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THE EFFECTS OF ESTROGEN ON DORSAL ROOT GANGLION NEURONS

by

Sheila A. Scoville
B.S. 1969, University of Wisconsin, La Crosse, Wisconsin
M.S. 1971, The Florida State University, Tallahassee, Florida

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Approved by:

Francis J. Liuzzi, Ph.D. (Director)

David E. Scott, Ph.D. (Member)

Charles W. Morgan, Ph.D. (Member)

Keith A. Carson, Ph.D. (Member)

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ABSTRACT

EFFECTS OF ESTROGEN ON DORSAL ROOT GANGLION NEURONS

Sheila A. Scoville
Eastern Virginia Medical School and Old Dominion University, 1997
Director: Francis J. Liuzzi, Ph. D.

Skin sensation is mediated by dorsal root ganglion (DRG) neurons. Data indicates that skin sensitivity in female rats is estrogen-dependent. Some DRG neurons have estrogen receptors (ERs) which are regulated by estrogen. In these cells, nerve growth factor (NGF) and estrogen receptors colocalize. Regulation of NGF receptors and neuronal sensitivity to NGF may allow estrogen to regulate NGF-dependent genes. The goals of the present study were to determine which DRG neurons express the ER gene and to analyze the effects of long-term estrogen administration on the interrelated expression of tyrosine kinase A (trkA), preprotachykinin (PPT), and 68kD neurofilament (NF) genes and to compare warm thermal withdrawal latencies in these animals. The hypothesis tested by the current study was that estrogen upregulates trkA and by doing so, increases the sensitivity of NGF-receptive neurons to the growth factor.

Three groups of ovariectomized (OVX) Sprague-Dawley rats were used: OVX (no replacement), 1X (low dose) and 10X (high dose). The replacement groups received daily Premarin injections. Rats from each group were killed at 56 or 90 days and their lumbar DRGs harvested.

ER mRNA localization was done by *in situ* hybridization. Results showed that ER mRNA was of low abundance, but was evident in smaller neurons. Quantification revealed that both trkA and PPT mRNA was downregulated in both estrogen

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replacement groups at both time points when compared to the OVX group. TrkA mRNA was localized predominantly in small DRG neurons. PPT mRNA was restricted to small neurons. Estrogen treatment slowed warm thermal plantar withdrawal, but not significantly. Neurofilament mRNA levels were dramatically increased by 56 days of estrogen treatment in a dose-dependent manner in all size populations of DRG neurons. Ninety days of estrogen treatment had a similar effect.

These data indicate that estrogen regulates DRG neuronal gene expression. An overlapping population of DRG neurons express the ER, trkA and PPT genes. Estrogen may regulate the trkA and PPT genes by altering NGF sensitivity. Since the ER gene does not appear to be expressed by all DRG neurons, the effect of estrogen on neurofilament gene expression must involve alternative mechanisms.

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To my husband, Marty, my daughter, Brooke, and my mother for their love, support and understanding

In memory of R. Bruce Masterton, 1931-1996.

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INTRODUCTION

BACKGROUND

According to US Census figures, by the year 2000 around 18.5 million women will be peri-menopausal and another 30 million women will be in their post-menopausal years (U.S. Census Bureau, 1995). Considering that the median age for menopause is around 50 years of age, and that the life expectancy for women is now 78, one can expect that more than a third of the female life span will be spent in the post-menopausal state (Judd, et al., 1983), that is, without cyclical naturally circulating estrogen. Although the role of estrogen in reproduction is comparatively well understood, its broader effects on non-reproductive organs are only now being discovered.

Effects of Estrogen Throughout the Body

The presence of estrogen receptors in the ovaries, endometrium, vaginal epithelium, pituitary, urinary tract and skin (Utian, 1989) is well documented. Additionally, estrogen receptors have been shown in vascular epithelium (Buonassisi and Colburn, 1980) and in liver (Wrange, et al., 1980).

