EFFECTS OF TESTOSTERONE AND SEX ON THE CYTOARCHITECTURE AND THE GAP-43 AND TAU-1 IMMUNOREACTIVE INNERVATION OF THE MEDIAL NUCLEUS OF THE AMYGDALA

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by

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ABSTRACT

The posterior dorsal division of the medial nucleus of the amygdala (MePD) is a target of gonadal hormone action. It is sexually dimorphic, and many of its neurons contain estrogen and androgen receptors. In adult male rats, its cytoarchitecture depends on testosterone (T), as the volume of MePD is reduced by 27% at 8 weeks post-castration (Malsbury and McKay, 1994). Experiment 1 of this thesis was based on the hypothesis that this atrophic response to castration includes the retraction of axons within MePD. If this hypothesis is correct, T replacement should stimulate axonal growth within MePD. In experiment 1, castrated male rats were given T, and two protein markers of axonal growth, GAP-43 and tau-1, were examined using immunocytochemistry.

Several recent studies have found sex differences in GAP-43 mRNA levels in the ventromedial nucleus of the hypothalamus (Shughrue and Dorsa, 1993a) and the frontal cortex (Lustig et al., 1993; Lustig et al., 1991) of adult rats. Based on these findings, experiment 2 investigated the possibility of sex differences in GAP-43 and/or tau-1 staining in the region of MePD.

Four groups of adult males were examined in experiment 1. Eight weeks after castration, controls received empty silastic capsules sc for either 2 (group CC-2) or 8 days (group CC-8),

while T-treated males received T-filled silastic capsules sc for either 2 (group C+T-2) or 8 days (group C+T-8), before being sacrificed. Alternate series of brain sections were stained for GAP-43, tau-1, and with cresyl violet. The relative optical densities (RODs) of GAP-43 and tau-1 staining in the region of MePD and part of the cerebral cortex were measured. The size of MePD was measured using cresyl violet-stained sections. As staining for both GAP-43 and tau-1 was darker in the region of MePD than in surrounding areas, the area of this darker staining was also measured. The results showed that T-treatment did not augment the density of GAP-43 or tau-1 immunoreactivity in the region of MePD or the cerebral cortex. However, it did increase the size of MePD, since the area of MePD in cresyl-stained sections was significantly larger in group C+T-8 than in group CC-8.

In experiment 2, brain sections of untreated adult males and diestrous females were stained for GAP-43, tau-1 or with cresyl violet as in experiment 1. Area and ROD measures were acquired as in experiment 1. No sex differences were found in the density of GAP-43 or tau-1 staining in the region of MePD or the cerebral cortex. However, there was a marked sex difference (male > female) in the size of MePD as well as in the areas of dense staining for GAP-43 and tau-1 in this region.

When data from castrated and intact males from experiment 1 and 2 were compared, it was found that castration did not reduce the density of GAP-43 or tau-1 staining in the region of MePD or the cerebral cortex. Castration did, however, significantly reduce the size of MePD as well as the areas of dense GAP-43 and tau-1 staining in this region.

In conclusion, the present experiments demonstrate that although T-treatment for 8 days is sufficient to produce a growth response within MePD in castrated male rats, this is not accompanied by any increase in the average density of staining for GAP-43 or tau-1 in the region of MePD. However, the size of MePD and the areas covered by the dense GAP-43 and tau-1 staining in the region of MePD are reduced by castration. Castration probably decreases the size of MePD at least partly by shrinking the dendritic trees of its neurons. This is probably accompanied by a loss innervation of MePD dendrites by GAP-43 and tau-1 immunoreactive axons. The reason for the lack of effect of castration on the average density of staining is not known. It may be that castration reduces both the synthesis and degradation of GAP-43 and tau-1, resulting in no change in their levels within the region where MePD dendrites are still present.

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INTRODUCTION

Gonadal hormones influence the structure of neural circuits in the CNS (reviewed in Arnold and Breedlove, 1985; Arnold and Gorski, 1984; Arnold and Jordan, 1988). The exposure of the brain to gonadal hormones can result in changes in such morphological characteristics as neuronal size, neuron number, dendritic length and branching, volume of defined neuronal groups, synaptic organization and the density of axonal innervation (reviewed in Toran-Allerand, 1984). The actions of gonadal hormones have been generally thought of as being either organizational or activational. Organizational effects are ones which occur during a "critical" period of development in early life that permanently "organize" the development of neural circuits. On the other hand, activational effects occur in adulthood and are thought of as being reversible and not permanent. They "activate" neural circuits which have already been organized. In this thesis only the activational effects of gonadal hormones were of interest. More specifically, the interest was in the activational effects of testosterone (T) in one area of the brain which has received much recent attention, the medial nucleus of the amygdala. The hypothesis was that T replacement in castrated males would stimulate axonal growth that could be detected by an increased density of staining for

protein markers of axonal growth, growth-associated protein 43 (GAP-43) and tau-1, within the medial amygdala.

The Medial Amygdala: Afferent and Efferent Connections

The medial amygdala contains mostly small neurons (5-10 microns) which have long and thick dendrites, and axons which give off collaterals that have limited branching (De Olmos et al., 1985). It receives input and projects to almost every major division of the brain. It is connected to areas in the cortex, thalamus, limbic system and brain stem.

At the most rostral level in the brain, the accessory olfactory bulb sends projections to (De Olmos et al., 1985) and receives input from the medial amygdala (Haberly and Price, 1978). The medial amygdala also receives a small number of afferent connections from the agranular insular cortex and the temporal subiculum (Ottersen, 1982). The thalamic projections to the medial amygdala are fairly diverse and come from the paraventricular nucleus, parataenial nucleus, medial division of the medial geniculate nucleus, principal medial geniculate nucleus, the ventrobasal complex (Ottersen and Ben-Ari, 1979) and the peripeduncular nucleus (Ottersen, 1981). The medial amygdala also sends efferent projections back to the mediodorsal nucleus of the thalamus (Siegel et al., 1977). It should be noted that the

connections between the medial amygdala and the thalamus are not extensive.

The hypothalamus and medial amygdala share an elaborate network of connections. The medial amygdala receives a rather heavy innervation from the ventromedial nucleus (Conrad and Pfaff, 1976b; Ottersen, 1980), a fairly dense input originates from the ventral premammillary nucleus (Ottersen, 1980), while a smaller number of projections come from the paraventricular (Conrad and Pfaff, 1976b; Ottersen, 1980), arcuate and dorsal premammillary nuclei (Ottersen, 1980). A small number of projections also originate from the anterior (Conrad and Pfaff, 1976b) and lateral (Ottersen, 1980) hypothalamic areas. Neurons in the medial amygdala also project axons back to innervate the ventromedial (Krettek and Price, 1978; Luiten et al., 1983), premammillary (Krettek and Price, 1978) and paraventricular nuclei (Silverman et al., 1981) of the hypothalamus.

Fairly extensive reciprocal connections between the medial preoptic area (MPOA) and the medial amygdala also exist. There is a substantial number of projections from the MPOA to the medial amygdala (Conrad and Pfaff, 1976a; Ottersen, 1980; Simerly and Swanson, 1988) and from the medial amygdala back to the MPOA (De Olmos et al., 1985). A less prominent afferent connection to the medial amygdala from the lateral preoptic area also exists (Swanson, 1976).

De Olmos et al. (1985) have advanced the view that the bed nucleus of the stria terminalis (BNST) can be considered as an extension of the amygdala. There are extensive reciprocal connections between the BNST and medial amygdala. The BNST projects heavily to the medial amygdala (Ottersen, 1980) and receives a large innervation from the medial amygdala (Krettek and Price, 1978; Weller and Smith, 1982).

Finally, the medial amygdala receives a small innervation from several areas of the brain stem. These areas include: the dorsal raphe, superior central nuclei, the parabrachial nucleus and the dorsal nucleus of the lateral lemniscus (Ottersen, 1981). Medial amygdala neurons also send projections back to several areas of the brain stem, including the ventral tegmental area, midbrain central gray and reticular formation (De Olmos et al., 1985).

As stated above, substantial reciprocal connections occur between the medial amygdala and the MPOA, ventromedial nucleus of the hypothalamus (VMH) and BNST. This is interesting due to the fact that these areas are sexually dimorphic in some respect and are hormone-sensitive. For example, a component of the MPOA, the sexually dimorphic nucleus of the preoptic area is sexually dimorphic with respect to its volume (Gorski et al., 1978; Gorski et al., 1980). The posterior medial BNST (also referred to as the encapsulated part of the BNST) is also sexually dimorphic with respect to its size (Hines et

al., 1992; Malsbury and Mckay, 1987). In the VMH, there is a sex difference in the number of synapses (Matsumoto and Arai, 1986). The medial amygdala is sexually dimorphic with respect to its synaptic organization, nuclear volume and neuropeptide concentration or immunoreactivity (see section below). The MPOA, BNST, VMH and medial amygdala all contain a high number of estrogen-concentrating (Pfaff and Keiner, 1973) and androgen-concentrating neurons (Sar and Stumpf, 1975). This has given rise to the concept of an interconnected network of steroid-sensitive neurons (Cottingham and Pfaff, 1986). This means that these areas contain neurons which are likely to be targets of the organizational and/or activational actions of gonadal hormones.

Sex Differences in the Medial Amygdala

One of the earliest studies demonstrating a sex difference in the medial amygdala was by Nishizuka and Arai (1981). Using the electron microscope, they observed that the synaptic organization of the rat medial amygdala was sexually dimorphic in that males had a significantly greater number of dendritic shaft synapses than females. This sex difference seemed to be the result of androgen exposure in the neonate. Several years later, Mizukami et al. (1983) demonstrated the existence of a sex difference in cytoarchitecture in the medial amygdala. They found that the nuclear volume of the

entire medial amygdala was markedly larger in male than in female rats and that this difference was also due to the organizational action of gonadal hormones in early postnatal life. The medial amygdala can be subdivided into several cell groups, two of those being the posterior dorsal and posterior ventral divisions. The volume of the posterior dorsal region of the medial amygdala is also much greater (85%) in male rats than in females (Hines et al., 1992).

Other studies have revealed sex differences (male > female) in the medial amygdala, but these involve differences in neuropeptide concentration or immunoreactivity. Two have found greater substance P concentration in male rats than in females (Frankfurt et al., 1985; Micevych et al., 1988b). Others have found greater numbers of cholecystokinin-immunoreactive cell bodies in the male rat (Micevych et al., 1988a). Studies conducted in this laboratory have demonstrated that the area of the fields of substance P-immunoreactive (SPir) fibers in the posterior dorsal division of the medial amygdala (MePD) in adult rats is over twice as large in males as in females (Malsbury and McKay, 1989). It has also been established that T organizes the development of this sex difference since injecting females on postnatal days 2 and 5 with T masculinizes the areas of SPir fiber staining in 27 day old prepubertal rats, while castration of newborn

males prevents the development of the male greater than female pattern of SPir fiber staining (Malsbury and Brown, 1989).

Activational Effects of T on the Medial Amygdala

The sex differences described above are in part the result of the organizational actions of gonadal hormones. However, gonadal hormones also produce activational effects. The activational actions of gonadal hormones are often investigated by looking at the effect of castration, thus showing that T has a regulatory role in the biochemical and/or structural maintenance of the CNS. Studies using this approach have demonstrated a drastic reduction in the immunoreactive levels of a number of different peptides in the medial amygdala. More specifically, castration of adult male rats results in a reduction in the number of vasopressin-immunoreactive (DeVries et al., 1985) and cholecystokinin-immunoreactive (Micevych et al., 1988a; Simerly and Swanson, 1987) cell bodies. This indicates a decline in the levels of these neuropeptides when the influence of T is removed. Similarly, there is a reduction in the intensity of staining and the area covered by SPir fibers in the medial amygdala following castration of adult male rats (Dees and Kozlowski, 1984; Malsbury and McKay, 1994).

The above findings show that T has a regulatory role and is necessary to maintain certain morphological characteristics

of the CNS. Activational actions of gonadal hormones, as have already been described, are thought to be reversible. As discussed above, the removal of T (the effect of castration) results in changes in the neural circuitry of the medial amygdala. Can replacement of T after castration reverse these changes, and if so, how soon does this occur? Studies which have replaced T after castration have shown, indeed, that the effects of castration can be reversed. For example, the reduction in the number of cholecystokinin-immunoreactive cell bodies in the medial amygdala 14 days after castration can be reversed by subsequent T-treatment for 14 days (Simerly and Swanson, 1987). Similar effects have been found in other locations in the brain. In the BNST (Van Leeuwen et al., 1985) and the lateral septum (De Vries et al., 1984) the drastic reduction in the number of vasopressin-immunoreactive cells (Van Leeuwen et al., 1985) and fiber density (De Vries et al., 1984) as a result of castration is reversed by 5 weeks of T replacement.

The effects of T replacement therapy after castration were of special interest to this thesis. This interest was sparked by the findings of a previous study conducted in this laboratory. These findings revealed that T regulates the SPir innervation of the medial amygdala in the adult rat, since 8 weeks after castration the area of the SPir fiber fields in MePD is reduced by as much as 42% compared to sham-operated

controls (Malsbury and McKay, 1994). In addition, castration also affected the cytoarchitecture of MePD. Gonadectomy reduced the size of MePD by approximately 27% (Malsbury and McKay, 1994). While these findings demonstrate that T has neurotrophic effects on this region of the brain, they do not give any indication of the mechanism(s) of hormone action.

Castration may lead to the retraction of axonal processes within MePD since the overall area of SPir fiber staining is greatly reduced. In fact, Malsbury and Mckay (1994) found that the greatest reduction in the area of SPir-staining occurs in the rostral portion of MePD. They suggest that this loss may be the result of the retraction of SPir axonal processes originating more caudally in MePD. The effect of replacing T after possible shrinkage of MePD has taken place has not been investigated as yet. Replacement of T may lead to regrowth of axons which may have retracted. A possible way of investigating such a question, is to look for induction of proteins whose levels are greatly enhanced when axons extend their processes. This may also indicate a possible mechanism through which T may cause growth of axonal processes. Two proteins which are involved with such growth are GAP-43 and tau. In this thesis these two proteins were used as axonal "growth markers".

What is GAP-43?

Growth-associated protein 43 as its name suggests is a protein which is associated with growth in both the peripheral and central nervous system. It is the same protein which has been referred to as GAP-48, pp46, F1, B50 (reviewed in Benowitz and Routtenberg, 1987) and P-57 (Wakim et al., 1987).

GAP-43 has 226 amino acids and is extremely hydrophillic (Basi et al., 1987). It has an acidic isoelectric point of 4.5 (Kalil and Skene, 1986; Nelson and Routtenberg, 1985; Skene and Willard, 1981a). GAP-43 is found only in the nervous system (Kristjansson et al., 1982) and its mRNA is expressed primarily in neurons (Basi et al., 1987; Neve et al., 1987). It is a component of axonal growth cones (Meri et al., 1986; Skene et al., 1986; Goslin et al., 1988), is membrane bound (Skene et al., 1986) and has an intracellular, non-uniform distribution on the membrane (Meiri and Gordon-Weeks, 1990). It seems to be associated with growing axons, but not dendrites (Goslin et al., 1988).

According to its migration on SDS-polyacrylamide gels it has an apparent molecular weight that varies from 43-57 kDa or greater, depending on the concentration of acrylamide in the gel (Jacobson et al., 1986; Benowitz et al., 1987). Using biophysical methods its true size in the presence of detergent was estimated as being 32.8 kDa (Benowitz et al., 1987). However, the predicted molecular weight from direct cDNA

sequence data gave a smaller value of about 24 kDa (Basi et al., 1987; reviewed in Benowitz and Routtenberg, 1987). Possibly the discrepancies in the estimated molecular weight of GAP-43 can be explained by the fact that the protein's amino acids may or may not be phosphorylated.

Before giving evidence which indicates that GAP-43 can be thought of as an axonal growth marker, it is important to consider the other functional roles which GAP-43 may play in the CNS. These include: being a substrate for protein kinase C and a calmodulin binding protein, being involved in phospholipid metabolism, being involved with long term potentiation, and being involved with structural remodelling in the CNS (see discussion below).

GAP-43 is a Substrate for Protein Kinase C and is a Calmodulin Binding Protein

GAP-43 can be phosphorylated by protein kinase C (Akers and Routtenberg, 1985; Aloyo et al., 1983; Nelson and Routtenberg, 1985) and thus serves as a substrate for this enzyme. This phosphorylation may be directly involved with neurotransmitter release (reviewed in De Graan et al, 1990; Dekker et al., 1989; Norden et al., 1991).

Andreasen et al. (1983) were the first to discover that GAP-43 was a unique calmodulin binding protein in that binding would only take place in the absence of calcium and not in its

presence. They suggest that one function of GAP-43 is to bind calmodulin in local sites within the cell making its concentration higher at these locations and releasing calmodulin when there are increases in intracellular free calcium. In this way, it would act as a 'calmodulin sponge' when calcium levels are low. However, calcium may not be the only regulator of calmodulin binding to GAP-43. Protein kinase C may also serve this function, since phosphorylation of GAP-43 by protein kinase C prevents GAP-43 binding to calmodulin (Alexander et al., 1987).

The Role of GAP-43 in Phosphatidylinositol Metabolism

GAP-43 participates in an important second messenger system in the CNS. This second messenger pathway begins with the phosphorylation of phosphatidylinositol 4-phosphate (PIP) to produce phosphatidylinositol 4,5-biphosphate (PIP_2), this is followed by receptor-activated hydrolysis of PIP_2 to produce two second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (reviewed in Berridge, 1987). IP_3 subsequently stimulates the release of calcium from intracellular stores and DAG activates protein kinase C (see Berridge, 1987). GAP-43 inhibits the activity of PIP kinase (Van Dongen et al., 1985) and in synaptic plasma membranes GAP-43 antibodies inhibit the phosphorylation of GAP-43, but also stimulate the production of PIP_2 (Oestreicher

et al., 1983). This suggests that the phosphorylation of GAP-43 may be a regulatory factor in the metabolism of phosphoinositides in the brain. It also suggests that by preventing the production of PIP_2, phosphorylated GAP-43 can inhibit protein kinase C activation and IP_3-activated calcium release.

Levels of Phosphorylated GAP-43
Change During Long Term Potentiation

GAP-43 may be involved in the synaptic changes that take place during learning. Long term potentiation in the hippocampus has long been used as a model of such synaptic changes. It is produced when the perforant pathway projection from the entorhinal cortex to the dentate gyrus is briefly stimulated at high frequency, the activated synapses show a long-lasting increase in their subsequent responsiveness to low frequency stimulation (Bliss and Lømo, 1973). Increased GAP-43 phosphorylation has been shown to occur after the induction of long-term potentiation in the intact rat hippocampus (Lovinger et al., 1985; Lovinger et al., 1986; Nelson and Routtenberg, 1985) and in the hippocampal slice (Gianotti et al., 1992). The increased phosphorylation of GAP-43 is correlated closely with the magnitude and duration of the enhanced responsiveness (Lovinger et al., 1985; Lovinger et al., 1986).

GAP-43 is Involved in

Structural Remodelling in the Adult CNS

GAP-43 is involved with the reactive synaptogenesis that occurs in the rat hippocampal formation following lesions to the perforant pathway. GAP-43 levels increase markedly in the inner molecular layer of the dentate gyrus between 2 and 4 days after such lesions (Benowitz et al., 1990). However, GAP-43 levels in the outer molecular layer decline after the same lesions and do not recover until up to 3 weeks after lesioning (Benowitz et al., 1990; Masliah et al., 1991). The width of the band of GAP-43 immunoreactivity in the inner molecular layer of the dentate gyrus also increases following perforant pathway lesions (Benowitz et al., 1990; Masliah et al., 1991). According to Benowitz et al. (1990), the increases in GAP-43 expression occur at the same time commissural-association fibers sprout axon collaterals onto dendritic segments which have been denervated by the lesions.

The pattern of decline and subsequent recovery of outer molecular layer GAP-43 expression about 3 weeks after perforant path lesioning also coincides with denervation and reinnervation of this area (Benowitz et al., 1990; Masliah et al., 1991).

The finding that increased levels of GAP-43 occur during reactive synaptogensis in the adult CNS shows that even in the adult (as opposed to the neonate) CNS, axonal sprouting (or

growth) leads to enhanced GAP-43 expression in the intact animal.

GAP-43 is Involved With the Growth and Development of Neuronal Processes

The main interest in GAP-43 has been in its relationship with the growth and development of neuronal processes. That is why it is thought of as a "growth associated" protein. This relationship seems to exist during development, during the regeneration of axotomized axons and in the growth of processes in cultured cells.

In general, GAP-43 immunoreactivity (Dani et al., 1991) and GAP-43 gene expression (De la Monte et al., 1989) are extremely high throughout the entire rat brain during the first week of postnatal life, but decline sharply thereafter. When more specific areas are considered there are still elevated levels of GAP-43 in the neonate as opposed to the adult. For example, in the rabbit, GAP-43 levels in retinal ganglion axons (Skene and Willard, 1981a) and its synthesis in rat cerebellum and cortex (Jacobson et al., 1986) are extremely high during the first week of postnatal life, but decline markedly by adulthood.

A similar situation exists in the hamster where the level of GAP-43 in the retinofugal pathway is very high at postnatal days 2 and 5, but declines drastically by postnatal day 12 and

thereafter (Moya et al., 1988). In addition, GAP-43 in hamster pyramidal tract neurons is at high levels during the first 2 weeks of life, but declines to low levels by 4 weeks and this decline occurs at the same time pyramidal tract axons stop elongating (Kalil and Skene, 1986). Taken collectively these studies indicate that high levels of GAP-43 seem to be correlated with periods of axonal elongation which occur during the first few weeks of postnatal life, and when axonal growth has ceased levels of GAP-43 decline drastically.

