).

We used an acetate buffer due to its ionic strength; the ions present in the buffer are sufficient to orientate the enzyme for maximum reactivity. This is due to the ability of the ions to expose the active site of the enzyme for better substrate addition (7).

Sodium nitroferricyanide is a stabilizing agent that was added to help the formation of the chromogen (TMB) precipitate. The chromogen precipitate is formed when the TMB is oxidized by the HRP-hydrogen peroxide complex. The sodium nitroferricyanide either adds to the stability of the reaction being formed or prevents the reaction-product precipitate form dissolving in the incubation media after being formed (7).Ethanol is used to dissolve the TMB which is not soluble in water.

9) Enzyme solution - After the TMB has been introduced into the neurons, the sections were placed in the enzyme solution where they were shaken gently for 20 minutes. The enzyme solution was composed of 4ml of 0.03% hydrogen peroxide in 100ml of incubation solution. The percentage of hydrogen peroxide was sufficient to form the enzyme-substrate complex with HRP, but not excessive enough to react with all of the peroxidases present in the brain tissue. Again, the peroxidases in the brain would form a precipitate with hydrogen peroxide and the TMB. This would form artifacts that would make it difficult to distinguish the actual labelled neurons (7).

10) Post-reaction storage and rinsing solution - In order to stop the activity of the HRP enzyme, the sections were immersed in the post-reaction storage and rinsing solution. The solution was composed of 5ml of acetate buffer and 95ml of distilled water.

The HRP activity was halted by washing the substrates away (TMB and hydrogen peroxide). The sections remained in the solution for 10-30 minutes or until mounted on slides (7).

11) Mounting - The sections were mounted onto subbed slides after being introduce into the post-reaction storage and rinsing solution. The subbed slides were made by submerging slides into a solution of 4g chromium potassium sulfate (CPS), 4g of gelatin, and 1 liter of distilled water. After being dipped into the gelatin solution, the slides were oven-dryed for 20 minutes. The gelatin along with the CPS acts as an adhesive to maintain the sections on the slide. The CPS is added to make the gelatin more soluble (7).

12) Dehydration and mounting - Next, the sections were dehydrated by placing the mounted sections through a series of solutions. 1) Distilled water - 10 seconds 2) 70% ethanol - 10 seconds 3) 95% ethanol - 10 seconds 4) 100% ethanol twice - 10 seconds each 5) Two baths of xylene - 2 to 5 minutes each. Dehydration is necessary to protect the reaction-product precipitate from undergoing further reaction. This protection was accomplished by removing most of the solutions from the tissue (7).

Counterstaining with a 1% neutral red solution is sometimes necessary to view the labelled neurons more thoroughly. In the sections analyzed in this study, however, no stain was needed to accent the neurons. Therefore, coverslips were placed, with permount, on top of the tissue to protect it from further dehydration and flaking (7).

13) Photomicroscopy - Photomicrographs were taken, at 40X, with a Leitz Ortholux II microscope equipped with an automatic camera. The film used was Kodak brand Kodacolor 200 ASA.

Results and Discussion

The injection site and diffusion zone were schematically represented after analysis of the dMCG (via light microscopy) at 7.52mm posteriorally from Bregma. The location of the analysis was in the center of the dMCG (from anterior to posterior). Since both the male and female's injection sites were in the same location, one computer image drawing is shown for both gender (figure 1).

The injection site in the dMCG is consistent with the initial injection and the primary diffusion of HRP (that occurs in the first 10 minutes after the initial injection of HRP). The diffusion zone, however, is the site of secondary diffusion that occurs in the second to eight hour after the initial injection. The injection site is considered, by Mesulam (1982), to be the only place that uptake of HRP occurs for detectable retrograde labelling.

According to Mesulam, the uptake of HRP into the synaptic terminal occurs within the first 2 hours after the initial injection. After 2 hours, the HRP is removed from the extracellular spaces in the area of initial injection and primary diffusion (injection site). Beginning at the second hour, secondary diffusion subsequently occurs from the HRP containing synaptic clefts into the area surrounding the injection site. The area is later known as the diffusion zone.