Other organs, such as heart and bone, are affected by estrogen, although specific receptors have not yet been identified in these organs (Utian, 1989). Intermediate mechanisms affecting serum lipids are believed to be the mode of action by which the heart is affected (Hazzard, 1989). Bone may also be affected by an intermediate mechanism. In this case, estrogen is believed to regulate interleukin-6 which supresses the action of osteoclasts (Jilka, et al, 1992).

The journal model used for this dissertation was the Journal of Neuroscience.

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Early studies of the CNS have shown estrogen receptive neurons within the hypothalamus and limbic system (Pfaff and Keiner, 1973), regions typically associated with sexual behaviors. However, more recent studies suggest that estrogen receptive neurons are more widely distributed in the CNS, in regions not primarily involved in reproductive behavior. Not only are there ERs in the hypothalamus, they are also expressed by some neurons within the hippocampus, (Bettini, et al., 1992) cerebral cortex and intralaminar nuclei of the thalamus (Simerly, et al, 1990). In a recent report, Toran-Allerand and her colleagues (1992) have demonstrated estrogen receptive neurons in the basal forebrains of neonatal female rats and adult female mice. Nuclei containing these neurons include the medial septum, the vertical and the horizontal limbs of the diagonal band, the substantia inominata, and the nucleus basalis of Meynert. More interestingly, the estrogen receptors in these regions co-localize with low-affinity NGF receptors (p75). These investigators concluded that their data suggest that estrogen and NGF might act synergistically upon these neurons (Toran-Allerand, 1992).

To determine whether ER expression is a general feature of NGF receptive neurons, Toran-Allerand and her colleagues (Sohrabji, et al., 1994) extended their studies to the dorsal root ganglion (DRG) neurons of adult female rats. They reported that all DRG neurons of the female rat express ER mRNA. Another laboratory, employing different investigative methods, (Sarajari et al, 1995, and Sarajari and Oblinger, 1996) has reported finding estrogen receptors only within smaller DRG neurons.

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The Effects of Aging on Somatic Sensation

Menopause, the cessation of estrogen production by the ovaries, is characteristic of normal female aging. Moreover, there is ample evidence showing an age-related decline in peripheral nervous system function. However, relatively little attention has been paid to gender differences in this decline. The ability to make light touch discriminations, as measured by von Frey hairs, is decreased in elderly populations on the palms and thumbs and on the cornea (Kenshalo, 1979). Gender was not a parameter in these studies. Falls and consequent fractures have a high morbidity in the elderly. They may, in part, result from a loss or decline in proprioception. Increased sway, indicative of proprioceptive disturbance, is more severe in elderly females than in elderly males (Overstall, et al., 1977).

It is well established that pain and thermal thresholds increase dramatically with age (Tucker, et al., 1989), yet the hormonal influences on these changes have not been explored. As early as 1931, Critchley described in a clinical study, an age-related loss of vibratory sensitivity in the feet, as well as, loss of ankle jerk. Dyck and his colleagues (1993), more recently, showed a significant increase in the vibratory detection thresholds (VDTs) for the index finger with age. They further state that there are significant differences with age and a significant gender difference. Females of all ages have lower VDTs than age-matched males. Whether hormonal status has any role in the decline of vibratory sense was beyond the scope of these studies.

Vibratory sense is mediated by large diameter, myelinated Aβ axons (Martin, 1985). The diminution in vibratory sense and reflexes in humans may, in part, be related to slowing of nerve conduction velocities (NCVs) with age (Wagman and Lesse, 1952).

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NCVs are determined by two axonal characteristics, axon diameter and degree of myelinization. Hoffman and his colleagues (1987) have provided convincing evidence that these interrelated features are determined by the density of neurofilaments within axons. This density is determined by neurofilament gene expression and synthesis. (A more extensive review of the neurofilament literature is included towards the end of the introduction.) Anything that decreases neurofilament gene expression would decrease axon diameter and thereby decrease conduction velocity and myelin thickness.