Axotomy also affects levels of GAP-43 or its mRNA in various areas. When rat sciatic nerves are crushed, levels of GAP-43 significantly increase to a maximum value that is 60 times their normal level 14 days after axotomy (Bisby, 1988). GAP-43 mRNA expression also increases 11.5-fold 2 days after these nerves are crushed and remains at elevated levels for the next 12 days (Hoffman, 1989). Similarly, in CNS neurons of the toad which normally regenerate (retinal ganglion neurons), axotomy leads to a 15-20 fold increase of GAP-43 levels (Skene and Willard, 1981b). However, axotomy of the optic nerve of the adult rabbit, which does not regenerate, does not result in any significant changes in GAP-43 levels (Skene and Willard, 1981a). In general, axotomy induces increased GAP-43 expression in neurons which can regenerate, but cannot induce increased GAP-43 levels in neurons which do not regenerate (Skene and Willard, 1981a).

A correlation between elevated GAP-43 levels and growth of neuronal processes has been found in cultured cells as well. When PC12 pheochromocytoma cells are stimulated by nerve growth factor to produce neurite outgrowth, they exhibit 16-fold increases in GAP-43 levels after 2 days of exposure (Costello et al., 1991). When non-neuronal cells, that do not have endogenous GAP-43 or any other neuron-specific molecules, are transfected with expression vectors containing rat GAP-43 cDNA, they exhibit extended long processes that appear to have filopodia and the number as well as the complexity of these processes are correlated with the GAP-43 levels within the cell (Zuber et al., 1989).

Conversely, inhibition of GAP-43 suppresses neurite outgrowth. In NB2A/d1 neuroblastoma cells, GAP-43 is necessary for the active stage of growth of processes since antibodies to GAP-43 introduced into these cells stops process outgrowth in a dose-dependent fashion (Shea et al., 1991). Another study has used antisense oligonucleotides to block GAP-43 expression in chick embryo dorsal root ganglion neurons. This resulted in thinner and less branched neurites which had smaller growth cones when grown on a laminin substratum, and even prevented growth cone and neurite formation when plated on poly-L-ornithine (Aigner and Caroni, 1993).

In general, it seems that increased GAP-43 levels are expressed during those stages of development in which great axonal elongation is occurring throughout the entire CNS. In addition, increased GAP-43 expression occurs during regeneration of axotomized axons and the levels of GAP-43 correlate well with the initial extension of these damaged axons. Inhibition of GAP-43 results in the prevention of proper neurite formation in cultured cells. Overall, this indicates that there is a direct connection between growth of neuronal processes and enhanced GAP-43 expression and possible retarded neuronal processes formation in its absence. Since GAP-43 is primarily localized in axons (see above discussion) it can be used as a neuronal growth marker, exclusively for axons.

Gonadal Hormone Modulation of and Sex Differences in GAP-43 mRNA in the CNS

There have been several recent studies demonstrating that gonadal hormones regulate the expression of GAP-43 mRNA in various areas of the rat brain, and it is this regulation that could possibly affect axonal growth. Lustig et al. (1991) found that estrogen treatment of adult female ovariectomized rats increased the level of GAP-43 mRNA within the VMH at 2 hours and at 3 days after estrogen treatment. In the adult frontal cortex there exists a sex difference (male > female)

in GAP-43 mRNA (Lustig et al., 1993; Lustig et al., 1991). This sex difference may depend on aromatization of T to estradiol and subsequent binding to estrogen receptors since neonatal treatment of males either with an estrogen receptor antagonist or an aromatase inhibitor significantly decreases GAP-43 mRNA frontal cortical levels in adulthood, down to those seen in oil-treated females (Lustig et al., 1993). This suggests that it is exposure to estrogen and not T which organizes the development of the sex difference in adult GAP-43 mRNA levels in the frontal cortex. Shughrue and Dorsa (1993a) also demonstrated a sex difference (male > female) in GAP-43 mRNA in the VMH of the adult rat and that estrogen treatment of ovariectomized females significantly increased GAP-43 mRNA in both the MPOA and VMH to levels comparable to those seen in males.

Finally, in the 6 day old rat there exists a sex difference (male > female) in GAP-43 mRNA expression in the MPOA, BNST and frontal cortex (Shughrue and Dorsa, 1993b). Castration of neonate males significantly reduced GAP-43 mRNA levels in these areas, while T treatment of neonate females significantly increased GAP-43 mRNA to levels comparable to those seen in the males (Shughrue and Dorsa, 1993b). The above effects of T on GAP-43 mRNA levels in the 6 day postnatal rat brain are modulated by estrogen and androgen

receptor activation depending on the area of the brain being investigated (Shughrue and Dorsa, 1994).

It is not known whether gonadal hormones directly or indirectly influence the levels of GAP-43 mRNA in these areas of the brain. That is, there may be a direct relationship between the two such that gonadal hormones directly stimulate the production of GAP-43 mRNA, or there may be an indirect relationship in that gonadal hormones may stimulate the growth of neuronal processes and it is this enhanced growth which results in the increases in GAP-43 mRNA expression. Taken collectively, these studies indicate that gonadal hormones can stimulate GAP-43 gene expression, which supports the idea that T could possibly increase the levels of the protein itself in various areas, including the medial amygdala.

I have discussed the view that GAP-43 is an axonal growth marker in the CNS. In addition, hormones could possibly influence the levels of this axonal growth marker. Another quite different protein that can also be thought of as an axonal growth marker is tau.

What is Tau?

Tau is a neutral or slightly basic protein (Cleveland et al., 1977) which belongs to a family of microtubule-associated proteins. By using quick-freeze, deep-etch electron microscopy Hirokawa et al. (1988) demonstrated that tau

proteins are rodlike molecules in appearance and are approximately 50 nm long and bind to microtubules as periodic, short, armlike projections (< 20 nm long).

Adult tau appears as several electrophoretic species (how many depends upon what animal is being assayed) on one-dimensional SDS polyacrylamide gels having molecular weights of 55-62 kDa (Cleveland et al., 1977). However, the composition of tau changes during development. In the 3 day old rat tau appears only as two bands on SDS gels, but in the adult it appears as four bands (Mareck et al., 1980). Only one of these two bands in the 3 day old rat cross-reacts with both polyclonal and monoclonal antibodies raised against the adult tau species and it has an apparent molecular weight of 48 kDa and has been referred to as 'juvenile' tau (Brion et al., 1988). It is only present in the immature stages of development and is progressively replaced by adult tau proteins (Couchie and Nunez, 1985).

Initial studies showed tau-1 immunoreactivity seemed to be restricted to axons, as dendrites and cell bodies showed no immunoreactivity (Binder et al., 1985; Binder et al., 1986). However, a later study revealed that the specificity of tau-1 immunoreactivity to axons seems to depend on the state of phosphorylation (Papasozomenos and Binder, 1987). In rat nervous tissue treated with alkaline phosphatase (which dephosphorylates the tissue), tau-1 immunoreactivity is seen

in astrocytes, perineuronal glial cells, and the axons, cell bodies and dendrites of neurons (Papasozomenos and Binder, 1987). This illustrates the existence of two species of tau whose ability to bind with certain antibodies is determined by the phosphorylation state of the protein where one species is masked by phosphorylation.

Tau is thought of as a factor that is necessary for the assembly of microtubules from tubulin because it induces microtubule assembly in vitro (Weigarten et al., 1975; Witman et al., 1976). It is thought to induce microtubule assembly by binding to several tubulin molecules per tau molecule, thus increasing the local concentration of tubulin which leads to the formation of longitudinal filaments (Cleveland et al., 1977). Whether the growth of existing microtubules or the creation of new ones dominates, depends on the tau/tubulin ratio (Brandt and Lee, 1993).

Tau Affects the Assembly of Microtubules in Cultured Cells

Evidence that tau promotes the formation of microtubules from tubulin and can even cause neurite outgrowth has been shown using a variety of cultured cell types and using different techniques. By infecting nonneuronal cells (sf9 cells from the moth ovary) with a baculovirus containing a tau cDNA insert, Knops et al. (1991) demonstrated that tau infection of these cells which normally do not have it,

results in the expression of long processes which resemble axons that are immunoreactive for tau. Similarly, transfection of tau cDNA into fibroblast cells results in a significant increase in tubulin levels and polymerization of tubulin to form microtubules (Drubin and Kirschner, 1986; Kanai et al., 1989). Nerve growth factor promotes microtubule assembly and induces neurite outgrowth in PC12 rat pheochromocytoma cells which occur at the same time as massive increases in tau levels (Drubin et al., 1985). Thus, it seems that tau promotes the assembly of microtubules that is required for neuronal process extension in cultured cells. It has also been found to increase the stability of the microtubules once they are formed (Drubin and Kirschner, 1986; Ferreira et al., 1989) by decreasing their depolymerization rate (Drubin and Kirschner, 1986).

Another approach which has been used to study tau's involvement in microtubule formation and neurite growth is to examine the effect of tau inhibition in cells which normally express the protein. Reduction of tau levels by treatment with tau antisense cDNA oligonucleotides can inhibit or prevent neurite outgrowth and decrease the number of axonal neurites in cultured neuroblastoma cells (Shea et al., 1992). This treatment can also cause the retraction of existing neurites in neuroblastoma cells (Shea et al., 1992) and nerve growth factor induced PC12 cells (Hanemaaijer and Ginzburg,

1991). It can even prevent the establishment of an axon during normal neurite outgrowth (Caceres and Kosik, 1990) and cause the loss of existing axonlike neurites in cultured cerebellar neurons (Caceres et al., 1991).

When axons elongate, new cytoskeletal material must be added to the growing axon which would mean that a greater amount of microtubules, from which the cytoskeletal material is made, would be produced. Since microtubule formation is induced by increases in tau proteins (see above discussion), axonal elongation would possibly occur in the presence of an increase in the amount of tau. Thus, tau can be thought of as an axonal marker whose levels would most likely increase during periods of axonal growth.

Estrogen Can Induce Tau Expression

Estrogen has been shown to stimulate neurite outgrowth in dissociated cultured medial basal hypothalamic neurons (Ferreira and Caceres, 1991). This estrogen-enhanced neurite outgrowth may be mediated by an induction of tau proteins since adding estrogen to cultures results in a three-fold increase in tau levels (Ferreira and Caceres, 1991). This supports the idea that gonadal hormones can stimulate growth of neuronal process and that this growth involves the enhanced expression of tau proteins.

Does T Induce GAP-43 and/or

Tau Levels in the Medial Amygdala?

Castration of adult males reduces the SPir innervation of MePD and this can be prevented by replacement of T at the time of castration (Malsbury and McKay, 1994). Experiment 1 explored the possibility that replacement of T after shrinkage of MePD (due to the possible retraction of axons) has taken place, may result in the induction of greater levels of GAP-43 and/or tau, which would indicate that axonal regrowth has or will take place. This possibility was investigated by comparing the density of GAP-43 and tau immunoreactivity in the region of MePD at 8 weeks post-castration in controls to that in male rats treated with T for either 2 or 8 days.

Are There Sex Differences in GAP-43

and/or Tau Levels in the Medial Amygdala?

As discussed above, several studies have found sex differences in adult GAP-43 mRNA levels in several areas of the rat brain. In addition, gonadal hormones seem to regulate the levels of GAP-43 mRNA in these areas. Tau levels in cultured neurons can also be altered by the presence of estrogen. Taken collectively, this indicates that there may be sex differences in the levels of GAP-43 itself and tau in the CNS. The medial amygdala seems to be a prime target for the actions of gonadal hormones. Since there are many sex

differences in the medial amygdala which involve cytoarchitecture and neuropeptide immunoreactivity differences, it is possible that there are also sex differences in GAP-43 and/or tau immunoreactivity in the medial amygdala. This possibility was investigated in experiment 2 by comparing the density of GAP-43 and tau immunoreactivity in the region of MePD of normal male rats to that of normal diestrous females.

EXPERIMENT 1

METHODS

Subjects and Treatments

Sixteen adult male Sprague-Dawley strain albino rats (Charles River, Quebec) were used as subjects. All animals were kept on a 12/12 light/dark cycle with lights on at 0800 hours. Food and water were available ad libitum. At the time of surgery the males were approximately 61-67 days old and weighed 270-325 g (mean = 299.5 g).

Before castration animals were weighed and then anesthetized with sodium pentobarbital. Castrations were then performed using the inguinal approach. A small incision was made about 1 cm caudal and lateral to the penis on each side through which the testis was removed.

Eight weeks later, silastic capsules were inserted into all animals. Four groups (n=4/group) were examined. Castrated controls at 2-days (CC-2) and 8-days (CC-8) after silastic capsule insertion, and castrated plus testosterone at 2-days (C+T-2) and 8-days (C+T-8) after silastic capsule insertion. Subjects were randomly assigned to treatment conditions.

Before silastic capsules were inserted, they were incubated in 0.01 M phosphate buffered saline (PBS) for 2 days following the procedure of Smith et al. (1977) in order to eliminate an initial T surge which might have had an unwanted greater effect on group C+T-2 than on group C+T-8. This procedure involved placing each silastic capsule in a separate plastic vial which was filled with the PBS, and then placing all the vials in a warm water bath. The PBS was changed with a fresh solution 3 times each day.

Rats were anesthetized with sodium pentobarbital before silastic capsule insertion. A small incision was then made about 4 cm rostral to the penis just to one side of the midline and a hemostat was inserted subcutaneously (sc) in order to make a pouch for the silastic capsule. A silastic capsule (i.d. = 1.58 mm, o.d. = 3.18 mm , length = 45 mm) was inserted sc in this pouch. Animals in groups C+T-2 and C+T-8 received sc implants of silastic capsules that were filled with T crystals. It was expected that these implants would

produce constant plasma T levels within the normal physiological range (Damassa et al., 1977). Animals in groups CC-2 and CC-8 also received implants of silastic capsules, but these were empty.

Immunocytochemistry

Brains were processed for immunocytochemistry using the peroxidase-anti-peroxidase (PAP) method (Sternberger, 1986). Brains from a control and T-treated group were always carried through the staining procedures together to ensure that any variability would be equally distributed among groups.

Before perfusion, rats were deeply anesthetized with sodium pentobarbital. The inferior vena cava was exposed and 0.5 ml of 1% sodium nitrite was injected intravenously. Two minutes later, the heart was exposed and pressure perfusion was initiated via a butterfly needle inserted into the left ventricle. Rats were perfused with 4% buffered paraformaldehyde in about 15 minutes. Brains were removed and stored overnight in the 4% buffered paraformaldehyde. The next day, brains were placed in PBS and stored in the refrigerator.

All brains were transferred to a 10% sucrose in PBS solution for cryoprotection and stored in the refrigerator for 3 days before being frozen. A razor blade was used to make one coronal plane cut just anterior to the cerebellum to

produce uniform blocks. Then, brains were frozen by slowly lowering them into isopentane cooled in liquid nitrogen.

Brains were sectioned in the coronal plane in a cryostat at 40 microns. As they were cut, alternate sections were either placed in the wells of plastic trays that contained PBS or mounted on slides. The immunocytochemistry procedure was carried out on free-floating sections within plastic tubes which were placed in the wells of the plastic trays. These tubes had open bottoms covered with nylon netting and were used to transfer sections from one tray to another. When cutting was complete sections were transferred three times to new trays that contained fresh PBS.

To eliminate endogenous peroxidase activity, sections were then placed in 3% hydrogen peroxide in PBS for 30 minutes. This was followed by four washes in PBS, each lasting 10 minutes. In order to block non-specific binding of the second antibody, sections were then placed in 10% normal goat serum (Gibco) in PBS for 30 minutes and subsequently washed three times for 10 minutes each time with fresh PBS.

Sections from each brain were immunoreacted for either GAP-43, or tau-1, or stained with cresyl violet. Mouse monoclonal antibodies to GAP-43 and tau-1 (Boehringer-Mannheim) were used as the primary antibodies. According to Boehringer-Mannheim, the GAP-43 antibody used binds specifically to GAP-43 from rat, cat, hamster and human

regardless of the protein's phosphorylation state. Also, the tau-1 antibody used binds to all known electrophoretic species of tau in rat, human and bovine brain. Since brain sections were not dephosphorylated by alkaline phosphatase treatment in the present study, the tau-1 antibody stained axons only.

The primary antibodies were diluted in a solution of PBS, Triton X-100 and normal goat serum. Sections were transferred from PBS to either the GAP-43 or the tau-1 antibody. Sections were placed on a rotary agitator for 10 minutes at room temperature and thereafter stored in the refrigerator for two nights in the primary antibody solution.

Brains were stained for GAP-43 at a dilution of 1:1500 (n=2) or 1:2000 (n=14). The tau-1 dilutions were 1:5000 (n=8), 1:6000 (n=4), or 1:7000 (n=4). Negative control experiments were done previously to check for possible artifactual staining. In these experiments, normal adult male brains were sectioned at 40 microns and were carried through the immunocytochemistry staining procedure. Some sections were not exposed to the primary antibody (that is, it was omitted), but others were. The rest of the steps in the staining procedure were carried out as normal. For those sections in which the primary antibody was omitted, staining was virtually eliminated (see Figure 1). This indicates that the staining reported here represents the presence of GAP-43 and tau-1 immunoreactive proteins and not artifactual or non-

specific staining of other molecules due to binding of the secondary or tertiary antibodies. However, it is possible that the primary antibodies could bind to molecules that were structurally similar to either GAP-43 or tau-1, which would produce a different type of artifactual staining, one that is not easily controlled for.

Following incubation in the primary antibody, sections were washed six times in PBS during a 1 hour period. Subsequently, sections were placed in a goat-anti-mouse IgG antibody (Sternberger Monoclonals Inc.) at a dilution of 1:200 for 1 hour. PBS was used as the diluent. Four washes in PBS, each lasting 15 minutes were then completed. Sections were then placed in the PAP solution (mouse monoclonal PAP from Sternberger Monoclonals Inc.) at dilution of 1:800 for 1 hour. The diluent in this case was 1% normal goat serum in PBS. Sections were removed from the rotary agitator and left in PAP overnight in the refrigerator.

The next day, sections were washed four times in PBS for 1 hour and then placed in a solution containing diaminobenzidine tetrahydrochloride, nickel ammonium sulfate and cobalt chloride for 5 minutes. They were then transferred to wells containing the same solution as above except for the addition of hydrogen peroxide. Eight minutes later sections were transferred to PBS. Sections were immediately washed

three times in PBS, each wash lasting 5 minutes, and then stored overnight in the refrigerator.

Sections were mounted onto chrome-alum-gelatin-coated slides using distilled water. After being air dried, they were dehydrated in a series of alcohols, placed in xylene and coverslipped.

Relative Optical Density Measures

The relative optical density (ROD) of the GAP-43 immunoreactive (GAPir) and tau-1 immunoreactive (tauir) fiber staining in the region of MePD, part of the cerebral cortex and whole sections were measured using an image analysis system. This system consisted of an IBM-compatible microcomputer, software (M1 software from Imaging Research Inc.), a solid state video camera (VSP Inc.) which had a macro lens (Nikon) attached to it, a light box (Northern Light), an image monitor (Electrohome Ltd.) and a mouse (Mouse Systems Corp.). The camera was mounted on an adjustable stand, with the light box placed on the base of the stand directly under the camera.

ROD is a measure that is closely related to the perceptual judgements of density. That is, a sample having a ROD of 1.0 would look about twice as dark as a sample that has a ROD of 0.5. It is a semiquantitative density measure, in that ROD measures are taken without calibrating the system to

an external concentration reference. Since the ROD was not calibrated to an external reference, the ROD of a sample image would change with varying lighting conditions. To eliminate this possibility, an extensive effort was made to control for the light intensity entering the tissue sample and the camera through the macro lens. The lighting conditions within the room where the measures were taken were kept constant. The light box used is specifically designed for densitometry. Unlike microscope illuminators, it has a digital readout which allows for a precise setting of illumination level that stays constant (within ± 0.5% over a 12 hour period). The illumination level of the light box was kept at the same setting for the entire experiment.

Correction for shading error was also done before any measurements were taken. Any video densitometry image analysis system exhibits density variability over the field of view. That is, different places in a blank field of view will have somewhat different density readings. This is unwanted and correction for this "shading" error makes the blank field of view homogenous. When correcting for shading error the system scans a blank field of view and stores a pixel by pixel matrix of deviation values. The deviation matrix contains a density value (in gray levels) for each pixel in the image. After correcting for shading error the system reports an average density value (in gray levels) for the entire field of

view (average intensity of the image). This average intensity of the image was an indication of the intensity of light entering the camera and being digitized by the image analysis system. This value was constant for the entire experiment, indicating that the system did not vary with respect to the lighting conditions that it was being exposed to when taking ROD measures from animal to animal.

As an extra precaution, animals were also measured in pairs, one castrated control was measured along with its T-treated counterpart in each measurement session. This ensured that any uncontrolled variability in measurement conditions would be equally distributed among groups. The ROD of immunoreactive fiber staining was measured without knowledge of group membership. That is, within each measurement session even though it was known that in each pair there was a control and T-treated subject, it was not known which subject was the control and which was T-treated.