The diffusion progresses at such a slow rate, however, (over a six hour period) that most of the HRP is transported out of the extracellular spaces. The remaining HRP left in the diffusion zone

is such a small quantity that incorporation into the synaptic terminals, and subsequently the soma, is undetectable with light microscopy after enzymatic reaction. Therefore, the injection site is the only area that is considered to be contigent in retrograde transport of HRP.

The injection site and diffusion zone were distinguished from each other during light microscopic evaluation of the dMCG according to the Mesulam criteria (7). The injection site was determined to be the dark staining region in which no cell bodies nor axons could be seen through the dark blue TMB precipitate. In the diffusion zone, on the other hand, cell bodies and axons could be seen through the precipitate. A photomicrograph of the injection site in the female rat brain is shown in figure 2.

After determining the validity and location of the injection sites in the dMCG, sections of the mZI were analyzed for retrogradely labelled neurons in both male and female rats. Figure 3 is a photomicrograph of a labelled neuron in the mZI of a male rat. As seen from this photomicrograph and from microscopic histological analysis we performed, it was determined that neurons were labelled in the mZI of both male and female rats. In both the photomicrograph and the sections viewed on the microscope, we observed neurons stained with granular type precipitate located inside. This neuronal analysis corresponds with the criteria suggested by Mesulam for the determination of labelled neurons. (5)

Furthermore, efferent fibers were located extending from nerve cell bodies of the soma that were labeled with HRP. Again, via the photomicrograph seen in figure 4 and microscopic analysis

of other brain sections, the axons were determined by Mesulam's criteria. Therefore they were described as dark granular structures located in a line-type trail extending from the mZI (7).

The histological analysis performed at the dMCG and the mZI and the passage-way between them suggests retrograde labelling from the dMCG to the mZI occured in both the male and female rat. Since retrograde labelling was determined in both genders, a comparison was accomplished between the male and female rat. The results of this comparison are presented in table I.

Table I is a representation of the number of cells present at four sites from Bregma in the mZI (anterior to posterior- -2.56mm, -2.80mm, -3.14mm, and -3.30mm), and an estimate of the total number of neurons labelled in the mZI of the male and female rat analyzed. To estimate the total number of labelled cells, we used a systematic approach suggested by Akesson and Micevych (1). We estimated the number of labelled cells in the gaps between the sections that were analyzed. The mean count in the two sections surrounding the gap was multiplied by the missing sections. The number of cells counted in the tissue sections and the numbers estimated to be in all gaps were then summed for the total number of labelled cells. (1)

As can be seen from table I, the number of cells counted at the most rostral section (Bregma -2.56mm) was more in the male than the female. Subsequent sections analyzed proceeding rostrally (Bregma -2.80mm, -3.14mm, -3.30mm), however, were distinctly different. More labelled cells were counted in the female than in

the male. Also, in the female rat, the total number of estimated labelled neurons in the mZI was greater than that of the male rat.

Although the data collected from this analysis is supportive of our hypothesis that the pathway between the mZI and dMCG is greater in the female than the male, it is not statistically significant. Only one male and one female were available for analysis. In order for this study to have significant results, many more animals must be studied and compared. Also, the injection site should be expanded to the entire extent of the dMCG.

As mentioned before, we injected the HRP retrograde labelling solution into the area of the dMCG that is suspected to be most functional in the expression of female sexual behavior. We proceeded by these coordinates for injection of HRP in order to localize the area implicated in sexual receptivity in our comparison analysis. This is the area of our greatest interest since we based our hypothesis on the fact that the difference between the male and female connection for the mZI to the dMCG was due to the functional role of the mZI in sexual behavior. In order to make a more precise analysis of the difference in connectivity between the two brain structures, we must implicate the entire dMCG.

In order to accomplish this, we must try to expand the injection site and condense the diffusion zone so that the HRP injection site covers the entire dMCG but does not extend outside of it. We would need to use a different criteria for the injection of the HRP into the dMCG to do this. In this study, 0.5µl of HRP were injected via a Hamilton pressure syringe over a 5 minute interval. It may, however, be more efficient and precise to use smaller

amounts (5-20 nanoliters) of HRP that would be injected over a period of time (10-15 minutes). This may minimize the diffusion zone and allow for a more precise localization of HRP so it may fill the entire length of the dMCG.