Sharma and his colleagues (1980) identified some reduction in fiber size of the rat tibial and medial plantar nerves in male rats from 2 through 24 months of age. Since 24 months was the extent of this study, it is not known what further reduction in axon diameter might have been observed after this time. Similar age-dependent reductions in axon diameters have been reported for humans. Tohgi and his colleagues (1977) reported that the number of nerve fibers in the sural nerve decrease with age, with a greater change in larger diameter axons. While one interpretation would be that there is a reduction of the number of fibers, it might also represent a gradual reduction of the axon diameters as well as a loss of DRG neurons. As with many other studies of age-related changes in peripheral nerve, gender differences were not examined nor was hormonal status considered.

While both aging men and women suffer these peripheral nerve sensory deficits, little research has been done to specifically address the sensory changes brought on by menopause. Several problems come to mind. Many investigators specifically choose male animals for their subjects in order to remove the experimental variable of changing hormonal levels due to the menstrual or estrous cycle. As Berkley points out in her 1992

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paper, in a survey of prominent neuroscience journals, 45% of articles in recent peer reviewed journals failed to report the gender of the experimental subjects. Even less likely to have been studied are the differences between cycling and non-cycling females. The research reported in the following studies provides intriguing evidence for a link between menopause and declining somatosensory function in aging females.

Behavioral Evidence of Estrogen's Effect on Somatosensory Function

It is possible to trace a few threads of evidence from the past. In a human experiment conducted 65 years ago, women were measured on their ability to make two-point tactile discriminations during presumed different times of their menstrual cycle (Herren, 1933). Although this study documented sensitivity changes due to "the follicular hormone", it attributed the mechanism of change to upper motor neuron inhibition mediated by the sympathetic nervous system. This study was conducted before estrogen had been identified as a discrete entity.

One of the first well-documented studies implicating the connection between estrogen and sensation was reported 35 years ago. This study dealt with hormonal effects on the brood patch of the female canary (Hinde and Steele, 1964). Avian nesting behavior is associated with changes to a patch of skin on the breast. This region becomes more sensitive and defeathers in advance of egg-laying. It was found that exogenous administration of estrogen alone could induce an increase in sensitivity, while progesterone was found to be exclusively responsible for defeathering.

From 1972-1980, renewed interest in the topic produced three studies which demonstrated an increase in the cutaneous sensitivity of female rats which corresponded

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to reproductive accessibility by the males. In the first of the studies, genital skin was shown to be more sensitive at proestrous, the phase of the rat reproductive cycle with the highest circulating estrogen (Komisurak, 1972). Increased skin sensitivity in response to changes in circulating estrogen proved to be a much more generalized phenomenon than had been suggested by the earlier investigation. Skin from other portions of the rat also showed increased sensitivity during proestrus (Kow and Pfaff, 1979). A third study added several other important pieces of information. In that study by Bereiter and Barker (1980), receptive fields of the skin of rats were measured. At proestrus, electrophysiological measurements showed that the receptive fields of skin surface expanded an average of 22%. Furthermore, by cutting the dorsal roots, their procedure allowed them to rule out the possibility of CNS involvement, thus assuring that the phenomenon is a peripheral one.

Since the time of those studies, a great deal has been learned about DRG neurons, their gene expression and function. This new knowledge may provide the basis for an understanding of estrogen's role in modulating cutaneous sensation. Morphologists have traditionally described the DRG neurons as either "small dark" or "large light" (Lawson, 1979) and describe their axons by diameter and degree of myelination (Martin, 1985). More recent schemes describe DRG neurons in terms of their transmitters or neuropeptide content. For example, subpopulations of DRG neurons have been identified immuno-histochemically by their calcitonin gene-related peptide (CGRP) or Substance P content (Verge et al 1989b). Very recently, Wright and Snider (1995) categorized DRG neurons by the type of neurotrophin receptors they expressed. Still others have subdivided and characterized these sensory neurons by their

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sensory receptor type, such as the Pacinian corpuscle or the free nerve ending. Physiologists, based on conduction velocities and patterns of electrical activity, have categorized these cells into 22 types (Snider, personal communication).