When taking ROD measures a section was placed on the light box. The image of a stained MePD was digitized and displayed on the image monitor. Since GAPir- and tauir-staining were more dense in the region of MePD than in surrounding areas of the amygdala, ROD measures were taken using the semi-automatic mode in which the software is able to distinguish a darkly stained target from a lighter background. In this mode, the mouse was used to position the cursor on the

background (typically the optic tract) and then on the target. The area of tissue from which the ROD was measured was displayed as an overlay on the image. In those instances in which MePD measurements were more difficult, the mouse was used to draw the appropriate boundaries as needed. At the caudal levels, the fields of GAPir and tauir fibers were fairly distinct (see Figure 2 and Figure 3). However, at the more rostral levels the boundaries of darker GAPir- and tauir-staining in the region of MePD were much less distinct (see Figure 2 and Figure 3). At these levels, the software was used to adjust the contrast of the displayed image of MePD on the monitor. This would accentuate the boundaries of the darker GAPir- or tauir-staining so that a more accurate measure could be taken. This in no way affected the ROD measures obtained, it only affected how the image appeared on the monitor.

Three MePD measurements on each section were taken and the values were averaged for that section. The ROD values for all sections were averaged to give a mean for each hemisphere in each animal. These means were representative of the "raw" data for MePD.

In addition to MePD measurements, cortex and whole section measures were also taken. A sampling box (2.05 mm x 0.80 mm) was used in taking ROD measures for the cortex. The bottom corner of this box was placed at the top of the rhinal

fissure (see Figure 4). When in the semi-automatic mode, the mouse was used to position the cursor on the background and then on the target. The ROD of the area within the box was taken, except for areas of torn tissue. Cortex ROD measures were taken only on those sections which also contained MePD. On sections at the caudal levels of MePD, the part of the cerebral cortex measured included portions of the perirhinal and temporal (areas 1 and 3) cortex. On sections at the more rostral levels of MePD, portions of the perirhinal and parietal (area 2) cortex were measured. Three cortex measures on each section were taken and their respective values were averaged for that section. The ROD values for all sections were averaged to give a mean for each hemisphere in each animal. These means were representative of the "raw" data for the cortex.

Even though every attempt was made to keep the conditions within the staining procedures from week to week as constant as possible, there will always be some variation in staining from section to section within an animal and overall variation between animals. Therefore, ratios were calculated in order to control for extraneous sources of error variation. This would in essence lower the variation between different RODs from both within the same animal and between different animals in the same group. Whole section ROD measures were taken to be used in the denominator of calculated ratios. Again, using

the semi-automatic mode, the ROD of a whole section which contained MePD, was taken three times and their respective values were averaged for that section. The numerator of these ratios was the ROD for the corresponding MePD or cortex measurement which was contained within the same whole section. These ratios were used as dependent measures because it was felt that any slight differences in raw MePD or cortex RODs may have been due to uncontrollable sources of error variation within the staining procedures. For example, immunoreactive staining in one MePD may be darker than another MePD on a different section just because these sections were processed in different wells and as such were not exposed to the exact same conditions. In addition, overall staining in one animal may be different or the same as another animal because of the same sources of uncontrollable error variation.

The MePD/whole or cortex/whole ROD ratio for each section was used to calculate an overall mean MePD/whole ROD or cortex/whole ratio for each hemisphere in each animal. Also, the three most caudal sections through MePD were used to calculate a caudal mean MePD/whole ROD ratio for each hemisphere in each animal. Likewise, the three most rostral sections through MePD were used to calculate rostral mean MePD/whole ROD ratios. MePD was identified using the cresyl-stained sections to ensure that ROD measures were taken throughout the entire rostral-caudal extent of MePD.

Morphometry

The areas of GAPir and tauir fiber staining in the region of MePD were also measured at the same time density measures were taken using the semi-automatic mode of the image analysis system. The system was always calibrated before any area measures were taken. Again, three measures of each feature of interest were taken. That is, three consecutive measurements of the same area of staining on each section were taken and the values were averaged for that section. Areas of staining on all sections were added together to give a total for each hemisphere in each animal. The rostral boundary of MePD was determined using the cresyl material and it was this rostral section that was designated as the rostral boundary of the GAPir- and tauir-staining through MePD.

The area of the cell group MePD was also measured from the cresyl-stained material using the image analysis system. The atlas of Paxinos and Watson (1986) was used as an aid to define this cell group. In this case, the areas were not measured directly from digitized images of the actual sections. First, the outlines of MePD were drawn by hand on paper using a microprojector. The boundaries of MePD in the cresyl-stained material were more definitive using the microprojector than when using the image analysis system. The actual area of MePD was measured from the digitized images of the pencil drawings using the image analysis system. Again,

it must be noted that the image analysis system was always calibrated before any area measures were taken and that there was no knowledge of group membership when the drawings or the measures were being completed. Only one measurement was made for MePD on each section in this case. MePD area measures on all sections were added together to give a total for each hemisphere in each animal.

Using the image analysis system, the area of cresyl-stained whole brain sections in each group were also measured to provide an index of brain size at the level in which MePD was located. Sections were selected beginning at the most caudal level of MePD and extending rostrally for a total of six sections. The areas of the six sections were added together to give a total for each animal.

Statistical Analysis

Each of the histological dependent measures, except for whole section measures, was examined by a separate three-way between-within analysis of variance. This analysis involved a between subjects factor for hormone treatment effects, another between subjects factor for survival time effects and a within subjects factor for hemispheric effects. It was expected that the only significant main effects would be that of treatment or a treatment x survival time interaction. It was also expected that there would be no significant

hemispheric main effects. Planned comparisons between control and T-treatment means (that is, CC-2 vs C+T-2 and CC-8 vs C+T-8) were done using t-tests with the appropriate error terms from the overall analysis. These planned t-tests compared left/right means between groups. These left/right means were an average of both hemispheres. T-tests were evaluated at $\alpha = 0.05$, two-tailed.

The expectation of no hemispheric main effects with respect to density measures was not realized. Therefore, more conservative post-hoc comparisons between hemispheric means were conducted using Tukey's HSD tests. The α-level of significance for the post-hocs was 0.05.

Each of the whole section dependent measures was examined by a separate two-way analysis of variance. This analysis involved a between subjects factor for hormone treatment effects and another between subjects factor for survival time effects. It was expected that there would be no significant treatment or treatment x survival time interaction.

Planned comparisons were also made between the castrated control groups in this experiment and the normal males (that is, CC-2 vs normal males and CC-8 vs normal males) of experiment 2 with respect to ROD ratios and MePD areas using simple t-tests for independent samples. The results of these t-tests were evaluated at $\alpha = 0.05$, two-tailed. Ideally, non-castrated control (sham-operated) males would have been

included in experiment 1. However, the question of whether or not there was an effect of castration was not raised until after the results of experiment 1 were known. It must be noted that the normal males of experiment 2 were gonadally intact untreated animals. That is, they were not sham-operated and as such did not receive any empty silastic implants. In addition, brain sections from these animals were not processed at the same time as brain sections in this experiment. However, the same immunocytochemical procedures were completed on brain sections in experiment 1 and those of the normal males and diestrous females of experiment 2 (see methods section in experiment 2 for the antibody dilutions used for the normal males). It was felt that sham-operation and separate processing would not likely affect the dependent measures significantly. These comparisons gave at least some indications of the effect of castration on the dependent measures employed.

RESULTS

Density of GAPir-staining in the Region of MePD

As can be seen in Figure 2, there existed a fairly discrete area of GAPir-staining in the region of MePD which was quite dense, more so than the surrounding areas. Visual inspection of Figure 2 also indicates that the density of

GAPir fiber staining did not seem to be different between groups CC-8 and C+T-8. Analysis of ROD data confirmed this observation.

As shown in Figure 5 and Table 1, when either the overall raw ROD or the overall ROD ratio of GAPir-staining for MePD is considered, T-treated subjects did not differ from control subjects after 2 or 8 days of T-treatment [all L/R mean contrasts; absolute value of $t(12) \leq 0.696$, $p > 0.05$].

According to Malsbury and McKay (1994) the loss of SPir innervation within MePD occurs mainly in the rostral portion of MePD. Therefore, it was felt that possible ROD differences between T-treated and control groups in the present study may be more prominent in the rostral portion of the nucleus as opposed to the caudal portion. Thus, as explained in the methods section, caudal and rostral means were separately calculated in order to explore this issue. Investigation of the caudal and rostral components of MePD did not reveal any significant effects either (see Table 1). When considering the caudal or rostral ROD ratio means, planned t-tests using mean contrasts of the combination of both hemispheres revealed that there were no significant differences between T-treated and control groups at either time period, [all L/R mean contrasts; absolute value of $t(12) \leq 1.043$, $p > 0.05$].

Since T-treatment did not seem to augment GAPir-staining density in the region of MePD, the question of whether or not

there was a decrease of GAP-43 staining in the region of MePD as a result of castration was of interest. To shed light on this issue, normal males in experiment 2 were compared to the castrated control subjects in this experiment. As illustrated in Figure 5, the overall ROD ratio of GAPir-staining for MePD in normal males was not significantly different than castrated controls at either time period [both t-tests; absolute value of $t(6) \leq 1.28$, $p > 0.05$].

Density of Tauir-staining in the Region of MePD

Tauir fiber staining in the region of MePD was similar to that of GAPir-staining in that there was a fairly distinct field of staining that was denser than the surrounding areas, although it was not so intense as GAPir-staining (see Figure 3). Inspection of Figure 3 shows that there was no apparent effect of T-treatment on the density of tauir fiber staining in this region. The overall raw ROD and the overall ROD ratio of tauir-staining for MePD (see Figure 6 and Table 2) of T-treated groups was not significantly different from their respective control groups [all L/R mean contrasts; absolute value of $t(12) \leq 0.599$, $p > 0.05$].

Again, the caudal and rostral distinctions within MePD did not show any different results than the overall means (see Table 2), as the ROD ratio of caudal or rostral tauir-staining

for MePD was not affected by T-treatment [all L/R mean contrasts; absolute value of $t(12) \leq 1.224$, $p > 0.05$].

Castration did not seem to affect the ROD of tauir-staining. As shown in Figure 6, the overall ROD ratio of tauir-staining for MePD in normal males did not significantly differ from group CC-2 or group CC-8 [both t-tests; absolute value of $t(6) \leq 2.366$, $p > 0.05$].

An important issue to consider is whether or not the ROD measures were sensitive enough to actually detect differences in the amounts of immunoreactive staining. To shed light on this issue, overall raw MePD tau-1 ROD values from two subjects in group CC-2 stained with an antibody dilution of 1:5000 were averaged and the same was done for two subjects in group CC-8 stained with a tau-1 antibody dilution of 1:7000. One would expect that an antibody dilution of 1:5000 would result in darker staining than an antibody dilution of 1:7000. As expected, the 1:5000 antibody dilution produced a 20.2% greater ROD of MePD immunoreactive staining than did the 1:7000 antibody dilution. The respective left/right hemisphere mean (average of both hemispheres) ± SEM for the 1:5000 sample was 0.9345 ± 0.0097 and for the 1:7000 sample it was 0.7776 ± 0.0638. This provided evidence that the ROD measures could detect differences in the amount of immunoreactive staining.

Hemispheric Asymmetries of

GAPir-staining in the Region of MePD

Overall raw RODs and overall ROD ratios of both GAPir and tauir fiber staining for left and right MePDs are illustrated in Figure 7. A three-way analysis of variance on the GAP-43 overall raw MePD or overall MePD ratio data both revealed an unexpected main effect for hemisphere [both ANOVAs; $F(1,12) \geq 9.72$, $p < 0.01$]. Post-hoc comparisons showed that the right MePD had a significantly greater GAPir raw ROD and ROD ratio than the left [both mean contrasts; $q(2,12) \geq 4.41$, $p < 0.01$; where 2 = number of treatment means and 12 = the degrees of freedom for the error term]. Both of these differences were about 4.1% and seemed to be consistent since 14 out of the 16 animals showed the right > left hemispheric difference.

Tauir-staining in the region of MePD did not show the same hemispheric differences as GAPir-staining did. A three-way analysis of variance on either the tauir overall raw ROD or overall ROD ratio data did not show any significant main effect for hemisphere [both ANOVAs; $F(1,12) \leq 1.80$, $p > 0.05$].

Density of GAPir-staining in the Cortex

Mean raw RODs and mean ROD ratios of GAPir-staining for the cortex in the left, right and the combination of the two hemispheres for each group are shown in Table 3. Figure 8

graphically represents the mean ROD ratios of the combination of both hemispheres of GAPir-staining for each group. As can be seen in Table 3 and Figure 8, T-treated groups did not have greater raw RODs or ROD ratios of GAPir-staining as compared to castrated controls [all L/R mean contrasts; absolute value of $t(12) \leq 0.502$, $p > 0.05$].

In addition, castration did not produce any obvious reduction in GAPir staining in the cortex since castrated control subjects did not have lower GAPir ROD ratios (see Figure 8) than normal males [both t-tests; absolute value of $t(6) \leq 1.215$, $p > 0.05$].

Density of Tauir-staining in the Cortex

As was the case for GAPir-staining, the raw RODs and ROD ratios of tauir-staining for the cortex (see Table 4 and Figure 9) of T-treated groups did not significantly differ from their respective control groups at either 2 or 8 days after silastic capsule insertion [all L/R mean contrasts; absolute value of $t(12) \leq 1.399$, $p > 0.05$].

Again, castration did not seem to affect protein immunoreactivity. As illustrated in Figure 9, both castrated control groups did not significantly differ from normal males with respect to their tau-1 ROD ratios [both t-tests; absolute value of $t(6) \leq 1.319$, $p > 0.05$].

Hemispheric Asymmetries of

Tauir-staining in the Cortex

Raw RODs and ROD ratios of GAPir and tauir fiber staining for the left and right cortex are represented in Figure 10. The right cortex had about a 2.6% greater tauir raw ROD and a 2.0% tauir ROD ratio than the left. A three-way analysis of variance on either the tau-1 raw ROD or ROD ratio data gave a significant main effect for hemisphere in each case [both ANOVAs; $F(1,12) \geq 5.05$, $p < 0.05$]. Post-hoc comparisons revealed the existence of a right > left hemispheric difference for both the raw ROD and ROD ratio of tauir-staining [both mean contrasts; $q(2,12) \geq 3.18$, $p < 0.05$]. For the raw ROD data 10 out of 16 animals showed a right > left difference, while for the ROD ratio data 8 out of 16 showed the same right > left difference.

The density GAPir-staining in the cortex did not show any hemispheric asymmetries. Separate three-way analyses of variance on the raw ROD and ROD ratio means of GAPir-staining in the cortex did not show any significant main effects for hemisphere [both ANOVAs; $F(1,12) \leq 0.40$, $p > 0.05$].

Density of Whole Section GAPir- and Tauir-staining

It is important to consider whether or not T-treatment affected the denominator of the calculated ratios. The ROD of whole section GAPir- and tauir-staining is shown in Table 3

and Table 4, respectively. Separate two-way analyses of variance on the GAP-43 and tau-1 whole section ROD data indicated that there was no significant main effect for hormone treatment [both ANOVAs; $F(1,12) \leq 0.43$, $p > 0.05$] or a significant hormone treatment x survival time interaction [both ANOVAs; $F(1,12) \leq 0.07$, $p > 0.05$] in each case. This indicates that the RODs of GAPir and tauir-staining for the whole section did not differ among the four groups and that T-treatment had no effect on these measures.

Area of MePD GAPir-staining

As shown in Figure 11 and Table 5, the areas of dense GAPir-staining in the region of MePD were a little greater in T-treated groups than in control groups. Group C+T-2 had a 12.5% greater area than group CC-2, and group C+T-8 showed a 15.7% greater area than group CC-8. However, planned comparisons showed that these differences were not significant [both L/R mean contrasts; absolute value of $t(11) \leq 1.691$, $p > 0.05$].

Even though castration did not affect the density of GAPir-staining in the region of MePD, it did affect the cross sectional area of this staining, since group CC-2 had a 21.4% and group CC-8 had a 24.2% smaller area of GAPir-staining in the region of MePD than normal males (see Figure 11). These

differences were statistically significant [both t-tests; absolute value of $t(6) \geq 4.529$, $p < 0.01$].

Area of MePD Tauir-staining

The pattern between the four treatment groups in the area of MePD tauir-staining was similar to that of the MePD GAPir-staining, but the differences between groups were smaller (see Figure 12). Both T-treated groups had slightly greater areas of tauir-staining in this region (see Figure 12 and Table 5). The magnitude of the difference between group C+T-2 and CC-2 was 3.5%, while the difference between group C+T-8 and CC-8 was 9.0%. However, these differences were not statistically significant [both L/R mean contrasts; absolute value of $t(12) \leq 1.412$, $p > 0.05$].

As with GAPir-staining, the areas of tauir-staining in this region (see Figure 12) were significantly smaller for group CC-2 and group CC-8 than for normal males [both t-tests; absolute value of $t(6) \geq 4.953$, $p < 0.01$]. The magnitude of these decreases was 19.5% for group CC-2 and 22.1% for group CC-8. Thus, castration for these time periods produces a decrease in the area of tauir-staining in the region of MePD.

Area of MePD in Cresyl-stained Sections

Figure 13 graphically represents the mean of the area of MePD in cresyl-stained sections for each group (also see Table 5). Although MePD was slightly larger in group C+T-2 than in

group CC-2 (8.6%), this difference was not statistically significant [L/R mean contrast; absolute value of $t(12) = 1.048$, $p > 0.05$]. On the other hand, the difference between group C+T-8 and CC-8 was larger (28.0%), and was significant [L/R mean contrast; absolute value of $t(12) = 3.231$, $p < 0.01$]. This indicates that T-treatment for 8 days did increase the size of the cell group MePD.

Castration produced atrophy in this region of the brain. In both castrated control groups there was a marked decline in the area of MePD as compared to normal males [both t-tests; absolute value of $t(6) \geq 6.192$, $p < 0.001$]. The decline for group CC-2 was 27.9%, while for group CC-8 it was 32.0%.

The number of cresyl-stained sections in which MePD appeared for each group is shown in Table 5. Group C+T-2 did not significantly differ from group CC-2 with respect to this measure [L/R mean contrast; absolute value of $t(12) = 0.490$, $p > 0.05$). However, group C+T-8 did have a greater number of sections which contained MePD than did group CC-8 [L/R mean contrast; absolute value of $t(12) = 2.828$, $p < 0.02$]. This was a small but consistent difference. Thus, T-treatment for 8 days increased the length of MePD as well as its total area (above).

One may question whether or not the differences in the size of MePD between groups may just be a reflection of the brain being larger at the level of MePD. To rule out this

possibility, for each group an estimate of brain size at this level was obtained by measuring the area of six whole coronal sections stained with cresyl violet. A two-way analysis of variance showed that there was no significant main effect for hormone treatment [$F(1,12) = 0.11$, $p > 0.05$] or a significant hormone treatment x survival time interaction [$F(1,12) = 1.75$, $p > 0.05$]. Thus, whole section areas though the region of the brain which contained MePD did not differ among the four groups (see Table 5).

EXPERIMENT 2

METHODS

Subjects and Estrous Cycle Determination

Eight (four intact males and four intact females) adult Sprague-Dawley strain albino rats (Charles River, Quebec) were used as subjects. All animals were kept on a 14/10 reversed light/dark cycle with lights off at 0800 hours. Food and water were available ad libitum. At the time of perfusion, the males were approximately 74-88 days old, while the females were approximately 104-107 days old. Body weights of the four males and four females ranged from 321-452 g (mean = 384.3 g).

Based on the findings of Shughrue and Dorsa (1993a), it was felt that the changing levels of estrogen during the estrous cycle could affect the levels of GAP-43 in the female

brain. They found that ovariectomy reduced the levels of GAP-43 mRNA and that estrogen treatment of these ovariectomized females for 96 hours significantly augmented GAP-43 levels in several brain areas. They even mention that on the basis of unpublished data, levels of GAP-43 mRNA change throughout the estrous cycle. This would suggest that the levels of GAP-43 itself may change during the estrous cycle and would be at their highest at the point in the estrous cycle where estrogen levels are also high. In this experiment I chose to sacrifice the females when their estrogen levels were low. It was thought that if there were a sex difference it would be more likely seen when comparing normal males to diestrous females.

Estrogen levels are the highest early in proestrus and decline to low levels by early estrus, and are maintained at slightly lower levels over the metestrous and early diestrous portions of the cycle (Brown-Grant et al., 1970). Based on this, it was decided to sacrifice the females during the beginning of the diestrous part of the cycle, at the time when estrogen levels had been low for some time.

The estrous cycle of the females was determined by the use of sexual behaviour testing in the presence of active males. The sexual behaviour testing was conducted in rectangular test boxes (20L x 15W x 14H inches) from about 0900 to 1100 hours every day over a period of about 2 weeks.

During this sexual testing procedure a female and a male were placed in the testing box and the behaviour of the female was observed. The female's receptive and proceptive behaviours were of interest. When the female showed that she was clearly sexually receptive she was immediately removed from the testing box. If no signs of receptivity were shown during 15 minutes of observation then she was removed from the testing box and the test was ended. A female was considered receptive if she showed such behaviours as lordosis, hopping and/or darting, or ear wiggling (head shaking). The male was not permitted to mount the female.

The 4-day estrous cycle of a rat on a 14/10 light/dark cycle as shown by Brown-Grant et al. (1970) was used a guide in determining which part of the cycle the females were in. Receptive females were considered as being in the late proestrous part of the cycle (tested at 1-3 hours after lights off). Three of the four females were found to be receptive on 3 testing days separated by 3 days in which no sign of receptivity was seen (two complete cycles). One female only showed receptivity on one day and did not show it again, thus the timing of her estrous cycle could not be determined and therefore this female was dropped from the study.

