

**ANDROGEN RECEPTOR GENE EXPRESSION IN THE RAT CENTRAL  
NERVOUS SYSTEM: EVIDENCE FOR TWO mRNA TRANSCRIPTS.**

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**Supported by the Department of Veterans Affairs and National  
Institutes of Health grant NS20311, and by the NICHD Population  
Research Center of the University of Washington (P50 HD12629).  
Robert McLachlan is a Neil Hamilton Fairley Fellow of the Australian  
National Health and Medical Research Council.**

**Running title: Androgen Receptor Gene Expression in Brain**

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## ABSTRACT

In order to characterize androgen receptor (AR) gene expression in the central nervous system (CNS) and peripheral tissues of male rats we used cDNA probes encoding regions of the rat AR gene to measure AR mRNA by Northern blot analysis and by in situ hybridization. Using a 962 bp probe from the 5' untranslated region of the rat cDNA (AR1), a single mRNA species of approximately 11kb was seen in Northern blots of poly A + RNA from reproductive tissues, kidney, liver and muscle. A faint band of this size was seen in whole brain and spleen. Using a 532 bp probe from the 5' end of the coding region (AR2), in addition to the 11 kb band, a novel transcript was seen in whole brain at about 9.3 kb. In poly (A+) RNA from dissected brain regions, the 9.3 kb transcript was predominant in the cortex, cerebellum and brain stem while in the hippocampus, both transcripts were expressed to a similar degree. Studies using a probe, which comprised the 530 bp 5' to the DNA binding domain (AR3), showed the same pattern as the AR-2 probe. AR mRNA levels increased 2-3 fold in the prostate on days 1 and 3 following castration but no significant change was seen in either CNS transcript in whole brain or cortex. Specific in situ hybridization of an 35 S-labelled AR-2 riboprobe was observed in the medial amygdala, ventromedial nucleus, habenula, bed nucleus of the stria terminalis, medial preoptic area, lateral septum, pyriform cortex and hippocampus . We conclude that two AR mRNA species exist in the adult male rat. In the central nervous system, the 9.3 kb species is predominant but the relative proportion of the two species varies between brain regions. In contrast to observations in the prostate, brain AR gene expression is not regulated in the short term by androgen withdrawal . AR probe hybridization was observed in areas known to bind radiolabeled androgens. Based on the failure of AR1 to hybridize to the 9.3 kb species, the data suggest that a difference in the 5' untranslated region, arising perhaps by a splicing event, may account for the two AR mRNA transcripts.

## INTRODUCTION

Androgens and their specific receptor (AR) form a complex which is a transacting factor affecting the transcription of androgen-responsive genes. The AR is a member of the steroid/thyroid hormone superfamily of receptors (1) and shows structural homologies with other steroid receptors. An understanding of the localization and regulation of AR is essential in the study of androgen action. The cloning and structural analysis of the rat AR has allowed initial studies of the AR gene expression (2,3).

In addition to regulating the androgen-responsive tissues of the reproductive tract, androgen receptors are present in brain tissue and affect a number of central nervous system (CNS) functions including gonadotropin release, sexual and aggressive behavior (4,5) and various neuroendocrine systems (6-8).

The anatomical distribution of AR in brain has been determined autoradiographically and in vitro (9-14). Using 3H-DHT as ligand, AR appear to be concentrated primarily in neurons of the medial preoptic area, bed nucleus of the stria terminalis, medial amygdala, ventromedial nucleus of the hypothalamus, as well as other hypothalamic nuclei and in the pyramidal cell layers of the hippocampus, the lateral septum and the parietal cortex.

We have characterized AR gene expression in CNS and peripheral tissues of adult male rats, preliminary to studies of androgen effect on neuroendocrine systems. We present evidence for a novel second AR mRNA transcript in the CNS which may differ from the previously described transcript (2) in its 5' untranslated region. As this smaller species may be regulated differently, these data have implications regarding the selection of AR nucleotide probes for the study of the AR in the rat CNS.