From a behavioral point of view, somatic sensations are described as pain, thermal sensations of heat and cold, light touch, discriminative touch, vibration and proprioception. The field of psychophysics seeks to document sensations and changes in these sensations through batteries of sensory testing. The von Frey test, for example, documents tactile sensitivity by applying a series of hairs in graded diameters.

All of these methods of characterizing DRG sensory neurons have begun to provide a broad understanding of primary sensory neurons and the sensations subserved by them. A new challenge, however, is to begin to understand the role of estrogen, and potentially other hormones, in the regulation of DRG neuronal gene expression, function and, ultimately, cutaneous sensation.

Regulation of DRG Neuronal Gene Expression and Function by NGF

Since the characterization of NGF by Levi-Montalcini (reviewed by Bendiner, 1992), scientists have sought to understand the role of the neurotrophic molecule and its relatives in neuronal development, maintenance and function. To date, NGF is known to be important developmentally for the survival of neural crest-derived neurons. Furthermore, it is known to promote neurite outgrowth in tissue culture (Grafstein and McQuarrie, 1978) and support the survival of axotomized sensory neurons in the adult (Rich, et al., 1987). NGF regulates the production of neurofilaments in NGF-dependent DRG neurons (Verge, et al., 1990) as well as the neuropeptides, CGRP and substance P

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in those cells (Lindsay and Harmar, 1989). Additionally, the receptors for NGF, high affinity (trkA) and low affinity (p75) have been characterized (reviewed by Chao, 1992).

About 40% of DRG neurons bind NGF and express the high affinity NGF (trkA) receptor gene (Verge, et al., 1989a). These neurons also express the low affinity NGF receptor (p75). However, other neurons in the DRG express the p75 gene only and fail to bind NGF (Verge, et al., 1989a). These other neurons express trkB and trkC (Wright and Snider, 1995) and bind other members of the neurotrophin family, BDNF and NT3 (reviewed by Chao, 1992).

A study using transections of the sciatic nerve and infusions of exogenous NGF has shown that NGF regulates p75 and trkA in those neurons that bind NGF (Verge, et al, 1992). Cutting the sciatic nerve removes target-derived NGF and causes a dramatic decline in DRG neuronal high affinity NGF receptor binding as well as in trkA and p75 mRNA. Intrathecal infusion of exogenous NGF restores receptor binding and returns the receptor mRNA levels toward normal.

Not only does NGF regulate its receptors, it has been shown to regulate other genes in those neurons that bind NGF with high affinity. There is, for example, a well established relationship between NGF and neuropeptide gene expression in DRG neurons. An *in vitro* study by Lindsay and Harmar (1989) showed that NGF regulates calcitonin gene-related peptide (CGRP) and preprotachykinin (PPT) gene expression in adult DRG neurons. The PPT gene encodes the precursor to substance P. This undecapeptide is a putative nociceptive neurotransmitter (Duggan, et al., 1987; 1988) and its depletion results in thermal analgesia (Yaksh, et al., 1979). A later, *in vivo* study

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showed that the decline in substance P following sciatic nerve transection is reversed by infusion of NGF to the proximal cut end of the nerve (Wong and Oblinger, 1987).

In the same study, Wong and Oblinger (1987) reported that exogenous NGF did not reverse the decline in lumbar DRG neurofilament gene expression that follows transection of the sciatic nerve. Contrary to this report, however, is substantive indirect, (Gold, et al., 1991) as well as, direct evidence that NGF regulates the synthesis of intermediate filaments in, at least, the NGF binding subpopulation of DRG neurons (Verge et al., 1990).

Neurofilaments are a major structural component of neurons and are particularly abundant in large diameter axons (Friede and Samorajski, 1970). These intermediate filaments are comprised of three protein subunits, which are encoded by separate genes (Julien et al, 1986). Neuronal size and axon diameter are proportional to the number of neurofilaments (Hoffman, et al., 1988). Paul Hoffman and his colleagues, based on substantive experimental evidence, have argued that neurofilaments are essential to the maintenance of axon diameter and thereby, govern conduction velocity (Hoffman, et al, 1984, 1985, 1987). Moreover, since myelin thickness is correlated with axon diameter, it follows logically that axonal neurofilament content determines myelin thickness.