Once it was determined that the females had a 4-day cycle and that the stages of their cycle could be predicted based on

the sex behaviour testing, they were sacrificed at the beginning of the diestrous part of the estrous cycle.

Immunocytochemistry

Perfusion, fixation of tissue and its preparation were the same as described in experiment 1. Brains were sectioned in the coronal plane at 40 microns and stained for GAP-43 (dilution 1:2000), tau-1 (dilution 1:6000), or with cresyl violet. Sections were immunocytochemically stained in the same manner as described in experiment 1. A male and a female brain were always processed together, except for one male brain which was processed alone because his female counterpart was excluded from the study.

ROD and Morphometry Measures

The ROD of GAPir and Tauir fiber staining in the region of MePD, part of the cerebral cortex and whole sections were measured using the image analysis system in the same manner as described in experiment 1. The image analysis system settings were the same as in experiment 1. Three measurements of each feature of interest were taken and the values were averaged for that section as was done in experiment 1. Again, there was a raw MePD or cortex ROD mean and a MePD/whole or cortex/whole ROD ratio mean that was calculated using all sections through MePD for each hemisphere in each animal.

Also MePD was subdivided into caudal and rostral portions in the same manner as described in experiment 1.

The areas of GAPir and tauir fiber staining through the region of MePD of the intact males and females were also measured as described in experiment 1. Three measures on each section were averaged and the values on all sections were again added together to give a total for each hemisphere in each animal. As in experiment 1, the cresyl-stained sections were used as a guide in determining the rostral boundary of MePD.

The area of the cell group MePD was also measured in the same manner as in experiment 1 using the cresyl-stained sections. The area values on all sections were again added together to give a total for each hemisphere in each animal. In addition, the area of cresyl-stained whole sections were measured in the same manner as in experiment 1, except that five sections were measured and added together to give a total for each animal.

Statistical Analysis

Density and area measures, except whole section measures, were examined by separate two-way between-within analyses of variance. The between factor in these analyses was the sex of the animal and the within factor was hemisphere. Planned comparisons between males and females were made using t-tests

with the appropriate error term from the overall analysis. Whole section density and area measures were examined by simple t-tests for independent samples. The results of all t-tests were evaluated at $\alpha = 0.05$, two-tailed.

Once again for the density measures, hemispheric main effects in the two-way analyses of variance were found. More conservative Tukey's HSD post-hocs were used in testing differences between left and right hemisphere means. The results of these post-hocs were evaluated at $\alpha = 0.05$.

RESULTS

Density of GAPir-staining in the Region of MePD

Matched sections of GAPir-staining through the region of MePD from a male and a female are shown in Figure 14. As was seen in experiment 1, GAPir-staining in this region of males and females was fairly dense. Howeve_, the distinctiveness and density seems to decline as one goes rostrally through the nucleus. Inspection of Figure 14 shows no obvious density differences in GAPir-staining between males and females.

The overall ROD ratios of GAPir-staining in this region for males and females are depicted in Figure 15. Corresponding, overall raw ROD means are given in Table 6. There were no notable differences in either the overall raw RODs or ROD ratios of GAPir-staining between males and females

[both L/R mean contrasts; absolute value of $t(5) \leq 0.979$, $p > 0.05$].

It was possible that sex differences may exist in different parts of MePD. So the nucleus was split up into a caudal and a rostral portion (see Methods). This did not produce any different findings (see Table 6), as no differences were found between males and females with respect to their caudal or rostral ROD ratios of GAPir-staining for MePD [both L/R mean contrasts; absolute value of $t(5) \leq 0.694$, $p > 0.05$].

Density of Tauir-staining in the Region of MePD

As was seen with GAPir-staining, male and female tauir-staining in the region of MePD was again dense, but the density and the distinctiveness of the borders declined further rostrally in the nucleus (see Figure 16). Also, there did not seem to be any apparent differences in the density of tauir-staining between males and females (see Figure 16). Males did not differ from females (see Figure 15 and Table 6) in their overall raw ROD and ROD ratio of tauir-staining in this region [both L/R mean contrasts; absolute value of $t(5) \leq 0.646$, $p > 0.05$].

Similar results were found with respect to the caudal and rostral ROD ratios of tauir-staining for this area (see Table 6). No sex differences were apparent for either of these

dependent measures [both L/R mean contrasts; absolute value of $t(5) \leq 0.848$, $p < 0.05$].

Hemispheric Asymmetries of
GAPir-staining in the Region of MePD

Hemispheric means of the overall raw ROD and overall ROD ratio of GAPir-staining for MePD in the left and right hemisphere are illustrated in Figure 17. As in experiment 1, the results of separate two-way analyses of variance on the GAP-43 overall raw ROD and overall ROD ratio data, yielded the finding of only a significant main effect for hemisphere in both cases [both ANOVAs; $F(1,5) \geq 10.88$, $p < 0.025$]. Post-hoc comparisons showed that there was a right > left difference (5.1%) in the overall raw ROD of GAPir-staining [mean contrast; $q(2,5) = 5.50$, $p < 0.05$], and a similar right > left difference (5.3%) in the ROD ratio of GAPir-staining [mean contrast; $q(2,5) = 5.86$, $p < 0.01$]. This effect was consistent as 7 out of 7 animals showed this difference in both cases and was not dependent on the sex of the animal.

Hemispheric Asymmetries of
Tauir-staining in the Region of MePD

Left and right hemisphere overall mean raw RODs and ROD ratios of tauir-staining for MePD are also graphically represented in Figure 17. Unlike experiment 1, separate two-way analyses of variance on the overall raw ROD or overall ROD

ratio data yielded only a significant hemispheric main effect in each case [both ANOVAs; $F(1,5) \geq 7.53$, $p < 0.05$]. Again, post-hoc comparisons showed that MePDs in the right hemisphere had significantly greater overall raw RODs and overall ROD ratios of tauir-staining than those in the left hemisphere [both mean contrasts; $q(2,5) \geq 3.85$, $p < 0.05$]. The magnitude of these differences were 3.5% for the raw ROD data and 3.0% for the ROD ratio data, with 6 out of 7 subjects showing the right > left difference in both instances. Again, these hemispheric differences were not related to the sex of the animal.

Density of GAPir-staining in the Cortex

As illustrated in Figure 18, for the cortex the ROD ratio of GAPir-staining was not different between males and females [L/R mean contrast; absolute value of $t(5) = 0.020$, $p > 0.05$]. The corresponding raw ROD means were also not sexually dimorphic [L/R mean contrast; absolute value of $t(5) = 0.077$, $p > 0.05$].

Density of Tauir-staining in the Cortex

The ROD ratio of cortex tauir-staining for both sexes is also shown in Figure 18 and the corresponding raw ROD means are presented in Table 7. Like the GAPir-staining, the raw RODs and ROD ratios of cortex tauir-staining were not

significantly different between males and females [both mean contrasts; absolute value of $t(5) \leq 0.454$, $p > 0.05$].

No Hemispheric Asymmetries of GAPir- or Tauir-staining in the Cortex

The raw RODs and ROD ratios of cortex GAPir- and tauir-staining for the left and right hemisphere are depicted in Figure 19. Separate analyses of variance on the GAP-43 raw ROD and ROD ratio data showed that there were no significant main effects for hemisphere in both instances [both ANOVAs; $F(1,5) \leq 4.78$, $p > 0.05$]. However, two-way analyses of variance revealed that there was a significant hemispheric main effect for the tau-1 ROD ratio data [$F(1,5) = 6.70$, $p < 0.05$], but not for the tau-1 raw ROD data [$F(1,5) = 2.75$, $p > 0.05$]. Although it almost reached significance, a post-hoc analysis showed that there was no significant difference in the ROD ratio of tauir-staining between the right and left cortex [mean contrast; $q(2,5) = 3.63$, $p > 0.05$]. It should also be noted that in this case the tauir-staining in the left hemisphere was slightly greater than in the right by about 4.8%. In experiment 1, the right was greater than the left.

Density of Whole Section GAPir- and Tauir-staining

To demonstrate that the ROD ratio measures were not invalidated by possible sex differences in the denominator of the calculated ratios, the ROD of whole section GAPir- and

tauir-staining for males were compared to that for females. As can be seen in Table 7, males and females did not differ with respect to these measures [both t-tests; absolute value of $t(5) \leq 0.554$, $p > 0.05$].

Areas of MePD and GAPir- and Tauir-staining in the Region of MePD

Means of the areas of GAPir- and tauir-staining through the region of MePD, and the area of MePD as measured in cresyl-stained sections for the left, right, and the combination of both hemispheres (L/R) of males and females are given in Table 8. The magnitude of the differences between males and females with respect to these measures are portrayed in Figure 20.

Males had a markedly greater area of dense GAPir-staining [L/R mean contrast; absolute value of $t(5) = 9.060$, $p < 0.001$] and tauir-staining [L/R mean contrast; absolute value of $t(5) = 12.500$, $p < 0.001$] in this region. The size of MePD in cresyl-stained sections was also much larger in males than in females [L/R mean contrast; absolute value of $t(5) = 18.286$, $p < 0.001$]. The difference (male > female) for GAPir-staining was 79.7%, for tauir-staining it was a little larger (90.6%), and it was the largest for the cresyl-stained sections (106.9%).

The number of cresyl-stained sections which contained MePD for males and females is shown in Table 8. It is apparent that males had a larger number of sections which contained MePD than females [L/R mean contrast; $t(5) = 5.116$, $p < 0.01$], indicating that MePD was longer in males.

To show that the above sex differences were not due to the males having greater brain size at the level in which MePD is located, an estimate of the whole section area at the level of MePD was obtained by measuring the area of five cresyl-stained whole sections which contained MePD. The summed cross-sectional areas of the five sections for males and females are shown in Table 8. As one can see, the area of the whole sections for males was not different from that for females [$t(5) = 0.082$, $p > 0.05$].

DISCUSSION

GAP-43 and Tau-1 Staining: Unexpectedly Distinct and Discrete

Fields of GAP-43 and tau-1 immunoreactive fiber staining in the region of MePD were quite dense and fairly discrete. This was unexpected since a previous study, which looked at the distribution of GAP-43 in the adult rat brain, found that the only portions of the amygdala which contained very dense immunoreactivity for GAP-43 were the central, basolateral and

lateral divisions (Benowitz et al., 1988). The medial amygdala seemed to show only moderate GAP-43 immunoreactivity in that study, much less than that found in the present study, and it was not distinct and discrete. One reason for this discrepancy may have been that Benowitz et al. (1988) used a polyclonal primary antibody (raised in sheep or rabbits), whereas in the present study a monoclonal primary antibody (raised in mice) was used. The polyclonal antibodies used by Benowitz et al. (1998) may only bind either phosphorylated or non-phosphorylated GAP-43 (they give no information about specificity), whereas the monoclonal primary antibody used in the present study binds to GAP-43 regardless of its phosphorylation state (product information supplied by Boehringer-Mannheim).

Experiment 4: GAP-43 and Tau-1
Immunoreactive Densities are Not Regulated by Testosterone

The results indicated that T did not increase the average density of GAP-43 or tau-1 staining in the region of MePD in castrated male rats. T-treatment of castrated males also failed to affect GAP-43 and tau-1 staining in the part of the cerebral cortex which includes portions of the perirhinal, temporal and parietal cortices. This indicates that T does not seem to regulate the expression of these two proteins in the region of MePD or the above region of the cortex, at least

for short periods of T exposure. These results are puzzling in light of the fact that estrogen which is a metabolite of T, has been found to regulate GAP-43 mRNA expression in several brain regions, those being the VMH (Lustig et al., 1991; Shughrue and Dorsa, 1993a) and the MPOA (Shughrue and Dorsa, 1993a) of the adult rat. In addition, estrogen has been found to enhance the outgrowth and length of axons of cultured hypothalamic neurons (Díaz et al., 1992), which has been shown to possibly involve the induction of tau proteins (Ferreira and Caceres, 1991).

The question of why T did not induce greater densities of GAP-43 or tau-1 staining remains unanswered. One could question whether or not the dose of T was high enough. However, according to Damassa et al. (1977) a 45 mm long T-filled silastic capsule releases a dose of T that is within the high end of the normal physiological range. As mentioned in the introduction, other studies (De Vries et al., 1984; Simerly and Swanson, 1987; Van Leeuwen et al., 1985) which have looked at the activational effects of hormones, used T-treatments of 14 days or 5-weeks. It is possible that the 8-day T-treatment was not long enough. There may be a delayed response to T. This would mean that a longer T-treatment may lead to increased levels of GAP-43 or tau-1 throughout MePD. However, short periods of hormone treatment have been found to enhance the levels of GAP-43 mRNA in several brain areas. As

mentioned in the introduction, Lustig et al. (1991) found that estrogen treatment for as little as 2 hours or 3 days increased the levels of GAP-43 mRNA in the VMH of ovariectomized adult female rats. Similarly, Shughrue and Dorsa (1993a) used a 4 day estrogen treatment to augment GAP-43 levels in both the VMH and MPOA of ovariectomized adult female rats. Furthermore, in the present study T-treatment of the castrated males for 8 days did increase the size of MePD by 28% as compared to controls. This demonstrates that the T-treatment was both large and long enough to affect the cytoarchitecture of this region and thus could have affected the densities of GAP-43 and/or tau-1 immunoreactivity.

An important question is whether or not GAP-43 and tau-1 levels were affected by castration. The comparisons of the density of GAP-43 and tau-1 immunoreactivity between both castrated control groups in experiment 1 with those of the normal males in experiment 2, showed that castration did not reduce the average density of these proteins in either the region of MePD or the part of the cortex which includes portions of the perirhinal, temporal and parietal cortices. This finding for the cortex is similar to that of another study which found that 4 weeks of castration did not reduce GAP-43 mRNA levels in the frontal cortex (Lustig et al., 1991). Since castration did not reduce GAP-43 or tau levels, then one would not expect T to restore them. This suggests

that there was no "loss" to restore. This also provides evidence that castration may not have affected the axonal morphology of neurons in MePD, since the immunoreactive densities of two axonal growth markers were not affected by castration.

GAP-43 and tau-1 are axonal markers only, they are not concentrated in dendrites. If gonadal hormones do not affect MePD axons, but do affect MePD dendrites then this would explain why castration and subsequent replacement of T did not have any affect on GAP-43 and tau-1 densities in the present study.

Several studies using different species of rodents have found that gonadal hormones affect the dendrites of neurons. Estrogen treatment of newborn mouse MPOA explants results in a greater number of dendrites with more dendritic branching from the soma of the neuron (Toran-Allerand et al., 1983). Three days after ovariectomy of adult female rats there is a dramatic reduction in the number of dendritic spines in CA1 pyramidal cells of the hippocampus, and subsequent estrogen treatment for 2 days reverses the loss (Gould et al., 1990). Of particular relevance to the present study, castration of adult male hamsters for 12 weeks results in dendritic atrophy in the posterior medial amygdala (Gomez and Newman, 1991). Finally, Lorenzo et al. (1992) found that estrogen increased the total dendritic length of medial amygdala neurons in vitro

by mainly increasing the number of branches which results in a larger dendritic tree. This resulted in a profound increase in the immunoreactive staining for microtubule-associated protein-2 (MAP-2). They also found that estrogen did not affect medial amygdala axons.

The results of these studies suggest the possibility that T may only affect dendrites of medial amygdala neurons. That is, castration would lead to dendritic atrophy of MePD neurons and subsequent replacement of T would enhance dendritic, but not axonal growth. Thus, possible T enhanced growth of dendrites could be assayed by measuring the induction of proteins which are compartmentalized mainly in dendrites and whose levels are increased during periods of neuronal growth. One such protein is MAP-2 which is found in dendrites and with much weaker labeling in the cell bodies and little or none in axons (Bernhart and Matus, 1984). It would be interesting to see if castration would result in a decline in MAP-2 levels in MePD and whether T replacement would augment their levels.

What would be the functional significance of possible shrinkage of the dendrites within MePD? Liu et al. (1994), have found a mismatch between the location of substance P (SP) and its binding site, the SP receptor, in many sites in the CNS. They found that SP receptors are mainly located on the somatic and dendritic surfaces of neurons, covering 70% of the surface membrane. More importantly, they found that most of

the immunocytochemically stained SP receptor membranes were not directly opposite SP terminals. They suggest that SP released from terminals can diffuse substantial distances before binding to its receptors. This concept has been referred to as volume transmission (Agnati et al., 1992). If dendrites within MePD shrink in response to castration, then there would be less surface area and therefore a lower number of SP receptors. Functionally this would mean that if the amount of SP release remained constant, there would be a reduced response to SP because of less dendritic membrane and therefore a decline in the number of SP molecules binding to their receptors at any one point in time.

Hemispheric Asymmetries in GAP-43
and Tau-1 Levels: Fact or Fiction?

Unexpectedly, the density of GAP-43 staining in the region of MePD in the right hemisphere was slightly greater than in the left, in both experiments. The right > left hemispheric differences of MePD GAP-43 levels were not large. Both experiments gave similar differences. That is, in experiment 1 the difference was about 4% and in experiment 2 it was about 5%. The results for tau-1 for both experiments were not so complimentary. In experiment 1, there was no significant hemispheric asymmetry of tau-1 levels in the region of MePD. However, in experiment 2 there was a

significant right > left hemispheric difference. In experiment 1, the levels of tau-1 were a little greater for MePDs in the right hemisphere (about 1%), whereas in experiment 2 they were larger (about 3%).

For the cerebral cortex, both experiments gave similar results of no hemispheric differences in GAP-43 levels. However, the results for cortical levels of tau-1 were inconsistent. In experiment 1, there was a right > left hemispheric difference in tau-1 density of about 2%, whereas in experiment 2, although there was an almost significant difference, the mean tau-1 density in the left cortex was greater than the right by about 5%.

In general, the hemispheric difference (right > left) in GAP-43 levels in the region of MePD have more credence than corresponding tau-1 levels. The hemispheric difference (right > left) in tau-1 levels in the region of MePD found in experiment 2 should be interpreted with caution. Since this effect is based on a smaller number of subjects, it may not be real. The same situation existed for the tau-1 levels in the cortex. The hemispheric difference (right > left) found in experiment 1 again may not be a real effect. Even though the data are based on more subjects than in experiment 2, the non-significant difference in experiment 2 was in the opposite direction. The reason for these hemispheric asymmetries is not clear. Further experimentation is needed to resolve the

issue of whether or not true hemispheric differences of GAP-43 and tau-1 levels exist in the region of MePD and the cortex.

Experiment 1: Neurotrophic Effects of T on the Medial Nucleus of the Amygdala

The novel finding of experiment 1 was that 8 day T-treatment of male rats castrated for 64 days changes the cytoarchitecture of MePD in that its size increases by 28%. In addition, castration of male rats for 58 days resulted in a reduction in the size of the cell group MePD by 27.9%. This change is almost identical to the value found by Malsbury and McKay (1994), a 27% reduction in size at 56 days after castration. Castration for 64 days resulted in a slightly greater decline in the size of MePD of about 32.0%. This suggests that the maximum loss may not be reached at 58 days, and that the nucleus would continue to atrophy if longer post-castration periods were examined.

Even though an 8 day T-treatment affected the cytoarchitecture of MePD, it did not significantly increase the size of the dense fields of GAP-43 or tau-1 staining in this region. The areas of GAP-43 and tau-1 staining did increase by 15.7% and 9%, respectively. The moderate increase in the area of GAP-43 staining almost reached significance, suggesting that there was a general trend in the expected direction. It is possible that the neurotrophic effects of T

on this area were delayed for the GAP-43 and tau-1 areas of staining.

However, castration did reduce the size of the fields of GAP-43 and tau-1 staining in the region of MePD below that seen in normal males at both time periods after castration. Reductions in the area of dense GAP-43 staining after 58 days of castration was 21.4% and after 64 days it was 24.2%. For tau-1 staining, reductions were 19.5% after 58 days and 22.1% after 64 days of castration. It is possible that the reductions in the areas of GAP-43 and tau-1 staining could have resulted from the retraction of axons. These reductions were considerably smaller than the 42% loss in the size of SPir fiber fields in the region of MePD found by Malsbury and McKay (1994). As mentioned in the introduction, other studies have found reductions in the number of vasopressin-immunoreactive (Devries et al., 1985) and cholecystokinin-immunoreactive cell bodies (Micevych et al., 1988a; Simerly and Swanson, 1987) in the medial amygdala as a result of castration. In the closely related BNST, castration also leads to a reduction in the numbers of vasopressin- (Devries et al., 1985; Van Leeuwen et al., 1985), cholecystokinin- (Micevych et al., 1988a; Simerly and Swanson, 1987), and substance P-immunoreactive (Swann and Newman, 1992) cell bodies.

The magnitude of the decreases in the areas of GAPir- and tauir-staining in the region of MePD were similar to the reduction in the size of the cell group MePD. This indicates that the decrease in the area of GAPir and tauir-staining may be largely due to the similar reduction in the size of MePD which is likely to be the primary target of this innervation.

It is interesting to note that the decline in the size of the fields of SPir-staining in the region of MePD found by Malsbury and McKay (1994) was similar to the gradual decline in male sexual behavior which follows castration (Feder, 1971; Whalen and Luttge, 1971). In relation to the present experiment, it is also interesting to note that the rapid effect of T in increasing the size of MePD (8 days) is also similar to the rapid restoration of anogenital explorations of 3 week prepubertally castrated male rats (Singer, 1972), and the rapid restoration in the ejaculatory behavior of 4 week adult castrated male rats (Feder, 1971) after 1 week of T-replacement.