## RESULTS

### Tissue Distribution of AR mRNA Species

Fig. 1 represents the regions of the rat AR cDNA used to generate the probes used in these studies. Poly (A+) RNA from various peripheral tissues and brain regions was studied by Northern blot analysis. Using the AR-1 probe a single mRNA species of approximately 11 kb was seen in all tissues (Fig. 2, top pannel). The prostate and epididymis showed extremely high levels of expression, testis, kidney, muscle showed intermediate levels while low but detectable levels were seen in liver and spleen. In the brain regions, the level of the 11 kb species was highest in the hippocampus and was barely detectable in the cortex, cerebellum and brain stem. Using the AR-2 probe, 2 mRNA species were apparent (Fig. 2, bottom pannel); the 11 kb species was seen in an identical distribution and pattern to that of the AR-1 probe. However, an additional smaller 9.3 kb species was apparent in all brain regions. The 9.3 kb transcript accounted for approximately 90% of the hybridization signal in the cortex, cerebellum and brain stem and 55% of that seen in the hippocampus. The smaller species was also a minor component of the hybridization signal in the prostate, muscle and kidney; although the strong 11 kb hybridization signal in reproductive tissues precluded quantification of the 9.3 kb band. Using the AR-3 probe both the 11 and 9.3 kb bands were seen in the same pattern as AR-2 in all tissues studied (data not shown).

### Effects of Castration

Poly (A+) RNA was prepared from control rats and from rats 1 and 3 days post-castration with 3 pairs of rats at each time point. Northern blots were hybridized with the AR-2 probe and a representative blot is shown in Fig. 3. Blots were reprobred with mouse tubulin. Densitometric analysis of the data is shown in Table 1, and is normalized to the level of tubulin expression. A 4 fold rise in prostatic AR mRNA occurred on day 1 ( $p < .05$ ) with a return to control on day 3. Liver AR mRNA levels did not change

significantly. In the cortex, neither the total nor the relative levels of the 11 and 9.3 kb transcripts changed following castration.

### In situ Hybridization

Specific hybridization of the AR-2 riboprobe was observed in discrete regions of the rat brain. These included the medial preoptic area, lateral septum and pyriform cortex all of which showed moderate to strong hybridization (Fig. 4 a,b). In addition the CA1, CA2, CA3, and dentate gyrus of the hippocampus showed strong hybridization (Fig. 4c) .

### DISCUSSION

In this study we have shown that a cDNA probe (AR2, corresponding to the 5' end of the rat AR coding region hybridizes under high stringency conditions to two mRNA species in the rat central nervous system. The larger 11 kb species also hybridizes with a AR-1 cDNA probe from the published 5' untranslated region of the rat AR (2). The failure of the AR-1 probe to hybridize with the novel smaller 9.3 kb species implies that the smaller transcript differs in its 5' untranslated region. We have not yet assessed the homology of these transcripts in regions 3' to AR3. Thus it is possible that they may also differ in their DNA and/or ligand binding domains as well. If the latter is true, it may provide a possible explanation for previous reports of heterogeneity of androgen bind sites in rat brain (14).

The presence of two AR transcripts in brain may be explained either by the existence of two separate genes, or by alternate splicing (15) of transcripts of a single AR gene. We are currently examining both possibilities by performing Southern analysis of genomic DNA and isolating, cloning and sequencing DNA from rat genomic libraries. Multiple mRNA transcripts of other steroid hormone receptor genes have also been reported for the estrogen(16), and progesterone (17) receptors, as well others which are structurally related such as thyroid hormone receptors(18).

The important consequence of a difference in the 5' untranslated region between the two species is that they may be differentially regulated. Certainly they show different relative levels of expression between brain regions. The 9.3 kb transcript represents the dominant form in the cortex, cerebellum and brain stem, while each species is similarly expressed in the hippocampus. On the other hand, neither species shows significant regulation following androgen withdrawal as neither the total AR hybridization signal nor the relative level of each transcript was altered up to 3 days after castration in the cortex.

Previous reports on the expression of AR found a single transcript of 10 kb which increased in all tissues following castration (2,19). Our gels were designed to resolve high molecular weight transcripts, and revealed 2 transcripts, the larger of which has a relative distribution similar to that previously reported (2,19). We did find other minor differences, however. First, we calculated the molecular weight to be ~11 kb compared with 10 kb. Second, we were able to detect low levels of hybridization in splenic RNA. Finally, we did not observe a universal increase in AR mRNA levels in tissues in response to castration. Only the prostate showed consistent and significant (approximately 4-fold) increases in mRNA levels on day 1 following castration. We consistently found no significant increases in expression in other tissues including the liver, whole brain and cortex. If any change in AR mRNA levels occurs in these tissues it is beyond the level of resolution of our Northern blot/densitometric analysis.