Axotomy causes neurofilament synthesis to decline (Oblinger and Lasek, 1988). Reconnection of axons with their targets restores neurofilament gene expression and synthesis (Hoffman, 1987) suggesting that target-derived growth factors, such as NGF, regulate neurofilament gene expression. In cultured rat pheochromocytoma cells, NGF upregulates all three subunits (Lindenbaum, 1988). Moreover, NGF deprivation *in vivo* by antibody infusion causes sensory axonal atrophy (Matheson, 1989). This atrophy has

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been tied to decreased neurofilament synthesis (Gold, et al., 1991). Verge and colleagues (1990) found that after axotomy, neurofilament synthesis was significantly decreased in large DRG neurons. Administration of NGF to the DRG somata restored neurofilament production in only that small proportion of these large neurons that bind NGF with high affinity. They concluded that NGF regulates neurofilament gene expression in NGF-dependent DRG neurons through some intermediate mechanism. In the 60% of the DRG neurons that are not NGF-dependent, neurofilament synthesis must be regulated by other target-derived factors, perhaps, the other neurotrophins.

Estrogen-NGF Interaction in the Regulation of DRG Neuronal Gene Expression and Function

A recent study by Sohrabji and her colleagues (1994) shows that estrogen receptors co-localizes with neurotrophin receptors in DRGs. Moreover, circulating estrogen levels regulate DRG neuronal estrogen receptor gene expression. For example, at proestrus, when estrogen levels are highest, estrogen receptor mRNA is at its lowest. In ovariectomized (OVX; estrogen deficient) rats, estrogen receptor mRNA levels are highest. Additionally, estrogen replacement to proestrus levels restores estrogen receptor mRNA levels to their lowest levels. Thus, as in other systems, estrogen regulates its receptor.

More importantly, there is evidence that estrogen regulates DRG trkA and p75 gene expression (Sohrabji, et al., 1994). During proestrus, with the highest estrogen levels, trkA is up-regulated. Similarly, p75 is up-regulated during proestrus. Sohrabji and her colleagues (1994) found that OVX down-regulates both of these NGF receptor

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genes. Unfortunately, it is not entirely clear how to interpret the effects of replacement estrogen. They found that estrogen replacement transiently decreased p75 mRNA levels at four hours post-replacement, while at 52 hours there was no difference in p75 mRNA levels in DRGs of OVX rats and OVX rats receiving estrogen. These data suggest that estrogen, through its action on the NGF receptors, may alter the sensitivity of DRG neurons to NGF. In this way, estrogen might act in synergy with NGF to regulate genes necessary for primary sensory neuronal function.

Recent evidence supports the idea of such a synergistic interaction between estrogen and NGF in the regulation of CGRP and substance P. Sarajari and her colleagues (1995), using immunohistochemistry, reported that there were high levels of ER immunoreactivity in only the small DRG neurons, but not the larger cells. This finding is somewhat surprising in light of Sohrabji and her colleagues (1994) data showing ER mRNA in all large and small DRG neurons. The difference in these findings might be explained by the difference in experimental approaches used by the two groups. The *in situ* hybridization used by Sohrabji and colleagues (1994) may be a more sensitive method than the immunohistochemical method used by Sarajari and colleagues (1995). This controversial issue, i.e. which DRG neurons express the ER gene is examined in the current research.