Rapid effects of gonadal hormones on neurochemical measures have been reported. For example, one study revealed that the levels of cholecystokinin in the posterior medial amygdala and the levels of cholecystokinin and substance P in the BNST in female rats varies with the 4 day estrous cycle (Micevych et al., 1988b). Rapid changes in synaptic ultrastructure have also been reported following T-treatment.

Within the spinal nucleus of the bulbocavernosus, the loss of synaptic contacts on motor neurons after 6 weeks of castration can be almost completely restored after 48 hours of T-treatment (Leedy et al., 1987). However, the finding that only 8 days of T-treatment would significantly increase the size of MePD was unexpected since there have been no previous demonstrations of such a rapid effect of gonadal hormones on the size of nuclear groups.

The results indicated that the average density of GAP-43 and tau-1 immunoreactivity in the region of MePD does not change in response to T-treatment. However, the area of this innervation does increase moderately, although not significantly, and the size of the cell group MePD increases in response to T-treatment. It is possible that GAP-43 and tau-1 levels may have increased, but the increase did not result in greater average densities of GAP-43 and tau-1 staining because the GAP-43 and tau-1 proteins were spread over a slightly larger area of innervation. That is, possible increases in the levels of these two proteins may have coincided with increases in the area of their innervation making the increased levels undetectable by average density measures.

A similar argument can be put forward for the effect of castration on GAP-43 and tau-1 staining. That is, GAP-43 and tau-1 levels may have declined in response to castration, but

the average densities of GAP-43 and tau-1 staining remained constant because the GAP-43 and tau-1 proteins were spread over a smaller area of innervation. Thus, it is also plausible that the reduced areas of dense GAP-43 and tau-1 staining resulted from the retraction of axons and that the levels of GAP-43 and tau-1 decreased but these decreases were undetectable by average density measures.

T exposure failed to affect the average density of staining for GAP-43 and tau-1. The levels of these proteins at any one point in time must depend on both their rate of synthesis and degradation. Thus it is possible that the apparent lack of effect of T exposure could be a result of a T-induced increase in both synthesis and degradation of these proteins. However, there is no information about the factors that regulate the degradation of these proteins.

The main purpose of experiment 1 was to see if GAP-43 and/or tau-1 in the region of MePD are regulated by T and to test the idea that the loss of the SPir innervation of MePD, as a result of castration, is at least partly mediated by the withdrawal or general atrophy of axons in this region. Overall the results suggested that T does not regulate the densities of GAP-43 and tau-1 in the region of MePD since castration does not reduce the density of GAP-43 and tau-1 staining nor does subsequent T-treatment increase it. However, castration does lead to a decline in the area of

dense GAP-43 and tau-1 staining in the region of MePD, but subsequent 8 days of T-treatment does not seem to significantly reverse this decline. Thus, the results are difficult to interpret with respect to whether or not there was an axonal response to T.

Experiment 2: No Sex

Differences in GAP-43 and Tau-1 Levels

The results indicated that there were no sex differences in the density of GAP-43 and tau-1 immunoreactivity in the region of MePD or part of the cerebral cortex which includes portions of the perirhinal, temporal and parietal areas. Especially for GAP-43, these results are puzzling in light of the fact that some areas in the brain which are known to be sexually dimorphic in some way are also sexually dimorphic with respect to the levels of GAP-43 mRNA. For example, the thickness of the frontal cortex is sexually dimorphic (Juraska, 1990) and the level of GAP-43 mRNA in the frontal cortex is also sexually dimorphic (Lustig et al., 1993; Lustig et al., 1991). There is also a sex difference in the number of synapses in the VMH (Matsumoto and Arai, 1986) and the level of GAP-43 mRNA is also sexually dimorphic in the VMH (Shughrue and Dorsa, 1993a). Of particular relevance to this experiment, the posterior medial amygdala is also sexually dimorphic with respect to its size (Hines et al., 1992), the

size of the SPir fiber fields in this region (Malsbury and McKay, 1989), and the numbers of cholecystokinin-immunoreactive cell bodies (Micevych et al., 1988a). In light of this, it was surprising to see that there was no sex difference in the level of GAP-43 in the region of MePD.

However, the finding of no sex differences in the levels of GAP-43 and tau-1 in the region of MePD even though some morphological sex difference exists in this area is not unique. There is a large sex difference in the size of the sexually dimorphic nucleus of the preoptic area of the adult rat (Gorski et al. 1980), while Shughrue and Dorsa (1993a) have shown that there is no sex difference in the level of GAP-43 mRNA in the preoptic area of adult rats.

The situation is different for newborn rats as there is a sex difference in the levels of GAP-43 mRNA in the medial preoptic nucleus of the 6 day old rat (Shughrue and Dorsa, 1993b). On the other hand, the level of GAP-43 mRNA in the frontal cortex is sexually dimorphic in both the 6 day old (Shughrue and Dorsa, 1993a) and in the adult rat (Lustig et al., 1993; Lustig et al., 1991). Thus, the effect of age on sex differences in GAP-43 mRNA level seems to depend on the brain area examined. In light of this realization, even though the levels of GAP-43 in the region of MePD in this study were not sexually dimorphic in the adult, their levels may be sexually dimorphic in the neonate. In support of this

idea is the finding that GAP-43 mRNA levels in the BNST, which has strong connections to the medial amygdala (Ottersen, 1980; Krettek and Price, 1978; Weller and Smith, 1982) are sexually dimorphic in the neonate (Shughrue and Dorsa, 1993b).

In the present study, the effects of hormonal treatment and sex on GAP-43 levels in the medial amygdala were of interest. As mentioned above, the medial amygdala receives major input from the BNST and it probably also contains local axonal branches from medial amygdala neurons. It is likely that changes in GAP-43 mRNA levels in the medial amygdala and BNST would have to take place for there to be changes in the levels of GAP-43 itself in the medial amygdala. Hence, an important issue is whether or not GAP-43 mRNA levels in the medial amygdala and BNST of adults are sexually dimorphic or respond to hormone treatment, which to my knowledge no one has assessed.

Experiment 2: Morphological Sex Differences in MePD

The results indicated that there were marked sex differences in the size of the fields of dense staining for GAP-43 and tau-1 in the region of MePD. In addition, the size of MePD as revealed in cresyl-stained sections, was also highly sexually dimorphic. Interestingly, the magnitude of the male > female pattern in the size of the fields of MePD GAP-43 (79.7%) and tau-1 staining (90.6%) were moderately

smaller than for MePD itself (106.9%). Thus the sex difference in the area that the axonal processes cover in the region of MePD is not as great as the sex difference in the area covered by the cell bodies of MePD neurons. The reason for this discrepancy is not known.

Although the sex difference in the size of MePD found here replicates the findings of Hines et al. (1992) and Kerchner et al. (1994), there is a discrepancy in the magnitude of the difference. Hines et al. (1992) found that the volume of MePD was 85.2% greater in male than in female rats and Kerchner et al. (1994) found a similar dimorphism of 78%, whereas the present study found a greater magnitude of 106.7%. There may be two reasons for this discrepancy. First, there may have been differences in the way in which the nucleus was measured in the present study. Inspection of Figure 1 in the Hines et al. (1992) study indicates that they may not have measured as far rostrally through the nucleus as I did. Secondly, the females in both the Hines et al. (1992) and Kerchner et al. (1994) study were probably mixed with respect to the stage of the estrous cycle they were in. In the present study, all females were in the early portion of the diestrous stage of the estrous cycle. As stated in the methods, estrogen levels were low at that time. Although no study has looked at whether or not there are variations in the size of MePD throughout the estrous cycle, if there are such

variations then the size of MePD would probably be the smallest when estrogen levels have been low for some time, at the diestrous portion of the cycle. Thus, when comparing the size of the MePD in males to that in diestrous females, the magnitude of the differences would be larger than when comparing the male MePD to that in a group of females who are in different parts of the cycle.

The demonstration of a substantial sex difference in area of both the GAP-43 and tau-1 MePD staining in the present study adds to the previous finding of Malsbury and McKay (1989), who found a larger sex difference in the area of SP staining in the same region. Axons of the neurons in MePD which contain substance P probably also contain the proteins GAP-43 and tau-1. Thus, the sex differences in the areas covered by dense staining for GAP-43, tau-1 and SP may all be the result of the same sex difference in the innervation of MePD. Finally, the sex differences in GAP-43, tau-1, and SP staining area are all probably largely due to the sex difference in the size of MePD.

Conclusions

Immunoreactive staining for GAP-43 and tau-1 is particularly dense within the region of MePD, a sexually dimorphic and hormone-sensitive cell group within the medial amygdala. However, the density of GAP-43 and tau-1

immunoreactivity in the region of MePD is neither regulated by T nor sexually dimorphic in the adult. On the other hand, the areas covered by this dense GAP-43 and tau-1 staining are affected by castration and are sexually dimorphic. These effects on the areas of staining are probably largely a reflection of castration effects on and sex differences in the size of MePD. Castration probably decreases the size of MePD at least partly by shrinking the dendritic trees of its neurons. This is probably accompanied by a loss innervation of MePD dendrites by GAP-43 and tau-1 immunoreactive axons. Thus a reduction in the area covered by the dense staining for these proteins is seen, as MePD dendrites now cover a smaller area. The reason for the lack of effect of castration on the average density of staining within these smaller areas is not known. It may be that castration reduces both the synthesis and degradation of GAP-43 and tau-1, resulting in no change in their levels within the region where MePD dendrites are still present.

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Table 1. GAP-43 raw ROD measures for MePD and the MePD/whole section ROD ratio are shown for each group. Overall, caudal, and rostral means (SEM) are given for the left, right and the combination of the left and right hemisphere (L/R).

	CC-2	C+T-2	CC-8	C+T-8
MePD (Raw)				
Overall				
Left	0.8899 (0.0186)	0.8737 (0.0352)	0.8206 (0.0594)	0.8280 (0.0986)
Right	0.9194 (0.0345)	0.9112 (0.0348)	0.8550 (0.0462)	0.8647 (0.0806)
L/R	0.9046 (0.0257)	0.8924 (0.0345)	0.8378 (0.0516)	0.8464 (0.0894)
MePD/Whole S.				
Overall				
Left	1.4159 (0.0257)	1.3709 (0.0344)	1.3694 (0.0693)	1.3981 (0.0455)
Right	1.4624 (0.0588)	1.4248 (0.0324)	1.4359 (0.0594)	1.4616 (0.0088)
L/R	1.4391 (0.0421)	1.3978 (0.0322)	1.4026 (0.0600)	1.4299 (0.0254)
Caudal				
Left	1.4798 (0.0165)	1.4487 (0.0573)	1.4222 (0.0691)	1.5204 (0.0795)
Right	1.5346 (0.0670)	1.5380 (0.0483)	1.5084 (0.0752)	1.5616 (0.0322)
L/R	1.5072 (0.0397)	1.4933 (0.0512)	1.4653 (0.0627)	1.5410 (0.0501)
Rostral				
Left	1.3751 (0.0450)	1.3215 (0.0292)	1.3426 (0.0790)	1.3166 (0.0515)
Right	1.4248 (0.0418)	1.3538 (0.0427)	1.3456 (0.0522)	1.3794 (0.0220)
L/R	1.3990 (0.0409)	1.3377 (0.0311)	1.3441 (0.0587)	1.3480 (0.0288)

Table 2. Tau-1 raw ROD measures for MePD and the MePD/whole section ROD ratio are shown for each group. Overall, caudal, and rostral means (SEM) are given for the left, right and the combination of the left and right hemisphere (L/R).

	CC-2	C+T-2	CC-8	C+T-8
MePD (Raw)				
Overall				
Left	0.9346 (0.0305)	0.9222 (0.0222)	0.8239 (0.0439)	0.7907 (0.0596)
Right	0.9870 (0.0183)	0.9356 (0.0282)	0.7799 (0.0284)	0.8158 (0.0589)
L/R	0.9608 (0.0242)	0.9289 (0.0244)	0.8019 (0.0349)	0.8032 (0.0587)
MePD/Whole S.				
Overall				
Left	1.2899 (0.0548)	1.3184 (0.0299)	1.3373 (0.0549)	1.3181 (0.0124)
Right	1.3577 (0.0731)	1.3387 (0.0382)	1.2667 (0.0707)	1.3673 (0.0349)
L/R	1.3238 (0.0630)	1.3286 (0.0325)	1.3020 (0.0608)	1.3427 (0.0225)
Caudal				
Left	1.3042 (0.0619)	1.3626 (0.0407)	1.3654 (0.0557)	1.3359 (0.0495)
Right	1.3383 (0.0833)	1.3640 (0.0530)	1.3148 (0.0555)	1.3363 (0.0239)
L/R	1.3212 (0.0725)	1.3633 (0.0406)	1.3401 (0.0555)	1.3361 (0.0252)
Rostral				
Left	1.2560 (0.0541)	1.2988 (0.0354)	1.2942 (0.0625)	1.3095 (0.0204)
Right	1.3809 (0.0732)	1.3059 (0.0366)	1.2280 (0.1164)	1.4097 (0.0700)
L/R	1.3185 (0.0595)	1.3023 (0.0358)	1.2611 (0.0857)	1.3596 (0.0280)

Table 3. GAP-43 raw ROD measures for the cortex, the cortex/whole section ROD ratio and whole section RODs are shown for each group. Data are means (SEM) and for the cortex are given for the left, right and the combination of the left and right hemisphere (L/R).

	CC-2	C+T-2	CC-8	C+T-8
Cortex (Raw)				
Left	0.7186 (0.0429)	0.7399 (0.0221)	0.6717 (0.0499)	0.6796 (0.0704)
Right	0.7598 (0.0218)	0.7373 (0.0176)	0.6799 (0.0468)	0.6559 (0.0702)
L/R	0.7392 (0.0303)	0.7386 (0.0196)	0.6758 (0.0478)	0.6677 (0.0701)
Cortex/Whole S.				
Left	1.1391 (0.0439)	1.1623 (0.0230)	1.1158 (0.0254)	1.1536 (0.0222)
Right	1.2063 (0.0153)	1.1552 (0.0164)	1.1362 (0.0232)	1.1060 (0.0176)
L/R	1.1727 (0.0198)	1.1587 (0.0193)	1.1260 (0.0212)	1.1298 (0.0184)
Whole Section	0.6297 (0.0161)	0.6357 (0.0117)	0.5997 (0.0361)	0.5899 (0.0548)

Table 4. Tau-1 raw ROD measures for the cortex, the cortex/whole section ROD ratio and whole section RODs are shown for each group. Data are means (SEM) and for the cortex are given for the left, right and the combination of the left and right hemisphere (L/R).

	CC-2	C+T-2	CC-8	C+T-8
Cortex (Raw)				
Left	0.8450 (0.0487)	0.8049 (0.0254)	0.7157 (0.0761)	0.6662 (0.0678)
Right	0.8959 (0.0680)	0.8434 (0.0261)	0.7171 (0.0743)	0.6533 (0.0595)
L/R	0.8705 (0.0582)	0.8241 (0.0251)	0.7164 (0.0752)	0.6597 (0.0632)
Cortex/Whole S.				
Left	1.1591 (0.0086)	1.1494 (0.0234)	1.1437 (0.0241)	1.1037 (0.0290)
Right	1.2176 (0.0287)	1.2039 (0.0088)	1.1350 (0.0295)	1.0887 (0.0278)
L/R	1.1883 (0.0175)	1.1767 (0.0154)	1.1393 (0.0265)	1.0962 (0.0255)
Whole Section	0.7332 (0.0447)	0.7008 (0.0188)	0.6239 (0.0538)	0.5993 (0.0473)

Table 5. The area (mm^2) of GAP-43 and tau-1 staining in the region of MePD and MePD itself as defined using cresyl-stained sections are shown for each hemisphere and the combination of both hemispheres (L/R) for each group. The number of sections in which MePD appeared and the area (mm^2) of the whole section for the cresyl material are also given. Data are means (SEM).

	CC-2	C+T-2	CC-8	C+T-8
MePD: GAP-43				
Left	4.156 (0.274)	4.728 (0.601)	4.157 (0.316)	4.902 (0.277)
Right	4.347 (0.245)	4.818 (0.226)	4.036 (0.173)	4.573 (0.262)
L/R	4.252 (0.221)	4.783 (0.411)	4.096 (0.245)	4.738 (0.267)
MePD: Tau-1				
Left	4.007 (0.188)	4.071 (0.224)	3.975 (0.185)	4.467 (0.177)
Right	4.187 (0.157)	4.413 (0.219)	3.952 (0.115)	4.176 (0.215)
L/R	4.097 (0.160)	4.242 (0.221)	3.963 (0.145)	4.321 (0.181)
MePD: Cresyl				
Area				
Left	2.312 (0.138)	2.502 (0.078)	2.262 (0.213)	2.879 (0.163)
Right	2.428 (0.152)	2.644 (0.172)	2.207 (0.104)	2.843 (0.115)
L/R	2.370 (0.135)	2.573 (0.119)	2.235 (0.158)	2.861 (0.133)
No. Sections				
Left	6.75 (0.25)	6.75 (0.25)	6.00 (0.41)	7.00 (0.00)
Right	7.00 (0.00)	6.75 (0.25)	6.00 (0.00)	6.50 (0.29)
L/R	6.88 (0.13)	6.75 (0.25)	6.00 (0.20)	6.75 (0.14)
Cresyl Whole Section	682.614 (18.245)	699.584 (8.561)	700.950 (22.499)	672.621 (16.090)

Table 6. GAP-43 and tau-1 raw ROD measures for MePD and the MePD/whole section ROD ratio are shown for males and females. Overall, caudal, and rostral means (SEM) are given for the left, right and the combination of the left and right hemisphere (L/R).

	GAP-43		Tau-1	
	Males	Females	Males	Females
MePD (Raw)				
Overall				
Left	0.8950 (0.1121)	0.8338 (0.1293)	0.8430 (0.0810)	0.8789 (0.0629)
Right	0.9335 (0.1120)	0.8870 (0.1325)	0.8642 (0.0888)	0.9216 (0.0502)
L/R	0.9142 (0.1116)	0.8604 (0.1309)	0.8536 (0.0847)	0.9003 (0.0564)
MePD/Whole S.				
Overall				
Left	1.4677 (0.0449)	1.3881 (0.0462)	1.4974 (0.0713)	1.4134 (0.0841)
Right	1.5364 (0.0576)	1.4739 (0.0568)	1.5319 (0.0625)	1.4707 (0.1091)
L/R	1.5021 (0.0494)	1.4310 (0.0515)	1.5147 (0.0662)	1.4420 (0.0966)
Caudal				
Left	1.5682 (0.0816)	1.4583 (0.0299)	1.4629 (0.0577)	1.4117 (0.0887)
Right	1.6261 (0.0729)	1.5958 (0.0807)	1.5161 (0.0580)	1.5128 (0.1198)
L/R	1.5971 (0.0763)	1.5271 (0.0537)	1.4895 (0.0559)	1.4623 (0.1040)
Rostral				
Left	1.3771 (0.0420)	1.3168 (0.0634)	1.5086 (0.0779)	1.4105 (0.0782)
Right	1.4621 (0.0420)	1.3518 (0.0746)	1.5373 (0.0667)	1.4413 (0.1049)
L/R	1.4196 (0.0343)	1.3343 (0.0648)	1.5229 (0.0721)	1.4259 (0.0912)

Table 7. GAP-43 and tau-1 raw ROD measures for the cortex, the cortex/whole section ROD ratio and whole section RODs are shown for males and females. Data are means (SEM) and for the cortex are given for the left, right and the combination of the left and right hemisphere (L/R).

	GAP-43		Tau-1	
	Males	Females	Males	Females
Cortex (Raw)				
Left	0.7169 (0.1182)	0.6919 (0.1360)	0.6843 (0.1168)	0.7263 (0.1016)
Right	0.6846 (0.1077)	0.6834 (0.1236)	0.6310 (0.1069)	0.7275 (0.0814)
L/R	0.7009 (0.1128)	0.6876 (0.1298)	0.6577 (0.1117)	0.7269 (0.0909)
Cortex/Whole S.				
Left	1.1513 (0.0416)	1.1326 (0.0544)	1.1797 (0.0414)	1.1486 (0.0304)
Right	1.1024 (0.0238)	1.1231 (0.0442)	1.0907 (0.0350)	1.1435 (0.0066)
L/R	1.1268 (0.0321)	1.1279 (0.0488)	1.1352 (0.0363)	1.1460 (0.0132)
Whole Section	0.6156 (0.0855)	0.6043 (0.0947)	0.5712 (0.0796)	0.6332 (0.0722)

Table 8. The area (mm^2) of GAP-43 and tau-1 staining in the region of MePD and MePD itself as defined using cresyl-stained sections are shown for each hemisphere and the combination of both hemispheres (L/R) for males and females. The number of sections in which MePD appeared and the area (mm^2) of the cresyl whole sections are also given. Data are means (SEM).

	Males	Females
MePD: GAP-43		
Left	5.599 (0.211)	2.952 (0.151)
Right	5.215 (0.120)	3.065 (0.372)
L/R	5.407 (0.128)	3.009 (0.260)
MePD: Tau-1		
Left	5.326 (0.183)	2.576 (0.110)
Right	4.850 (0.064)	2.761 (0.208)
L/R	5.088 (0.120)	2.669 (0.157)
MePD: Cresyl		
Area		
Left	3.414 (0.086)	1.635 (0.079)
Right	3.160 (0.084)	1.543 (0.065)
L/R	3.287 (0.060)	1.589 (0.071)
No. Sections		
Left	7.50 (0.29)	5.33 (0.33)
Right	7.00 (0.00)	5.00 (0.58)
L/R	7.25 (0.14)	5.17 (0.44)
Cresyl Whole Section	579.912 (8.353)	578.404 (18.606)

Figure 1. An example of a negative control in which the primary antibody was omitted from the immunocytochemistry procedure. The medial amygdala in the left hemisphere of a male is shown. Notice that staining is almost entirely eliminated. Secondary antibody dilution was 1:400 and tertiary antibody dilution was 1:400.