Using an AR2 riboprobe for in situ hybridization, we detected AR mRNA in several defined brain regions. The strongest signals were detected in the medial preoptic area, medial amygdala, bed nucleus of the stria terminalis and all cell body regions of the hippocampus. Previous studies using radiolabeled androgens have shown a similar pattern of distribution. Nonetheless differences exist between the relative abundance of androgen binding AR mRNA levels. This is most noticeable in the hippocampus where the mRNA levels are strikingly high while androgen binding has been reported to be only moderate with respect to other areas (13). Translational control and/or AR

protein processing and stability may account for these differences. Simerly et al (20) have recently reported similar findings with respect to distribution of AR mRNA in brain.

The use of a cDNA probe to sequences of the published coding region (such as AR-2) will detect both the 11 and 9.3 kb species. Our data suggest that these species differ in their 5'-untranslated and regulatory regions. In future studies of AR gene regulation, it will be important to identify the nature of these two different transcripts and develop probes that specifically identify each.

## **METHODS AND MATERIALS**

### **Animals**

Adult male Wistar rats (260-280 g) were obtained from Simonsen Labs. Inc., Gilroy, CA. Animals were used as sham or intact controls or were castrated under inhalational anaesthesia using Metofane (methoxyl fluorane, Pitman-Moore Inc., Washington Crossing, NJ) and killed 1, 3 and 8 days later by decapitation. The study was approved by the Animal Use Subcommittee of the University of Washington.

### **RNA Preparation**

Tissues were either processed immediately or frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  prior to RNA extraction. Tissues collected included liver, spleen, skeletal muscle, kidney, testis, epididymis, prostate and whole brain. In some animals, brain regions were dissected and separate preparations were made from the cortex, cerebellum, brain stem and hippocampus. Equal portions of a tissue from each rat (6 animals/group) were combined for the RNA extraction. Total RNA was extracted using guanidinium thiocyanate and phenol extraction as previously described (21). Poly (A<sup>+</sup>) RNA

was purified on oligo dT cellulose (Collaborative Research Inc, Bedford MA).

### Northern Blot Analysis

Poly (A+) RNA (5 mg/lane, OD 260) was fractionated on a 0.75% agarose 18% formaldehyde gel at 25 mV for 50 h. RNA was partially hydrolysed in 50mM NaOH, 100 mM NaCl for 30 min, prior to blotting onto a Nytran membrane (0.15  $\mu$ m, Schleicher and Schuell, Keene, NH). Prehybridization and hybridization were performed in 50% formamide, 5 X SSC, 1 X Denhardt's, 1% SDS, 10 mM Na<sub>2</sub> PO<sub>4</sub>, 100  $\mu$ g/ml herring sperm DNA for 16 h at 50°C.

Blots were washed (3 x 45 min) in 0.1 X SSC, 0.1% SDS at 65 °C and exposed for between 3 h and 11 d on Kodak X-OMAT AR film. An RNA ladder (BRL Inc.) was used as a size marker.

### AR Probes

The 3 cDNA probes were generously supplied by Dr. Frank French of the Department of Pediatrics, Univ. of North Carolina. AR-1 was a 960 bp sequence comprising the majority of the 5' untranslated region as published (2). AR-2 was a 530 bp sequence commencing immediately 3' to AR-1 and extending 500 bp into the coding region. AR-3 was a 919 bp fragment between AR-2 and the DNA-binding domain (see Figure 1). Isolated inserts were used to generate <sup>32</sup>P-labeled probes by the random primer method (22). Antisense riboprobes for *in situ* hybridization were produced using linearized pGEM plasmid containing the insert which had been subcloned and religated in the appropriate orientation for use of the T7 promoter and <sup>35</sup>S-UTP.