Another localization method that may be more efficient in labelling only the neurons in the mZI would entail an intercerebraly placed cannula and iontopheretic equipment. The cannula could be placed into the dMCG two days before the HRP would be administered (7). This would allow the axons destroyed during implantation to heal properly enough so no HRP with taken up by them(7). In the injection method used in this study the needle was placed in dMCG just prior to administration of HRP. The axons, consequently, would not be able to heal properly and HRP could have possibly been taken up. This could result in a misrepresentation of the cells labelled in the mZI if the broken axon had a soma terminating in the mZI, and the synaptic terminal was out of the dMCG.

Ionotophoresis could also be implicated in better analysis of the connections, in addition to cannilization, because it allows for a greater uptake of HRP. When neurons are electrically stimulated by intracerebrally implanted electrodes, the metabolism is known to increase (7). When metabolism is increased, the release of neurotransmitters and the endocytosis of denatured constituents of neurotrasmitter occurs. Along with the endocytosis of the neurotransmitter, the HRP could also be taken up into an endocytotic vesicle if it were present at the synaptic terminal.

Iontophoresis applies both of these principles in its methodology (7).

The micropipette that is placed into the brain region, from which HRP uptake will take place, contains a wire that can be induced to conduct on electric potential. This electric potential is transferred to the brain tissue in which it is implanted and stimulates neural activity. The micropipette also contains HRP inside of it. So when metabolism is increased by neural excitation, the HRP is present in synaptic terminals to be taken into the neuron via endocytosis. Since the neural activity is higher than in a normally functioning state, endocytotic activity is increased and more retrograde labelling is likely to occur. This leads to better labelled cells because of the greater amount of HRP that was transported to the neuron(7).

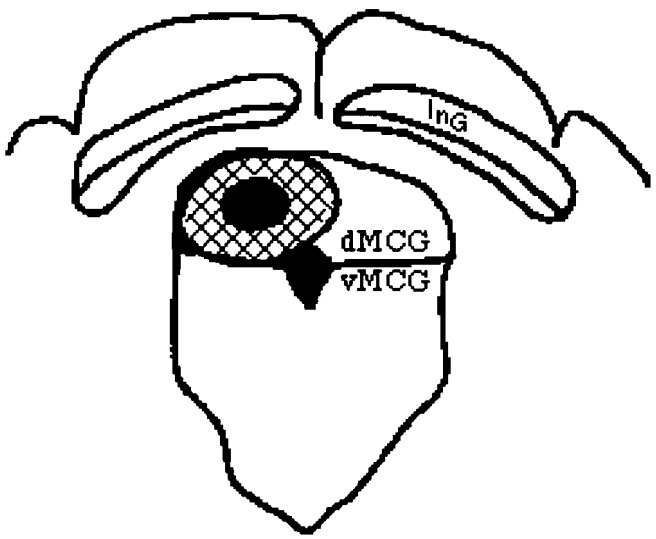
If all other methods are implicated and a statistically significant analysis can be made to determine that the female connections from the mZI is greater than that of the male, the dimorphic pathway could be analyzed to see if it is dependent on circulating hormones in the critical period of rat neural development.

The critical period is the time of the rat's life when changes in the neural development are permanent. In the rat, this period is immediately after birth (2). Hormones, mainly estrogen and testosterone, seem to play an important role in neural development during this critical period (11). Therefore, a change or fluctuation in the hormones could alter brain structures for the adult life of the rat. A study could be conducted on neonatal rats, altering

hormone balances. Upon examining the adult rat, the connections between the mZI and dMCG would be observed to see if there is a change in the connectivity, from the dimorphic pattern. If there is, it would be due to the flux in hormones in the critical period of neural development.

Figure 1: Schematic representation of an injection site and diffusion zone into the dMCG in both the female and male rat. The brain regions present were from the Paximos and Watson rat brain atlas (1982) at 7.52mm posteriorly to Bregma.