Regardless, Sarajari and co-workers (1995) were able to co-localize CGRP, substance P and ER in the smaller DRG neurons. They showed further that CGRP immunoreactivity was increased in OVX rats receiving estrogen compared to those not receiving estrogen replacement. Furthermore, they show that this pattern is present at very early post-natal stages (Sarajari, et al., 1996). Contrary, however, to what might

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be expected based on Lindsay's and Harmar's (1989) report showing up-regulation of both CGRP and PPT genes by NGF in cultured adult DRG neurons, Sarajari and her colleagues (1995) reported a decrease in substance P immunoreactivity in estrogen-treated OVX rats. If estrogen does indeed up-regulate the DRG neuronal NGF receptors, as reported by Sohrabji and her colleagues (1994), and thereby enhances the sensitivity of those neurons to NGF, it becomes difficult to understand the differential regulation of the two neuropeptides by estrogen reported by Sarajari and her colleagues (1995). The answer may depend on an understanding of the time course of the regulatory events.

By contrast, if, as Sohrabji and her colleagues (1994) have shown, trkA and p75 are up-regulated by estrogen, one might also expect substance P to be up-regulated. An up-regulation of substance P would, in turn, be expected to decrease pain and temperature thresholds. Evidence supporting this expectation was recently provided in a study by Frye and her colleagues (1992). They used the tailflick latency to a focused light beam as a measurement of thermally induced nociception to examine the effects of estrus cycle on pain and temperature. They found significant differences in sensitivity at different phases of the cycle. During proestrus (highest circulating levels of estrogen) they found the lowest tailflick latencies, while during metestrus (lowest circulating estrogen levels), the tailflick latencies were at their highest. In the current study the interrelationships among chronic estrogen administration, trkA gene expression, PPT gene expression and warm thermal withdrawal latencies are determined.

Not only does NGF regulate substance P gene expression, it also regulates Aδ and C fiber afferent collateralization in the skin (Diamond, et al., 1992). Thus, elevated

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circulating estrogen levels should, through their effects on DRG NGF receptor genes, cause expansion of the receptive fields for these fibers. This may be the case. Moreover, neurofilament gene expression and synthesis may be essential for maintenance of this collateralization.

Nearly 25 years ago, it was suggested that hormones acted peripherally to increase skin sensitivity (Komisaruk, et al., 1972: Kow and Pfaff, 1973). Three groups of investigators Bereiter and Barker (1980), Komisaruk and colleagues (1972), and Kow and Pfaff (1973) have all found that estrogen replacement increased cutaneous receptive fields in OVX rats. The mean receptive field size of the pudendal nerve in treated rats was 22% larger than that in untreated OVX rats (Kow and Pfaff, 1973). Bereiter and Barker, (1975) expanded our understanding of this phenomenon by demonstrating receptive field enlargement in skin of the facial region in OVX rats which were given replacement estrogen, in addition to the genital skin areas which had been studied by these earlier investigators.

Estrogen and the Estrogen Receptor

Because the work in this dissertation is focused on the effects of estrogen on one of its recently discovered target tissues, the DRG, not on the intricacies of genetic activation, little attention will be focused at the molecular level, as is true of the issues concerning gene activation. In this body of work, molecular biology is used as a tool for achieving the research aims of this study. Nonetheless, a basic description and an understanding of the current thinking on these mechanisms is necessary in order to

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interpret some of the apparently disparate results of previous research and is included at this time.

There are three categories of steroid hormones: the adrenal steroids, vitamin D3 and the sex steroids. Estrogen as well as progesterone and testosterone comprise the sex hormones. These and other steroid hormones are derived from cholesterol. Cholesterol is one of the most basic and one of the phylogenetically oldest molecules of life since it is an important component of the cell membrane (Evans, 1988). The ovary is responsible for the production of circulating estrogen in the adult female. The other major sex hormones produced by the ovaries are progesterone and the androgens which are comprised mostly of testosterone and androstenedione. The circulating levels of these hormones vary greatly depending on the stage of the menstrual cycle of the adult human female or the corresponding estrous cycle of the rat. The interactions of these sex steroids are extremely important but are well beyond the scope of the body of information presented in this paper.