Figure 2. Matched coronal sections showing the pattern of GAPir-staining in the region of MePD of an 8 week castrated male that was subsequently T-treated for 8 days and one which received an empty capsule for 8 days (control). Sections are arranged in rostral to caudal order (A-E, F-J). Notice that the boundary of the fields of staining are not as discrete at the rostral levels as they are at the more caudal levels. Primary antibody dilution was 1:1500. The scale bar in A is 1 mm. Abbreviations: PD, medial nucleus of the amygdala, posterodorsal.

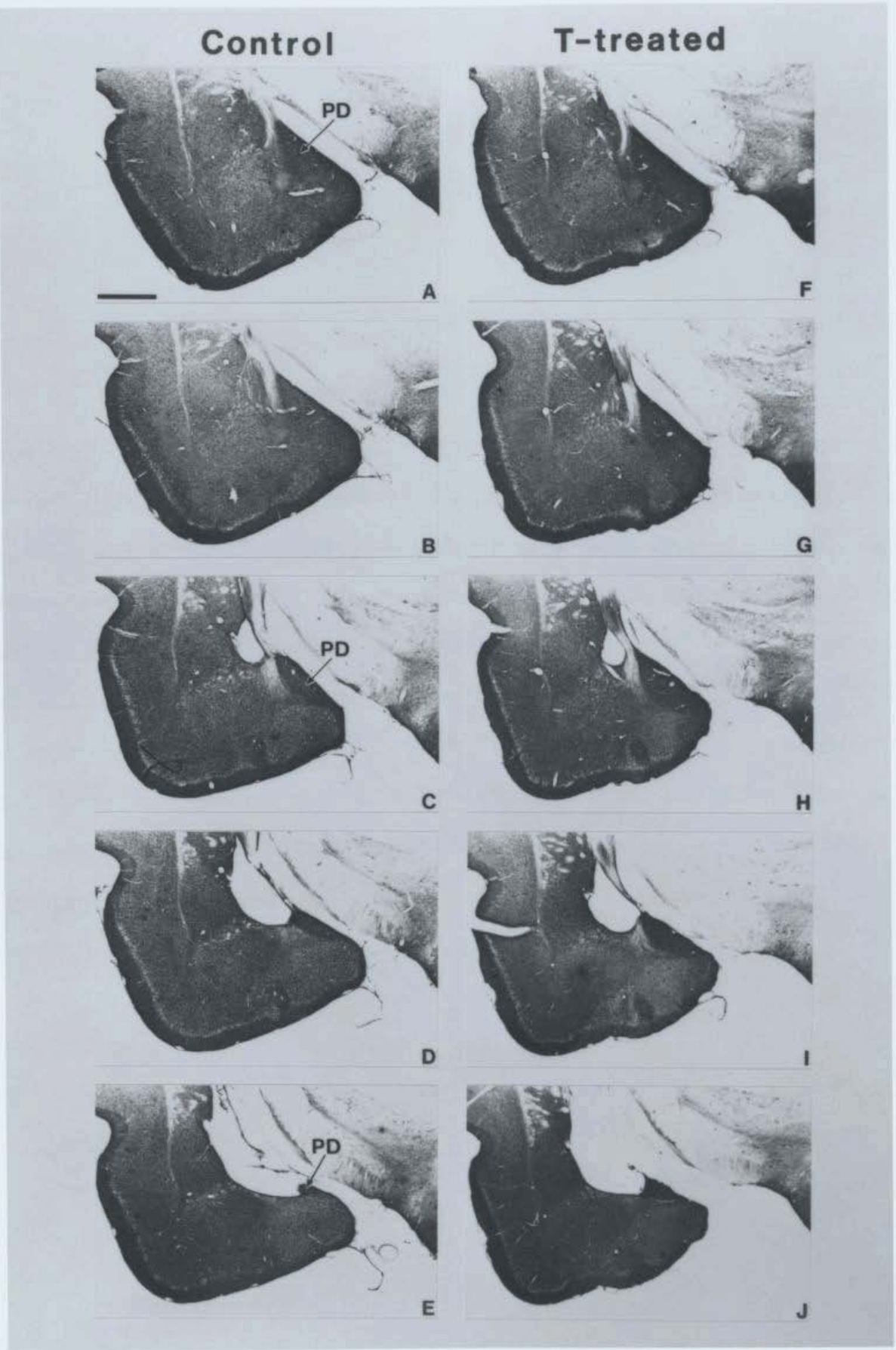
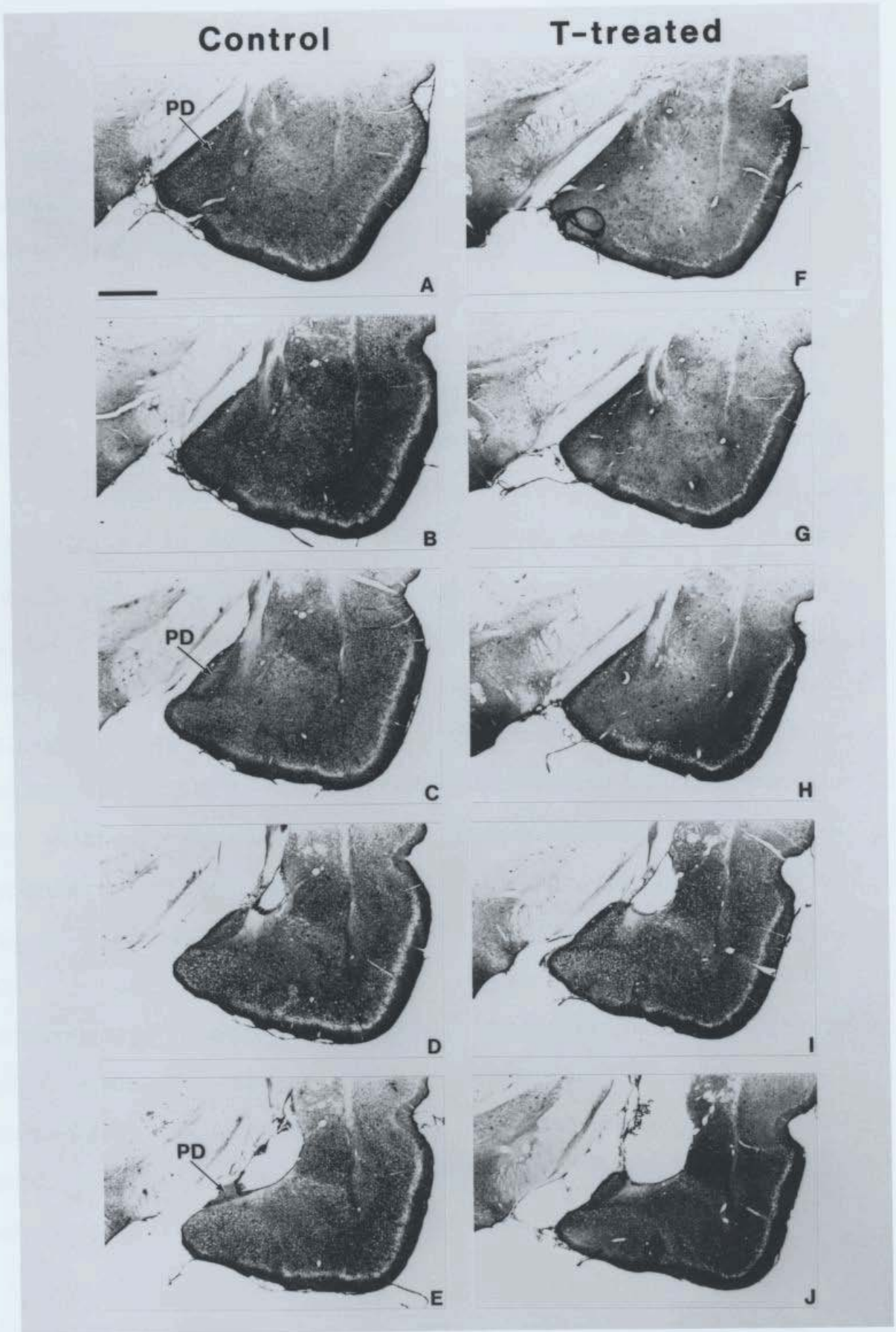

Control
T-treated
PD
PD
PD
A
B
C
D
E
F
G
H
I
J

Figure 3. Matched coronal sections showing the pattern of tauir-staining in the region of MePD of an 8 week castrated male that was subsequently T-treated for 8 days and one which received an empty capsule for 8 days (control). Sections are arranged in rostral to caudal order (A-E, F-J). Like the GAPir-staining, the boundary of the fields of staining are not as discrete at the rostral levels as they are at the more caudal levels. Primary antibody dilution was 1:5000. The scale bar in A is 1 mm. Abbreviations: PD, medial nucleus of the amygdala, posterodorsal.


Control
T-treated
PD
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PD
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I
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Figure 4. Schematic drawing of a coronal hemi-section of the rat brain modified from Paxinos and Watson (1986). The placement of the sampling box used to measure the ROD of GAPir- and tauir-staining in the cerebral cortex is illustrated. Abbreviations: BLA, basolateral amygdaloid nucleus, anterior; BLP, basolateral amygdaloid nucleus, posterior; BLV, basolateral amygdaloid nucleus, ventral; BPM, basomedial amygdaloid nucleus, posterior; BSTIA, bed nucleus of the stria terminalis, intraamygdaloid division; CA1-3, fields CA1-3 of Ammon's horn; CeL, central amygdaloid nucleus, lateral; DG, dentate gyrus; Fr1, frontal cortex, area 1; Fr2, frontal cortex, area 2; HL, hindlimb area of the cortex; I, intercalated nuclei amygdala; LaDL, lateral amygdaloid nucleus, dorsolateral; LaVL, lateral amygdaloid nucleus, ventrolateral; LaVM, lateral amygdaloid nucleus, ventromedial; MePD, medial amygdaloid nucleus, posterodorsal; MePV, medial amygdaloid nucleus, posteroventral; Par1, parietal cortex, area 1; Par2, parietal cortex, area 2; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; PMCo, posteromedial cortical amygdaloid nucleus; PoDG, polymorph layer dentate gyrus; PRh, perirhinal cortex; RSA, retrosplenial agranular Cx; RSG, retrosplenial granular Cx; VMHDM, ventromedial hypothalamic nucleus, dorsomedial; VMHVL, ventromedial hypothalamic nucleus, ventrolateral.

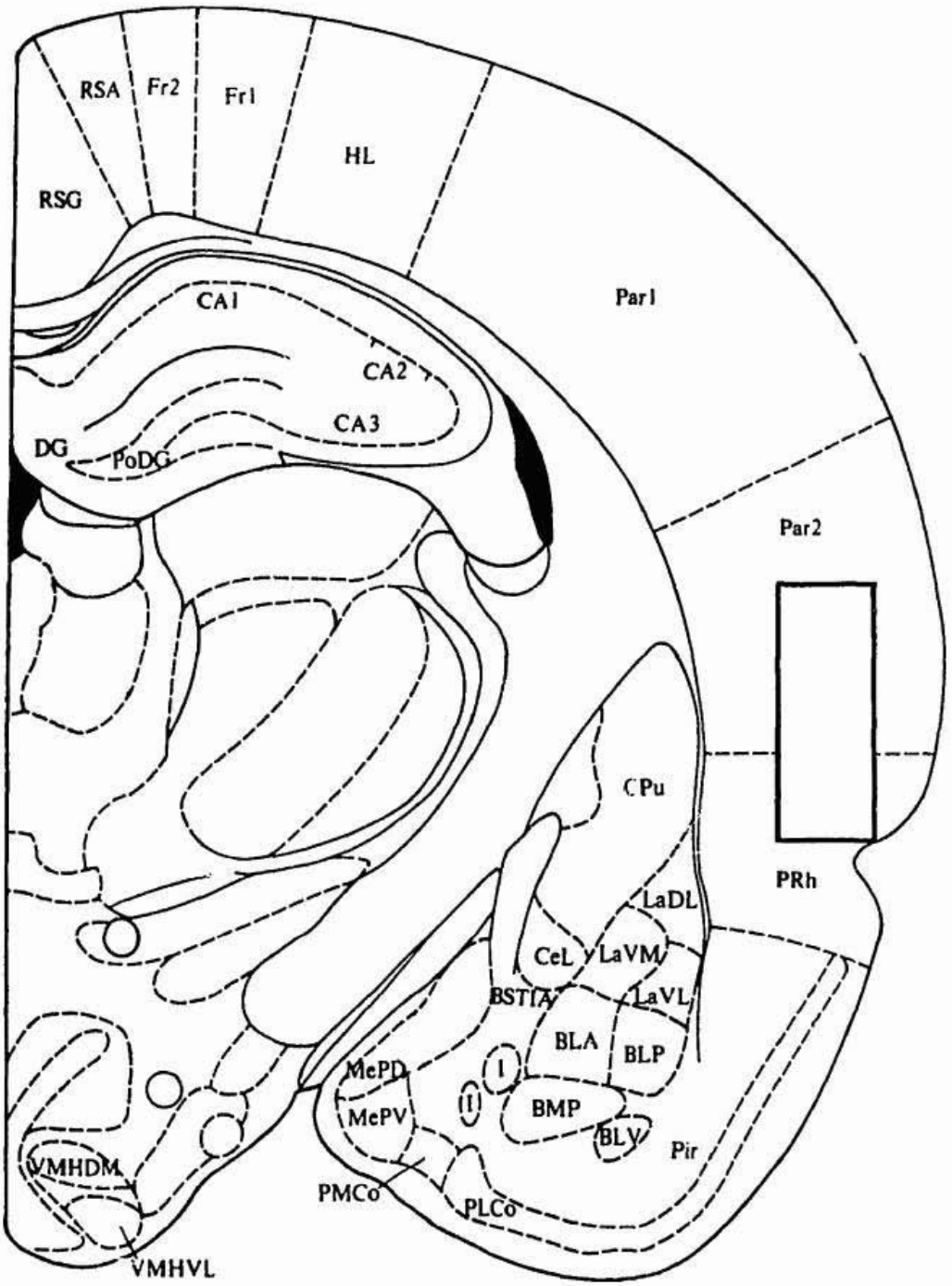

RSA
Fr2
Fr1
HL
RSG
Par1
CA1
CA2
CA3
DG
PoDG
Par2
CPu
PRh
LaDL
CeL
LaVM
BSTIA
LaVL
BLA
BLP
MePD
I
II
BMP
MePV
BLV
Pir
VMHDM
PMCo
PLCo
VMHVL

Figure 5. The overall MePD/whole section ROD ratio of GAP-43 immunoreactivity for normal males, castrated control males (CC ♂) and castrated plus T-treated males (C+T ♂). Castrated control and castrated plus T-treated males had silastic capsules inserted for either 2 days or 8 days. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat.

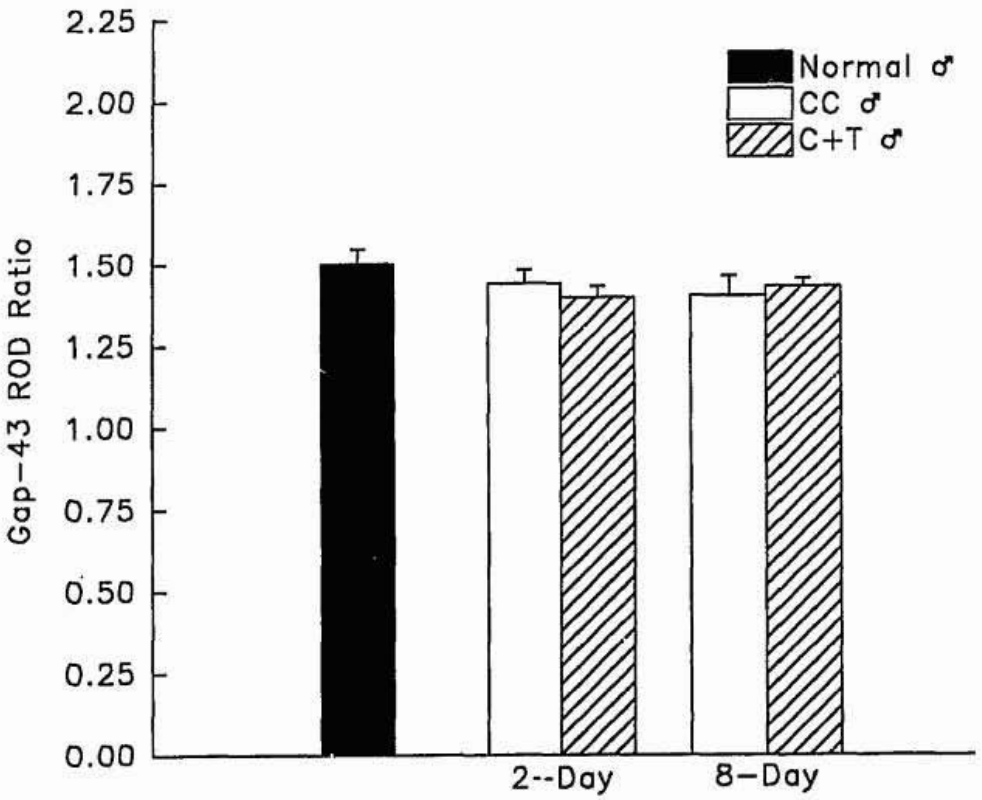

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Gap-43 ROD Ratio
Normal ♂
CC ♂
C+T ♂
2--Day
8-Day

Figure 6. The overall MePD/whole section ROD ratio of tau-1 immunoreactivity for normal males, castrated control males (CC ♂) and castrated plus T-treated males (C+T ♂). Castrated control and castrated plus T-treated males had silastic capsules inserted for either 2 days or 8 days. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat.

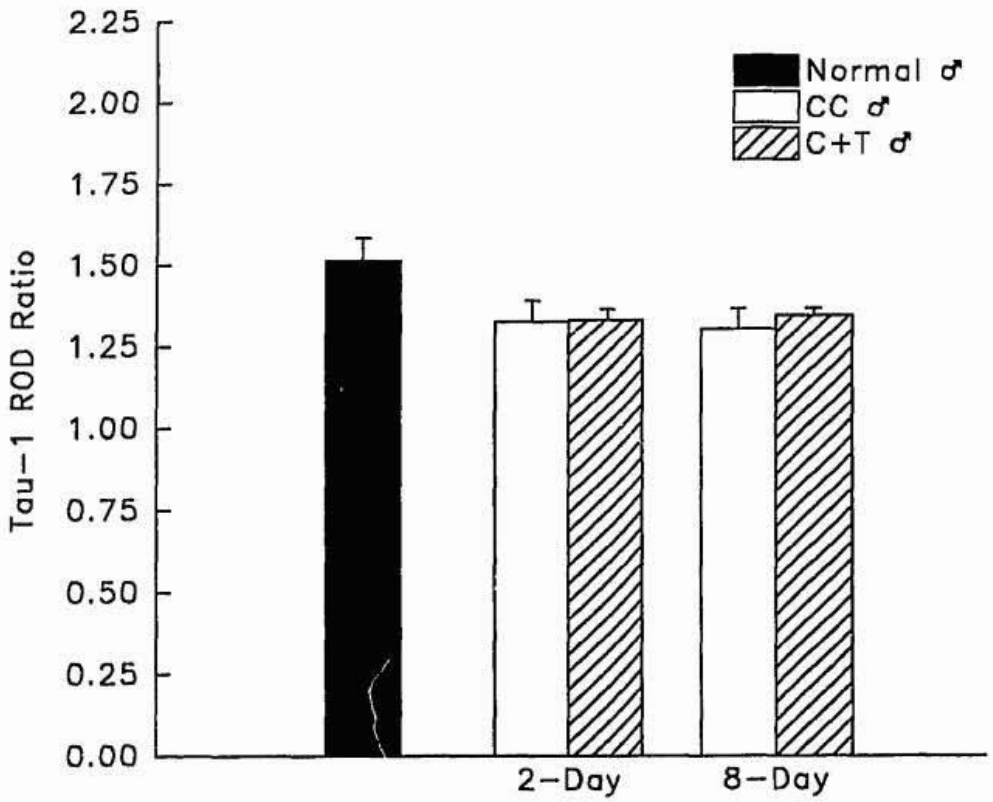

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Tau-1 ROD Ratio
Normal ♂
CC ♂
C+T ♂
2-Day
8-Day

Figure 7. The overall raw ROD and the overall ROD ratio (MePD/whole section) of GAP-43 and tau-1 immunoreactivity for MePD in the left and right hemisphere. Data were obtained from control and T-treated subjects and are hemispheric means (± standard error of the mean). An asterisk above the bars indicates significantly darker staining for GAP-43 in the region of MePD in the right hemisphere ($p \leq 0.05$).