Figure 1



1mm

Injection Site

Diffusion Zone

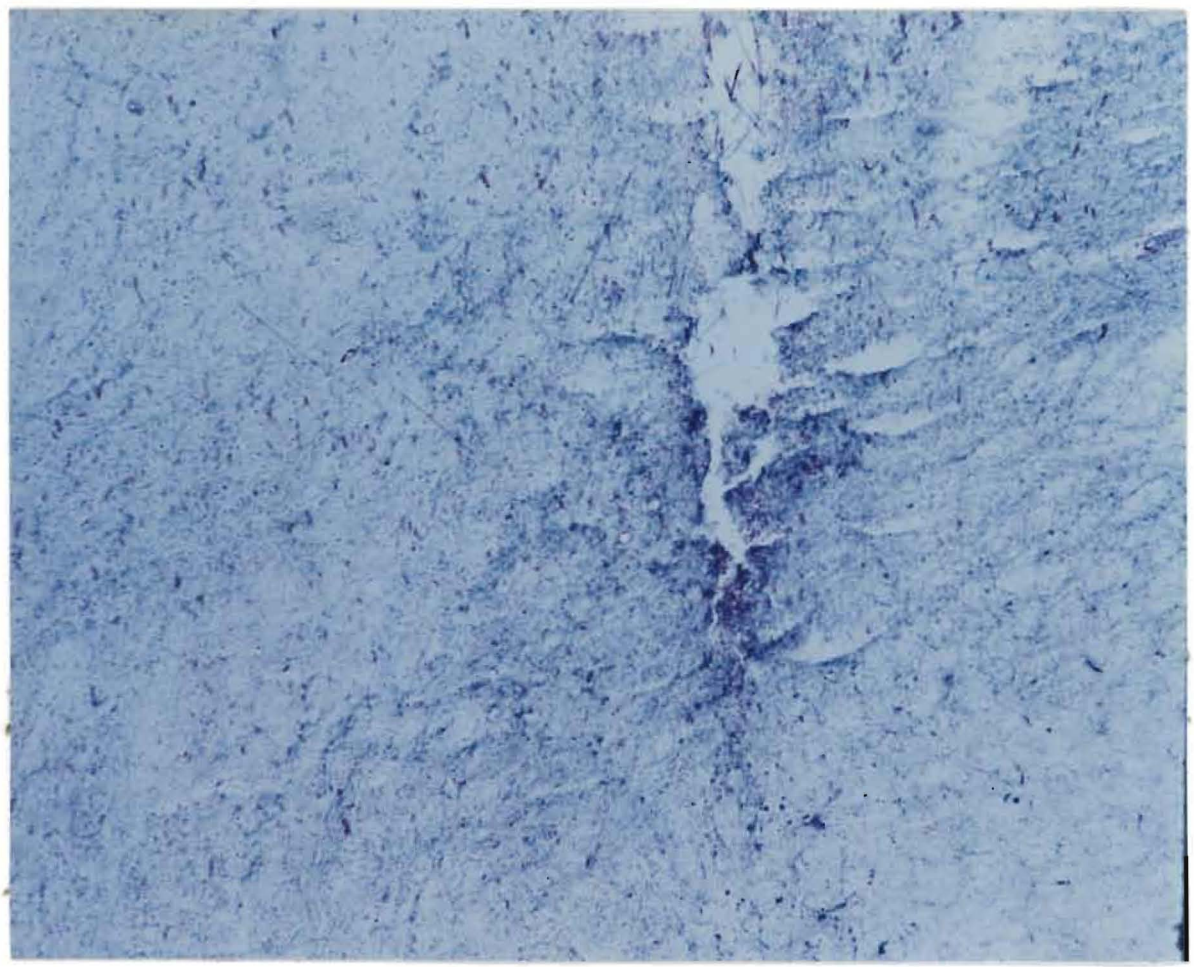
dMCG dorsal portion of the Midbrain Central Gray

vMCG ventral portion of the Midbrain Central Gray

InG intermediate gray layer of the Superior Colliculus

Figure 2: Actual photomicrograph, at 40X, of an injection site into the dMCG of a female rat.