### In situ Hybridization

In situ hybridization histochemistry was performed as described previously (23). Slide-mounted brain sections were warmed to room temperature, treated sequentially with 4% paraformaldehyde, 0.25%

acetic anhydride (in 0.1 M triethanolamine, pH 8.0), a graded alcohol series, and chloroform and air dried. Probe (2 pmol/ml) was applied in 45  $\mu$ l hybridization buffer [50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1mM EDTA, 0.2% BSA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.5 mg/ml yeast tRNA, and 10 mM dithiothreitol], and slides were incubated at 45°C in a moist chamber overnight. To remove unhybridized probe, slides were washed for 15 min at room temperature with 2 x SSC (1 x SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), followed by RNase-A digestion as described previously (24). Additional washes were performed in 2 x SSC for 1 h at room temperature, 0.1 x SSC for 30 min at 62C, and 0.1 x SSC briefly at room temperature. Sections were dehydrated in a graded alcohol series in which water was replaced by 0.6 M ammonium acetate and air dried.

To assess specificity of labeling, some sections were treated as described except for the substitution of labeled sense probe for labeled antisense probe. In addition, other sections were treated with a mixture of labeled antisense probe and excess (250-fold) unlabeled antisense RNA. Slides subjected to in situ hybridization were apposed to Hyperfilm-BetaMax (Amersham) for 44 h, and film was developed in D19 (Eastman Kodak, Rochester, NY). In situ hybridization labeling was considered specific if it was 1) bilaterally symmetrical, 2) consistent from section to section and from brain to brain, and 3) absent in sections treated with labeled sense probe or a mixture of labeled antisense probe and excess unlabeled antisense RNA.

### Densitometry

A computerized image analysis system (Bioimage VISAGE 2000, Ann Arbor, Michigan) was used to quantitate the hybridization signal. The system performs what is termed whole band analysis, and measures the optical density of all the pixels that lie within the boarder of a band area defined by a derivative method. The blots were subsequently hybridized with a  $^{32}\text{P}$ -labeled mouse tubulin cDNA and the amount of RNA loaded/lane normalized accordingly.

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Table 1

	CONTROL	CASTRATION	
		Day 1	Day 3
Prostate 11 kb	100 ± 23	408 ± 100 *	100 ± 28
Liver 11 kb	100 ± 38	191 ± 40	43 ± 33
Cortex 11 kb	100 ± 25	90 ± 17	121 ± 17
9.3 kb	100 ± 18	105 ± 18	112 ± 17
% 9.3 kb <sup>b</sup>	89	87	88

Densitometric analysis of AR-2 hybridization signal following castration. Data normalized according to <sup>32</sup>P-tubulin hybridization and expressed as % control levels. For cortex, each of the 2 transcripts were analyzed separately. b, the percentage of total AR-2 signal accounted for by the 9.3 kb transcript. Three pairs of animals/data point. Data expressed as mean ± SE. \*, p <0.05 compared with control.

## Figure Legends

1. Schematic representation of the rat AR gene adapted from Tan et al 1988 (2) showing the regions represented in the 960 bp AR-1 and 530 bp AR-2 cDNA probes. AR 3 comprises the span between AR2 and the DNA binding domain.
2. TOP: Poly A+ RNA (5 mg) from the tissues and brain regions indicated were size fractionated on a denaturing agarose - formaldehyde gel, transferred to a Nytran membrane and hybridized with the P-labelled AR-1 cDNA probe. A 11 kb band is seen in most tissue. Some non-specific and variable hybridization is seen to the 28S ribosomal RNA as previously described (2).

BOTTOM: The same blot was stripped, exposed for 7 days to confirm removal of AR1 probe, and reprobbed under identical conditions with the AR-2 cDNA probe. In addition to the 11 kb band, a 9.3 kb band is apparent in RNA samples obtained from all brain regions.

3. Poly (A+) RNA (5 mg) from the prostate, liver and cortex on days 0, 1 and 3 following castration was fractionated as discussed and hybridized with the AR-2 cDNA probe (top panels) and a <sup>32</sup>P labelled tubulin probe (bottom panels). Densitometric analysis showed a significant increase in AR mRNA levels only in the prostate (see Table 1). RNA from 2 animals/track, one of a triplicate experiment is shown.
4. Film autoradiograms of in situ hybridization for AR mRNA in rat brain using the AR-2 cRNA probe. Regions labeled include (a) the cerebral cortex (c), bed nucleus of the stria terminalis (BNST), pyriform cortex (PC), (b) the medial preoptic area (mPOA), and (c) the medial amygdala (MA), and the ventromedial nucleus (VMN). The CA1 layer of the hippocampus (H), is also indicated.