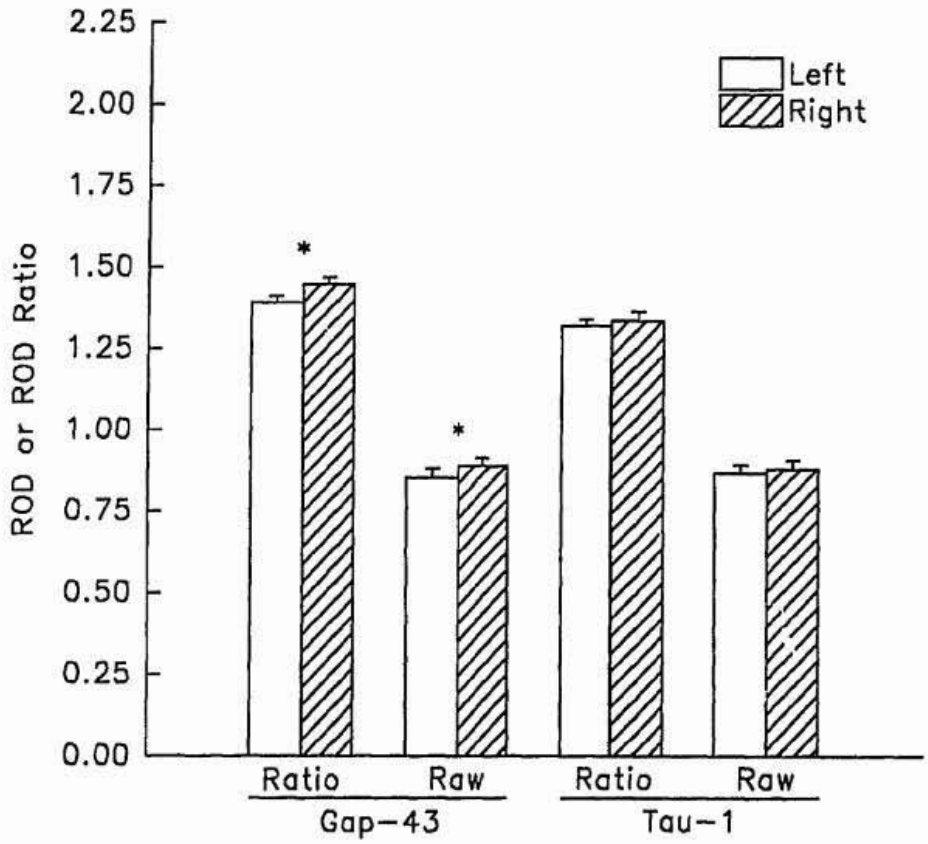

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ROD or ROD Ratio
Left
Right
*
*
Ratio
Raw
Ratio
Raw
Gap−43
Tau−1

Figure 8. The cortex/whole section ROD ratio of GAP-43 immunoreactivity for normal males, castrated control males (CC ♂) and castrated plus T-treated males (C+T ♂). Castrated control and castrated plus T-treated males had silastic capsules inserted for either 2 days or 8 days. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat.

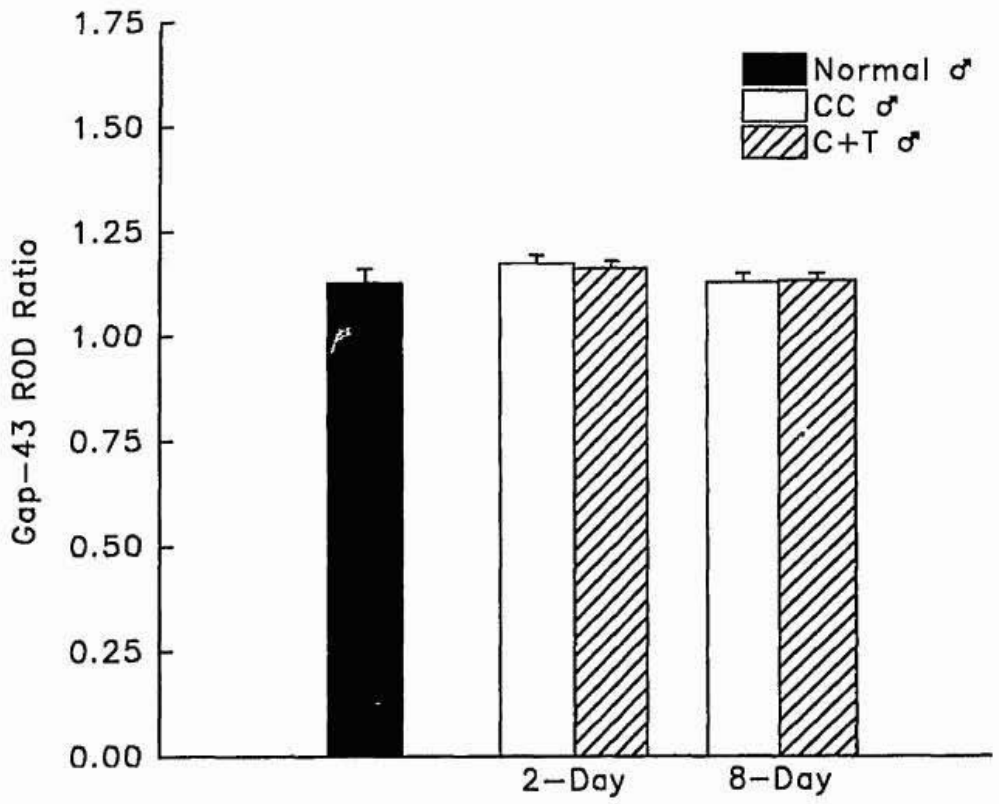

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Gap-43 ROD Ratio
Normal ♂
CC ♂
C+T ♂
2-Day
8-Day

Figure 9. The cortex/whole section ROD ratio of tau-1 immunoreactivity for normal males, castrated control males (CC ♂) and castrated plus T-treated males (C+T ♂). Castrated control and castrated plus T-treated males had silastic capsules inserted for either 2 days or 8 days. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat.

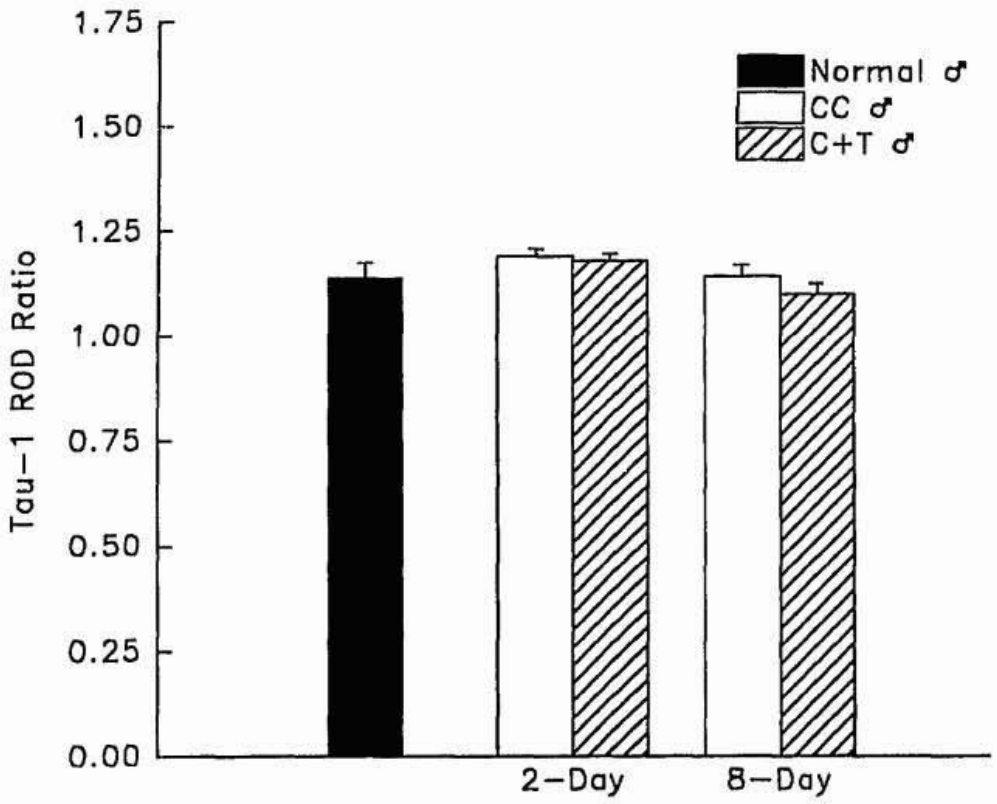

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Tau-1 ROD Ratio
Normal ♂
CC ♂
C+T ♂
2-Day
8-Day

Figure 10. The raw ROD and ROD ratio (cortex/whole section) of GAP-43 and tau-1 immunoreactivity for the cortex in the left and right hemisphere. Data were obtained from control and T-treated subjects and are hemispheric means (± standard error of the mean). An asterisk above the bars indicates significantly darker staining for tau-1 in the cortex of the right hemisphere ($p \leq 0.05$).

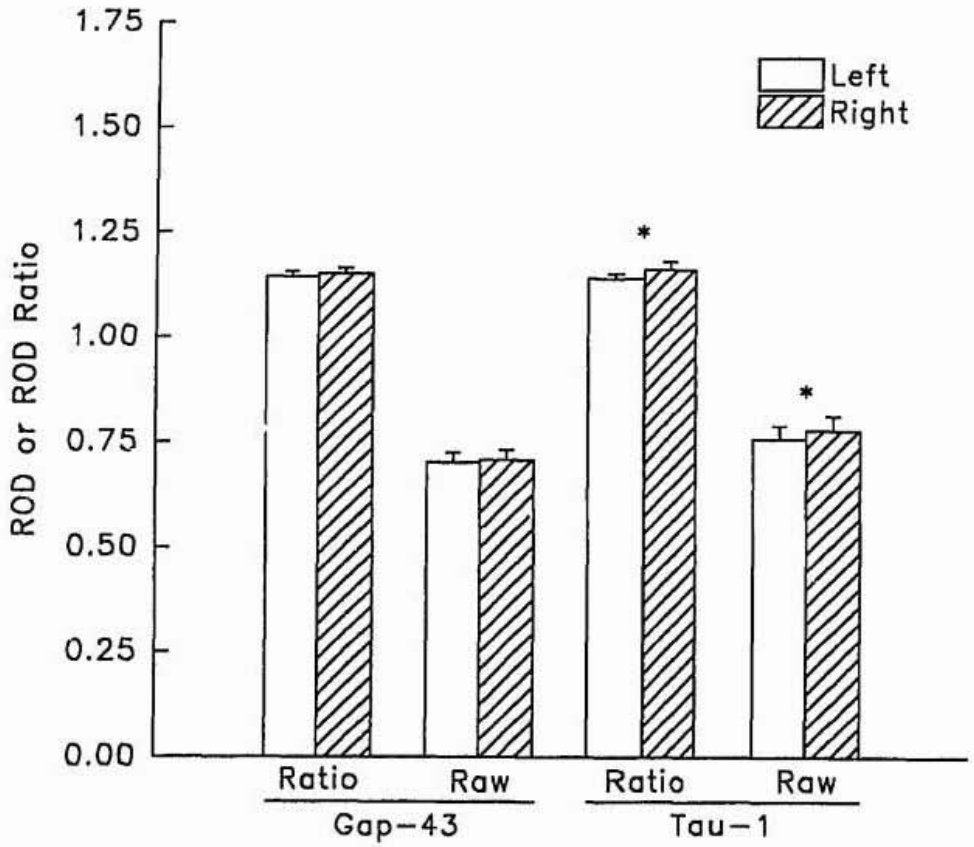

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ROD or ROD Ratio
Left
Right
*
*
Ratio
Raw
Ratio
Raw
Gap−43
Tau−1

Figure 11. The area (mm^2) of GAP-43 immunoreactive staining in the region of MePD for normal males, castrated control males (CC ♂) and castrated plus T-treated males (C+T ♂). Castrated control and castrated plus T-treated males had silastic capsules inserted for either 2 days or 8 days. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat. An asterisk above the bars indicates that normal males significantly differed from castrated control males at $p \leq 0.01$.

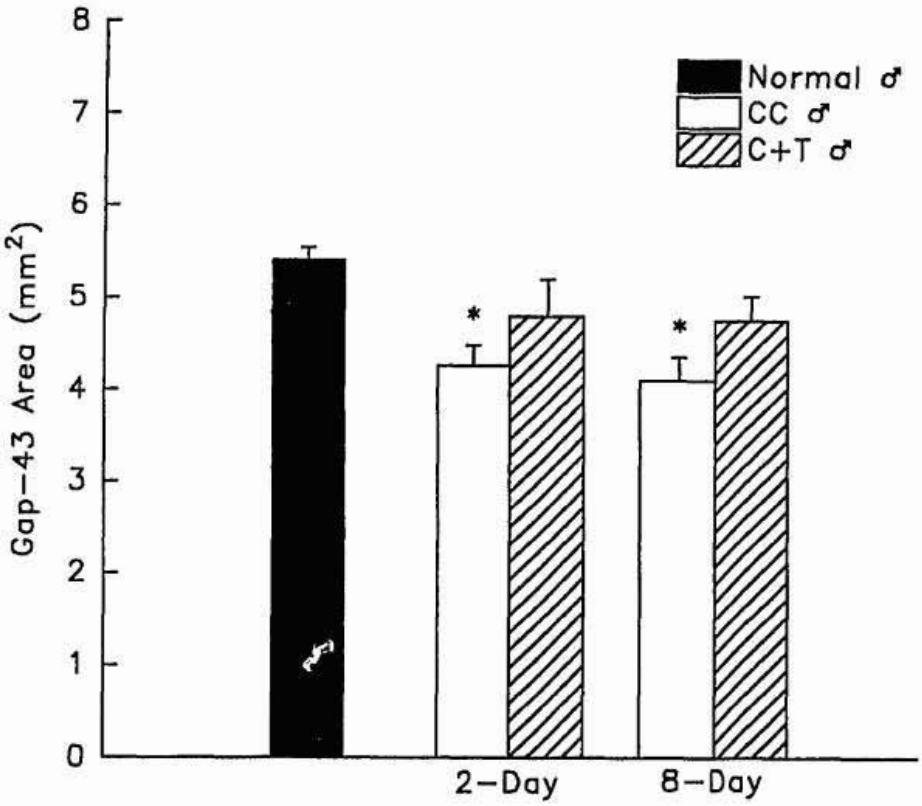

Gap–43 Area (mm^2)
0
1
2
3
4
5
6
7
8
Normal ♂
CC ♂
C+T ♂
*
*
2–Day
8–Day

Figure 12. The area (mm^2) of tau-1 immunoreactive staining in the region of MePD for normal males, castrated control males (CC ♂) and castrated plus T-treated males (C+T ♂). Castrated control and castrated plus T-treated males had silastic capsules inserted for either 2 days or 8 days. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat. An asterisk above the bars indicates that normal males significantly differed from castrated control males at $p \leq 0.01$.

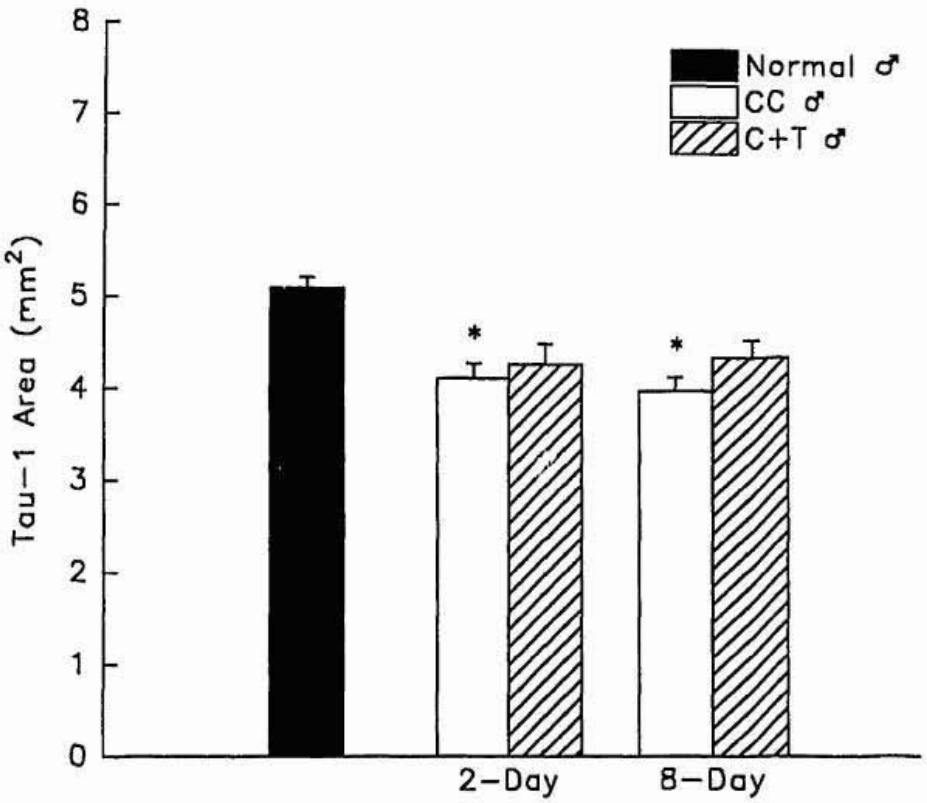

Normal ♂
CC ♂
C+T ♂
Tau-1 Area (mm²)
0
1
2
3
4
5
6
7
8
*
*
2-Day
8-Day

Figure 13. The area (mm^2) of the cell group MePD for normal males, castrated control males (CC ♂) and castrated plus T-treated males (C+T ♂). Castrated control and castrated plus T-treated males had silastic capsules inserted for either 2 days or 8 days. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat. A single asterisk above the bars indicates that normal males significantly differed from castrated control males at $p \leq 0.001$. A double asterisk above the bars indicates that castrated plus T-treated males significantly differed from castrated control males at $p \leq 0.01$.

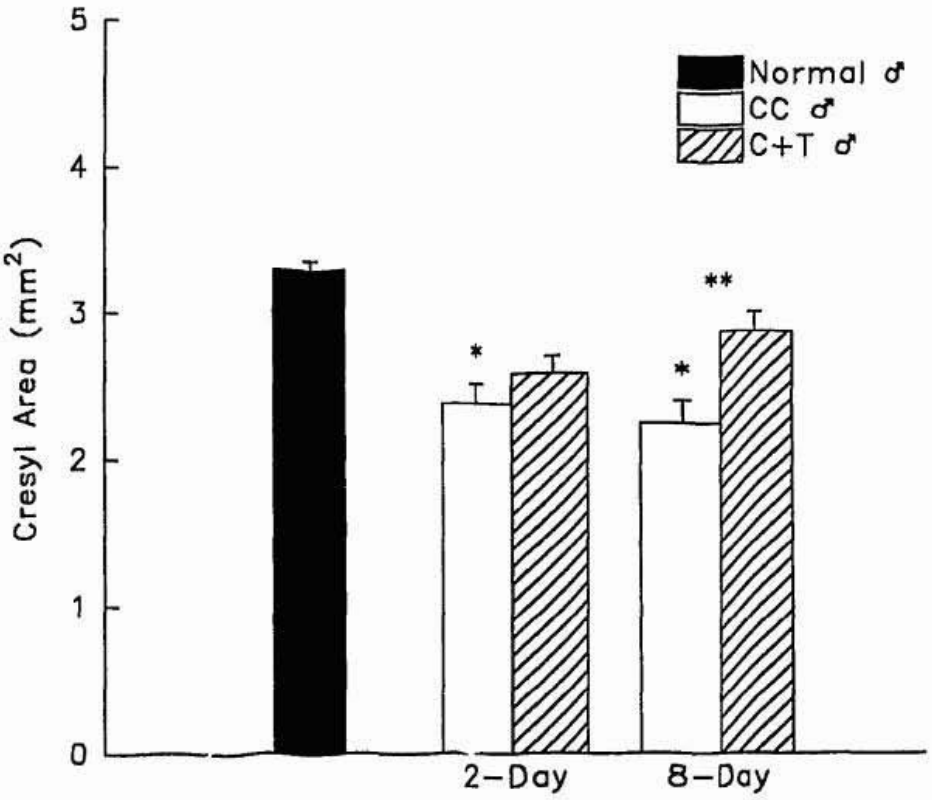

Normal ♂
CC ♂
C+T ♂
Cresyl Area (mm^2)
0
1
2
3
4
5
*
*
**
2-Day
8-Day

Figure 14. Matched coronal sections showing the pattern of GAPir-staining in the region of MePD of a male and a diestrous female. Sections are arranged in a rostral to caudal order (A-E, F-J). Staining is dense in both animals with the distinctiveness of the staining declining at more rostral levels. Primary antibody dilution was 1:2000. The scale bar in A is 1 mm. Abbreviations: PD, medial nucleus of the amygdala, posterodorsal.