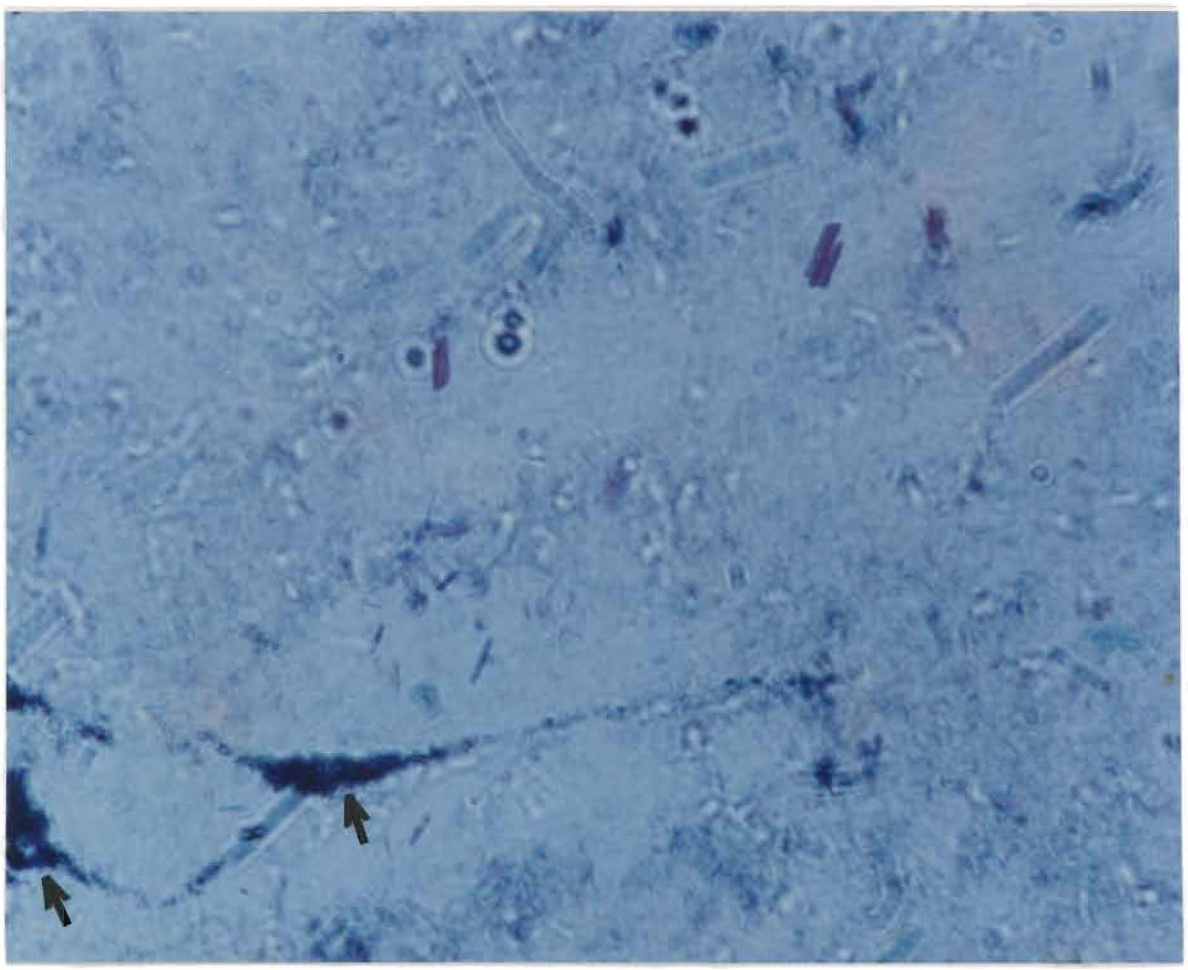
Figure 2



200um

Figure 3: Actual photomicrograph, 40X, of two HRP labelled neurons located in the mZI of a male rat. Arrows indicate the location of the neurons visible.