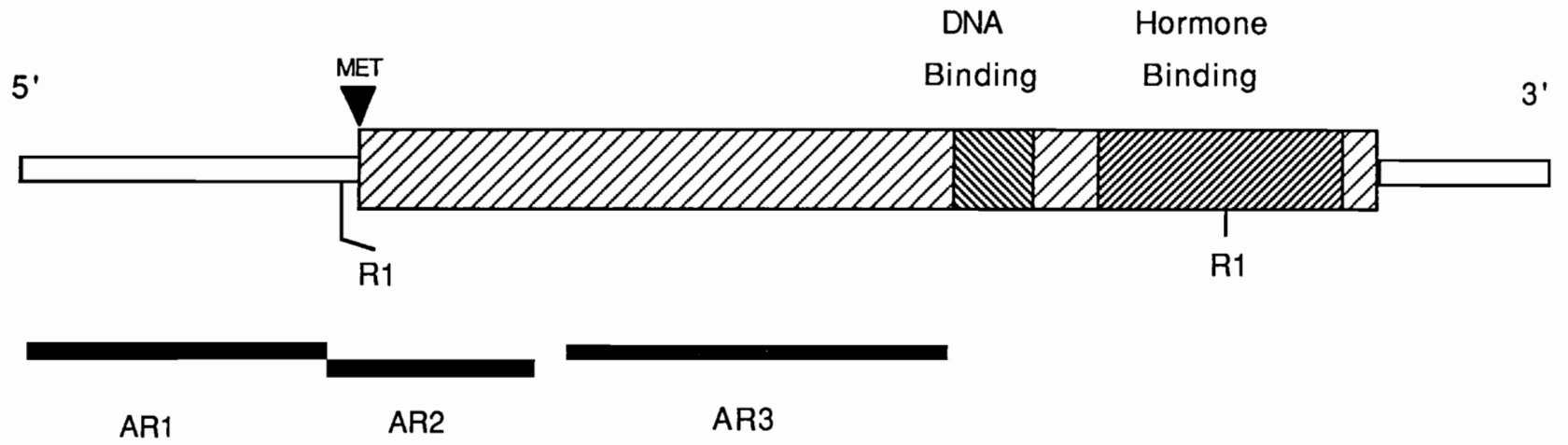


Fig 1

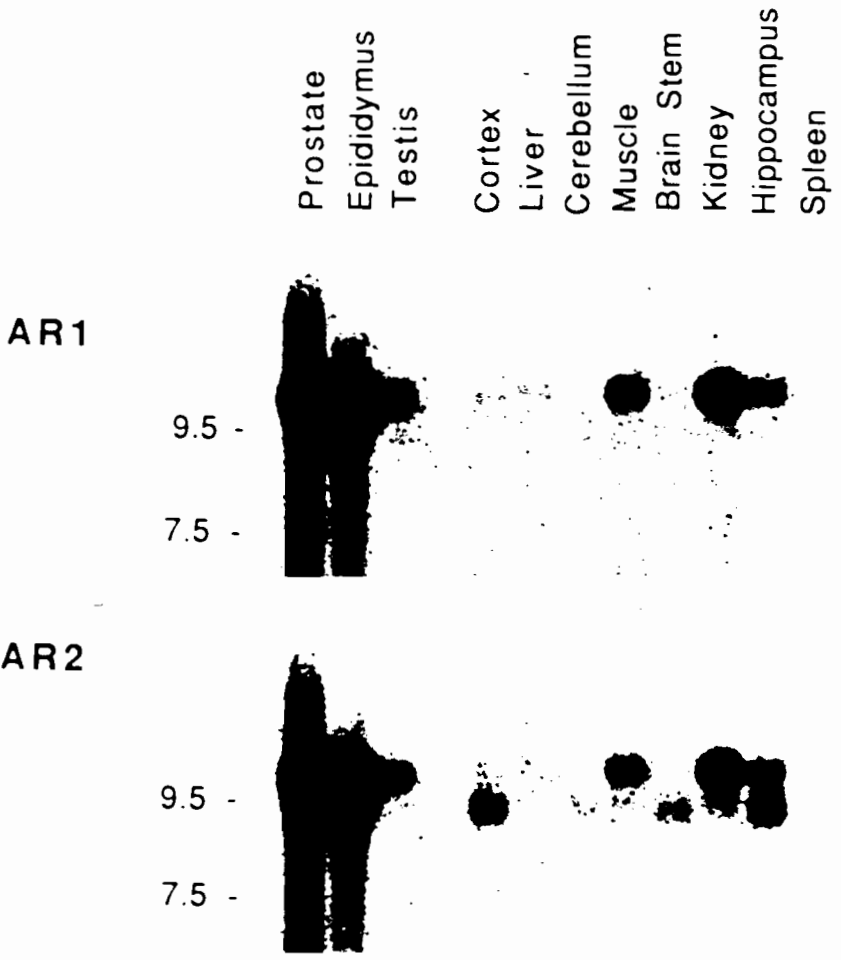


Fig 2

**EFFECT OF CASTRATION**

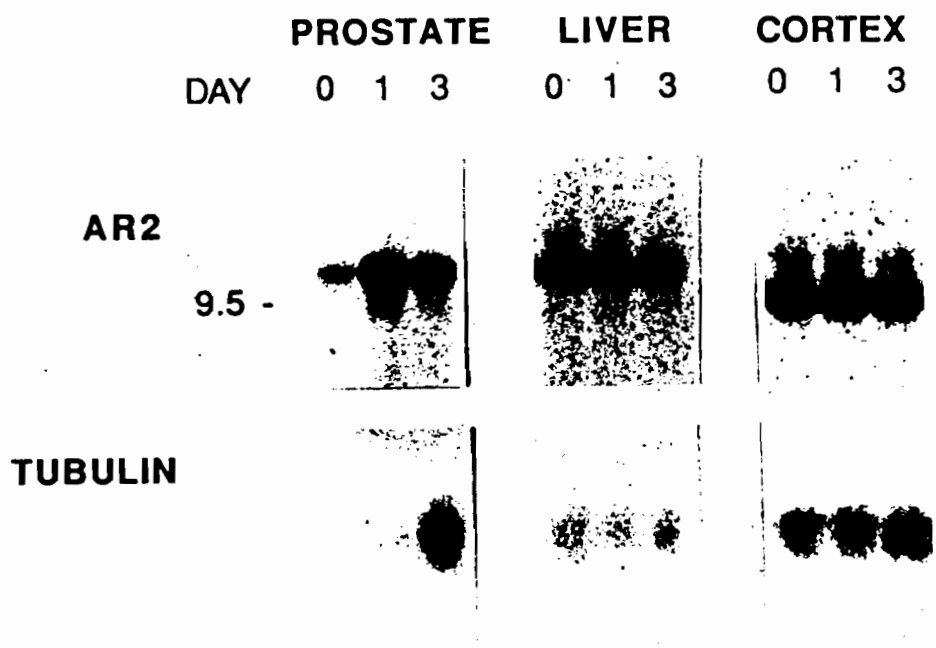