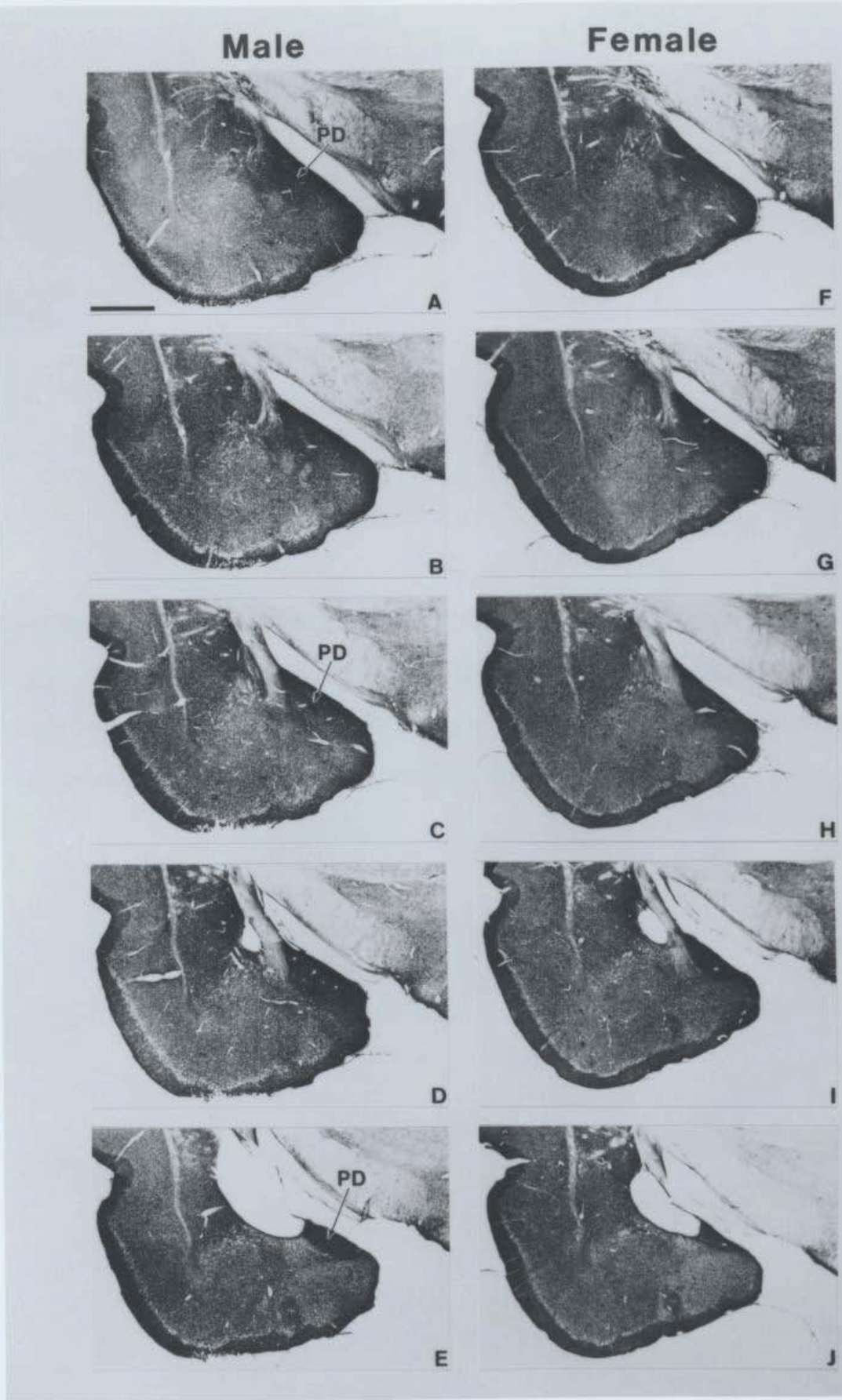

Male
Female
PD
PD
PD
A
B
C
D
E
F
G
H
I
J

Figure 15. The overall MePD/whole section ROD ratio of GAP-43 and tau-1 immunoreactivity for normal males and diestrous females. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat.

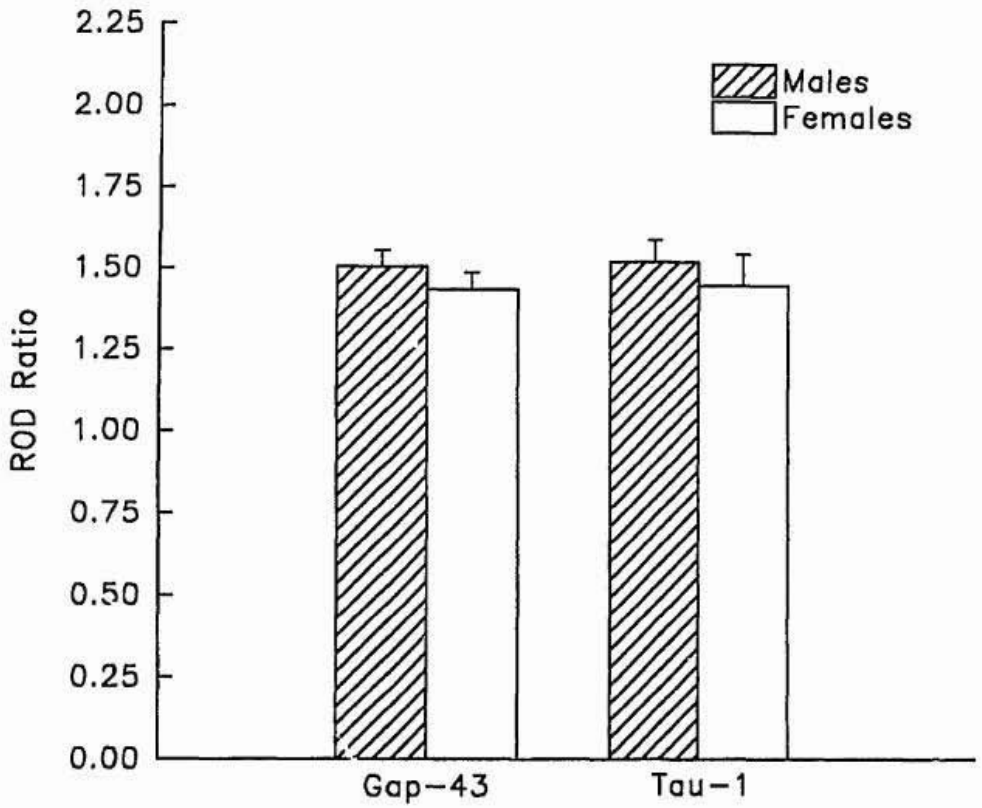

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ROD Ratio
Males
Females
Gap−43
Tau−1

Figure 16. Matched coronal sections showing the pattern of tauir-staining in the region of MePD of a male and a diestrous female. Sections are arranged in a rostral to caudal order (A-E, F-J). Like the GAPir-staining, tauir-staining is dense in both animals with the distinctiveness of the staining declining at more rostral levels. Primary antibody dilution was 1:6000. The scale bar in A is 1 mm. Abbreviations: PD, medial nucleus of the amygdala, posterodorsal.

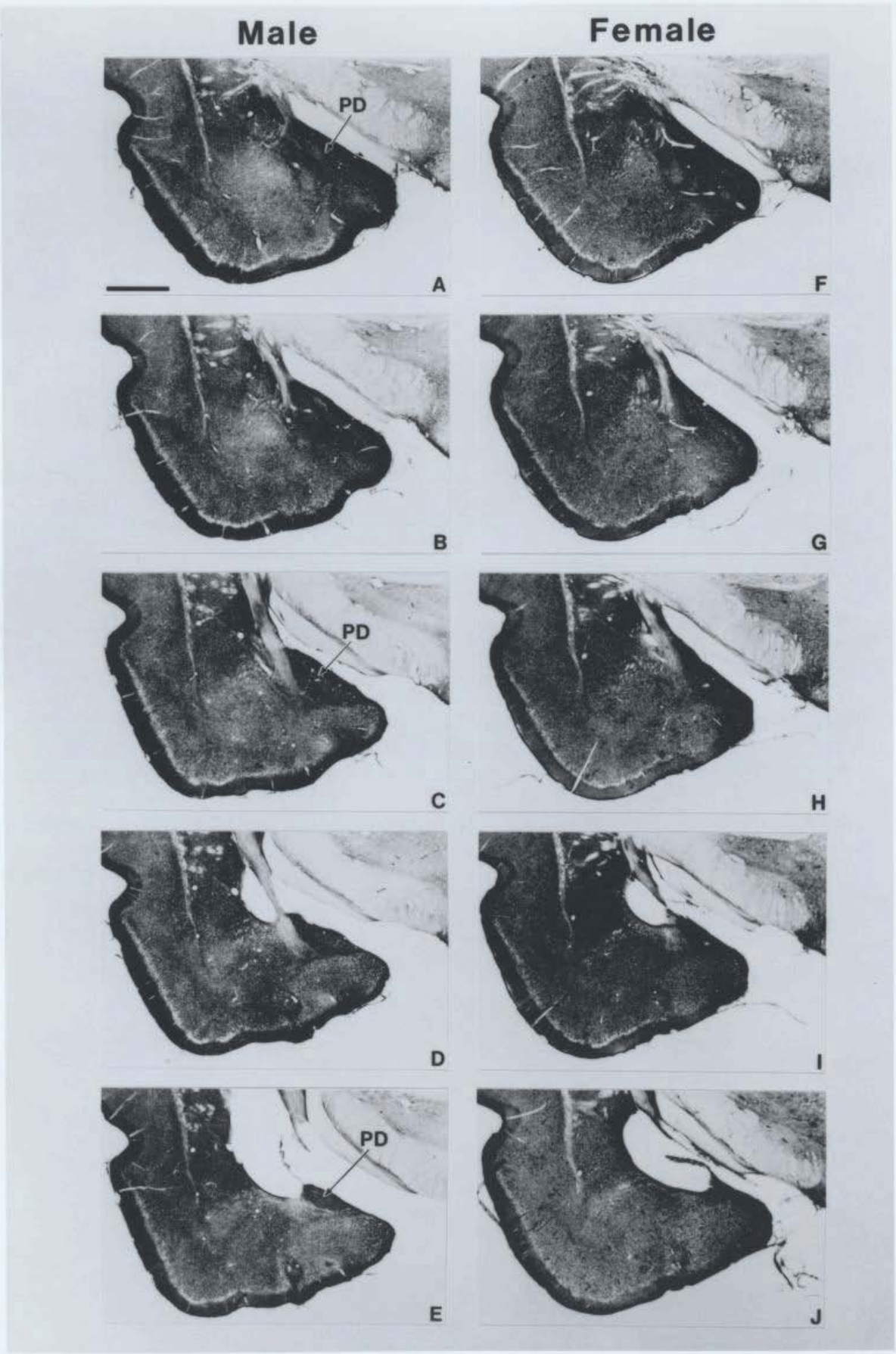

Male
Female
PD
PD
PD
A
B
C
D
E
F
G
H
I
J

Figure 17. The overall raw ROD and overall ROD ratio (MePD/whole section) of GAP-43 and tau-1 immunoreactivity for MePD in the left and right hemisphere. Data were obtained from normal males and diestrous females and are hemispheric means (± standard error of the mean). A single asterisk above the bars indicates that right significantly differs from the left hemisphere at $p \leq 0.05$. A double asterisk above the bars indicates that right significantly differs from the left hemisphere at $p \leq 0.01$.

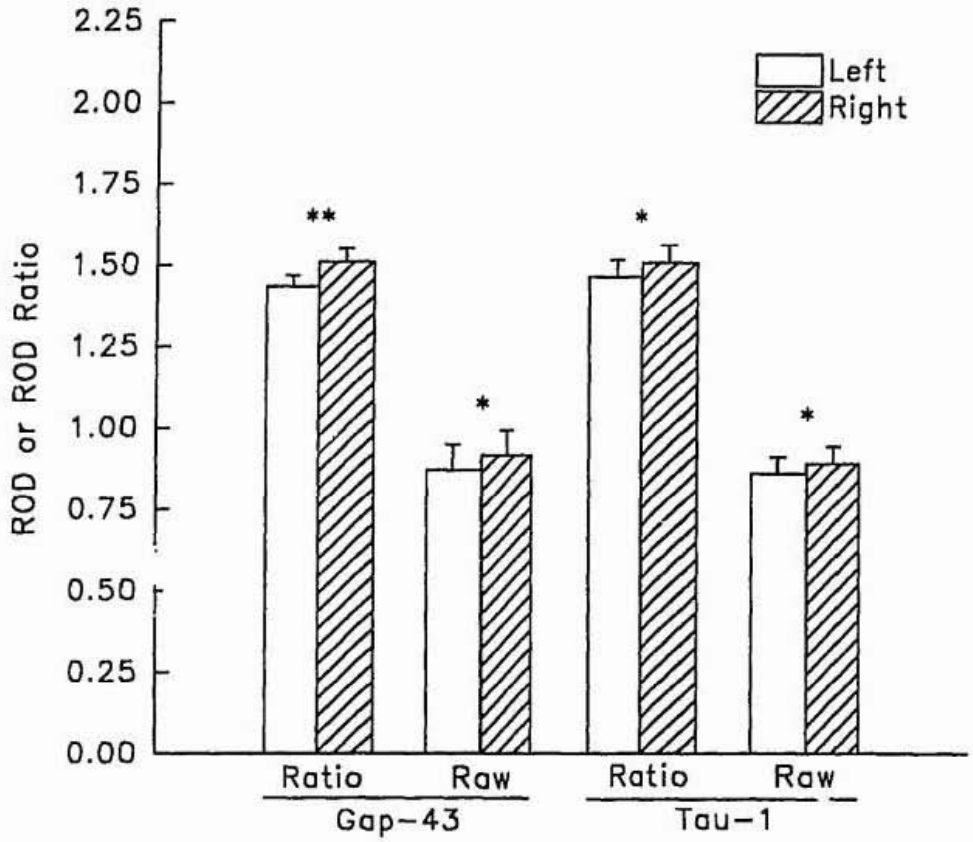

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ROD or ROD Ratio
Left
Right
**
*
*
*
Ratio
Raw
Ratio
Raw
Gap-43
Tau-1

Figure 18. The cortex/whole section ROD ratio of GAP-43 and tau-1 immunoreactivity for normal males and diestrous females. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat.

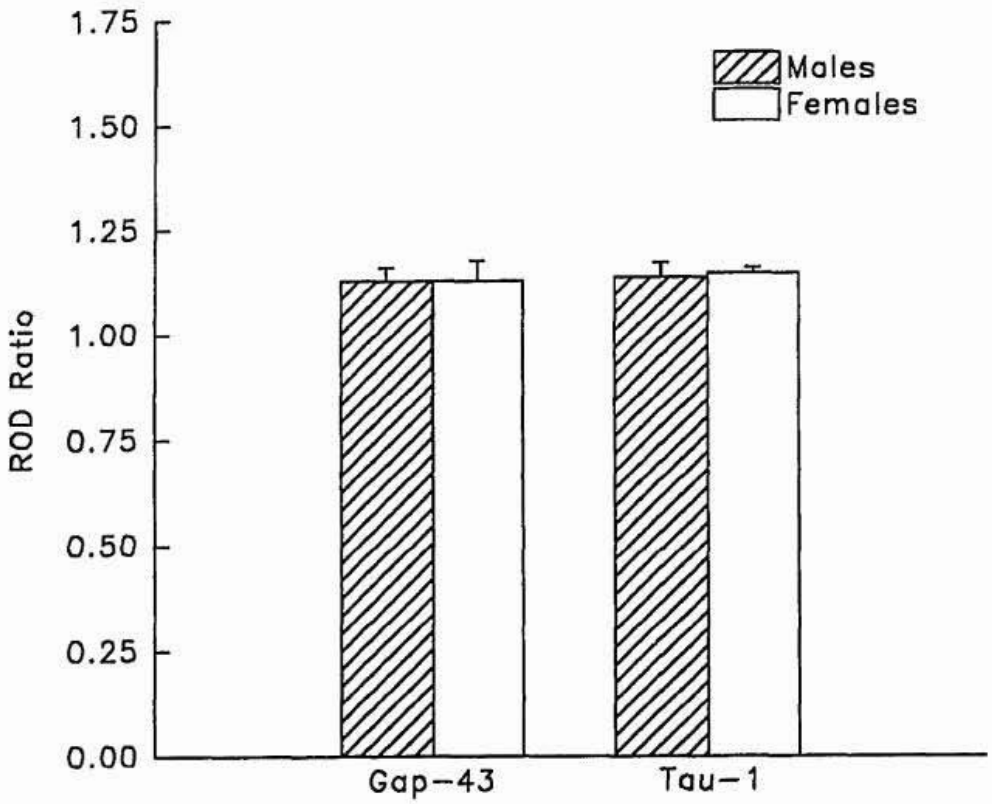

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ROD Ratio
Males
Females
Gap−43
Tau−1

Figure 19. The raw ROD and ROD ratio (cortex/whole section) of GAP-43 and tau-1 immunoreactivity for the cortex in the left and right hemisphere. *Data were obtained from normal* males and diestrous females and are hemispheric means (± standard error of the mean).

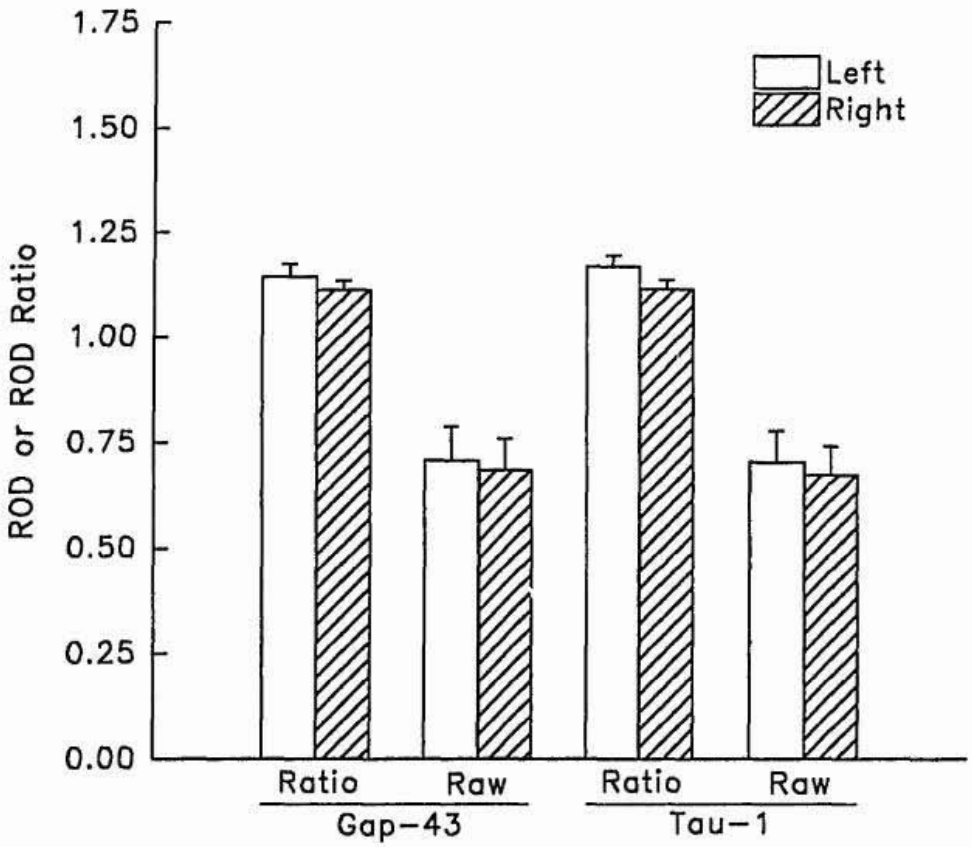
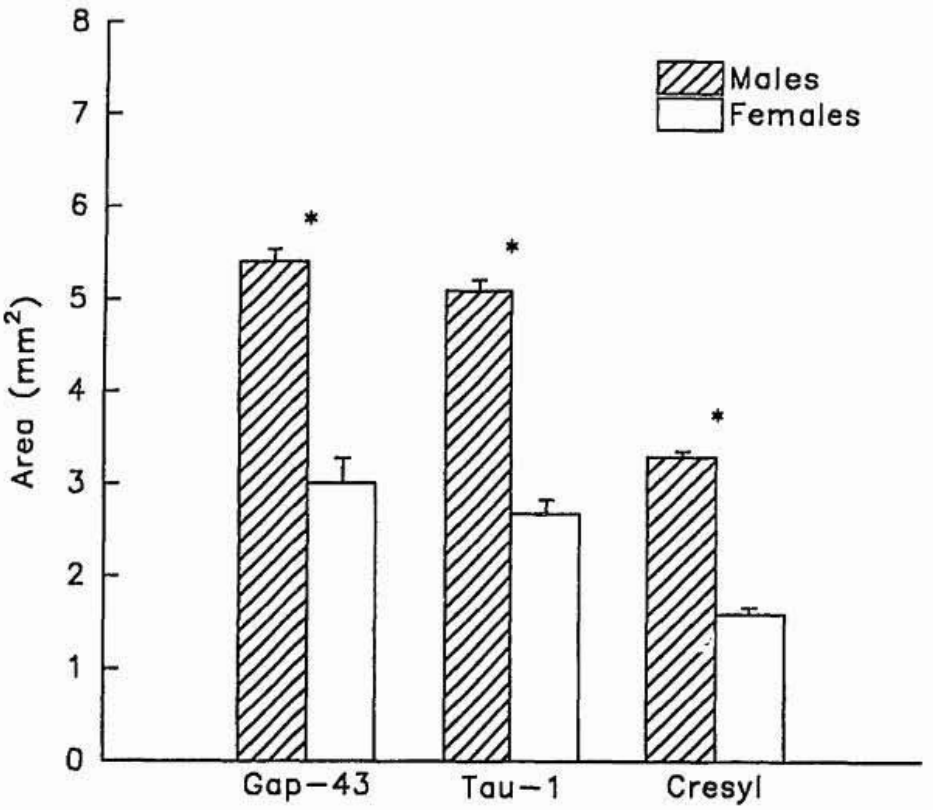

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
ROD or ROD Ratio
Left
Right
Ratio
Raw
Ratio
Raw
Gap-43
Tau-1

Figure 20. The area (mm^2) of GAP-43 and tau-1 immunoreactive staining in the region of MePD and the cell group MePD for normal males and diestrous females. Data are group means (± standard error of the mean) of the average of both hemispheres in each rat. An asterisk above the bars indicates male > female at $p \leq 0.001$.


8
7
6
5
4
3
2
1
0
Area (mm²)
Males
Females
*
*
*
Gap−43
Tau−1
Cresyl