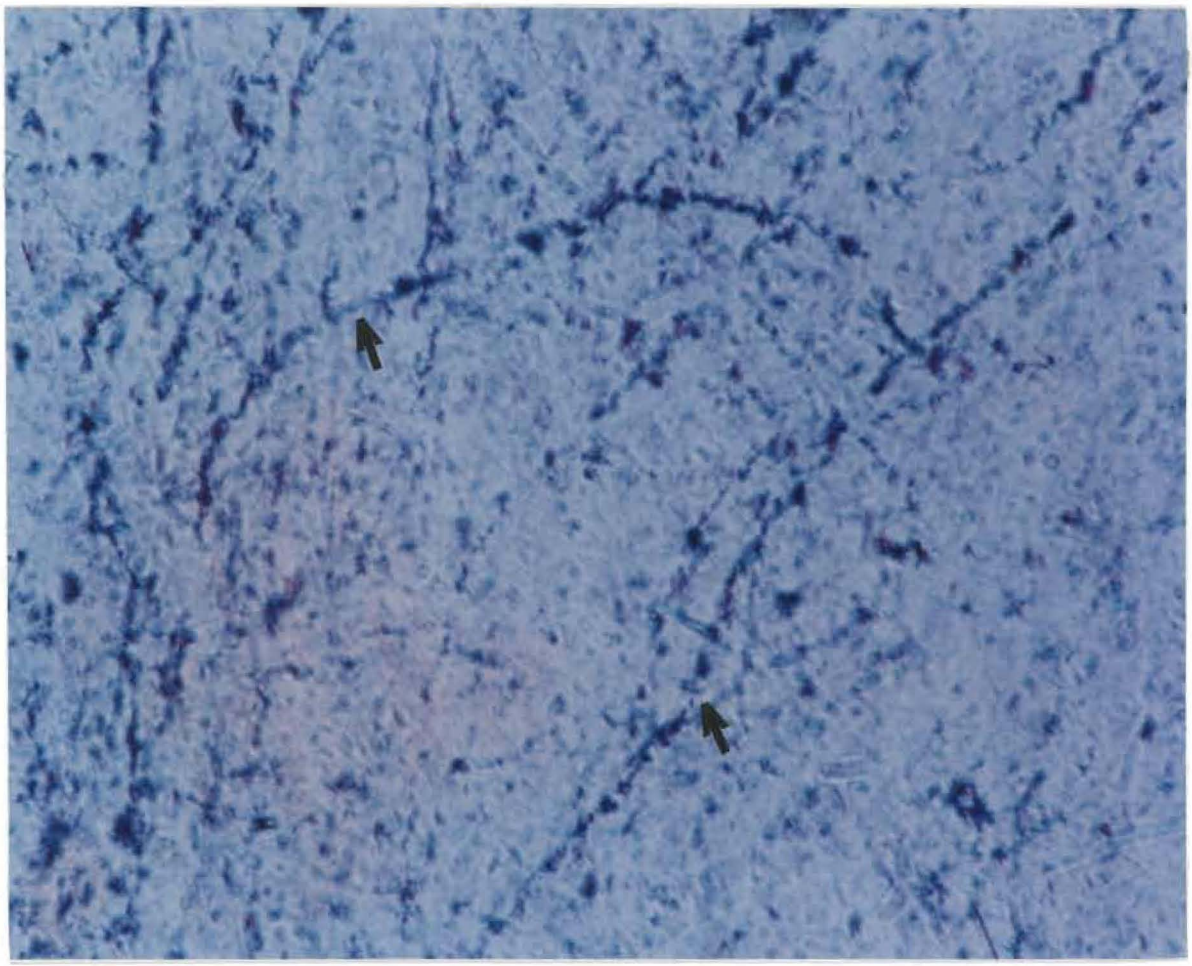
Figure 3



100µm

Figure 4: Actual photomicrograph of efferent axons from the mZI to the dMCG. Arrows indicate the location of the axons visible.

Figure 4



100µm

Table I: Estimate of the number of HRP labelled neurons in the mZI of a single male and female rat used in the analysis of the experiment. Table from anterior to posterior.

Table I

mZI	Labelled cells Bregma -2.56mm	Labelled cells Bregma -2.80mm	Labelled cells Bregma -3.14mm	Labelled cells Bregma -3.30mm	Labelled cells total
female rat	7	11	8	19	90
male rat	10	5	4	15	68

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