Fig 3

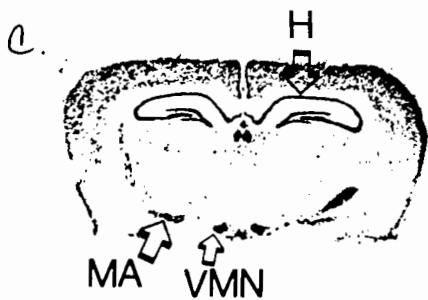
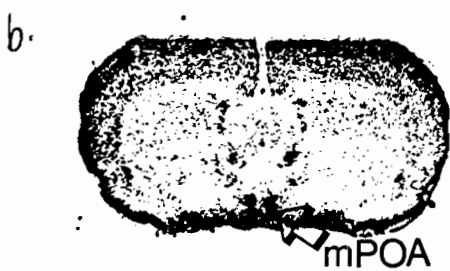
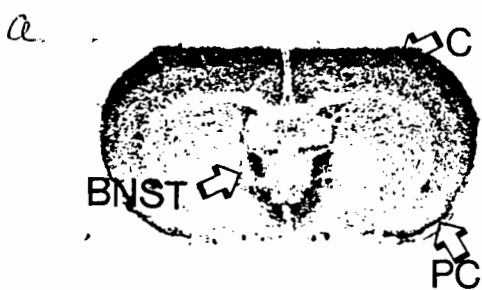


FIG 4