The two major components of estrogen are estradiol and estrone. Their molecular derivation, as well as that of testosterone, is depicted in Figure 1 (modified from Stryer, 1987). These two estrogens can be converted into 40 known metabolic products, although most of these are intermediate breakdown products destined for excretion (Erickson and Scheiber, 1995). A few of these metabolites are biologically active. If the estradiol or estrone molecule becomes hydroxilated at the 2 or 4 position, they become known as catechol estrogens. Although the significance of these molecules is not understood, one study finds relatively high concentrations of these estrogen forms in the pituitary and hypothalamus (Paul and Axelrod, 1977). Also, the molecular

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configuration (conformation) of estradiol (17-beta estradiol vs. 17-alpha estradiol) may prove to be important for biological activity.

How do hormones have such diverse effects within the body? In order for any circulating hormone to have an effect upon a particular target organ it binds to receptors of the cells of the various tissues. Until a few years ago, a discussion of the ER would have been quite simple. A newly emerging picture, however, is much more complicated. The ER, along with other steroid hormone receptors, glucocorticoid and mineralocorticoid receptors and the sterol vitamin D receptor comprise a "superfamily" of receptors. And although the ligands are dissimilar, the receptors for retinoic acid (Vitamin A) also show considerable homology to the hormone receptors. The first formulation of the nature of the ER occurred in the early 1970's with the use of radioactive ligands (Evans, 1988). It had been determined earlier that the action of estrogen was not due to local metabolism within the tissue, since when estrogen was administered at physiological doses, it was retained within the target tissue (Jensen, 1960). This original observation led to the search for hormone receptors in general, and specifically estrogen receptors.

A widely accepted view arose that ERs resided in the cytosol and, when bound by hormone migrated to the nucleus where the complex bound to the DNA strand. The now, more widely accepted view was presented in back-to-back Nature papers by King and Greene (1984) and Gorski and colleagues (Welshons et al., 1984). The first paper localized occupied and unoccupied ERs within the nuclei of cells. This evidence suggested that since unoccupied receptors can be found in the nucleus, cytosolic receptor binding was not a prerequisite for estrogen's action. The second paper, partially

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reinterpreting their earlier results, presented evidence that the presence of estrogen receptors in the cytosol was largely or wholly artefactual due to methodology. In summary, although the idea of translocation of estrogen bound to its receptor from the cytosol to the nucleus has a few proponents, that theory has been discarded. Current techniques are able to demonstrate few or no estrogen receptors in the cytosol. Instead, they are believed to be confined to the nucleus. Some evidence exists that the site of new receptor synthesis is on the outer surface of the nuclear membrane and that the newly formed receptor moves to the interior of the nucleus at, or shortly after the time of synthesis (Tumohimaa, et al., 1988). Another variation of this idea is that the newly bound receptors, which are molecular monomers, bind with another and pass to the inner side of the nuclear envelope as a dimer (Carson-Jurica et al., 1990).

Another evolving theory, which is gaining support, has to do with the nature of the unbound receptor. Steroid receptors are now referred to as being *inactive* when not bound by specific ligands (Carson-Jurica et al, 1990). When hormones enter the cell, the receptors become activated or transformed. The inactive state of the ER is now believed to be dependent upon the receptor's association with one or more intracellular proteins. Currently, heat shock proteins, especially HSP 90, are believed to be the particular proteins which competitively bind to the DNA binding location of the receptor complex (Ing and O'Malley, 1995). Additional evidence indicates that this binding apparently works by more than simple competition for binding sites since experimental removal of the HSPs from unbound receptors does not activate them (Meshinchi, 1988). A conceptualization of theorized interactions of the estrogen receptor with its ligand is presented in Figure 2 (modified from Carson-Jurica, 1990).

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Regardless of whether or not a different receptor type is present in the cytosol or nucleus, an entire new category of non-traditional steroid receptor is beginning to be elucidated. Evidence for a membrane receptor comes predominantly from physiological experiments (Mermelstein, 1996). Although previous investigations have been accumulating evidence that identified neural areas with no known cytosolic or nuclear estrogen receptors, few have postulated the heretical mechanism of a hormone membrane receptor to explain results. Mermelstein and coworkers (1996) have presented evidence that estrogen may also act on a membrane calcium channel in CNS neurons.

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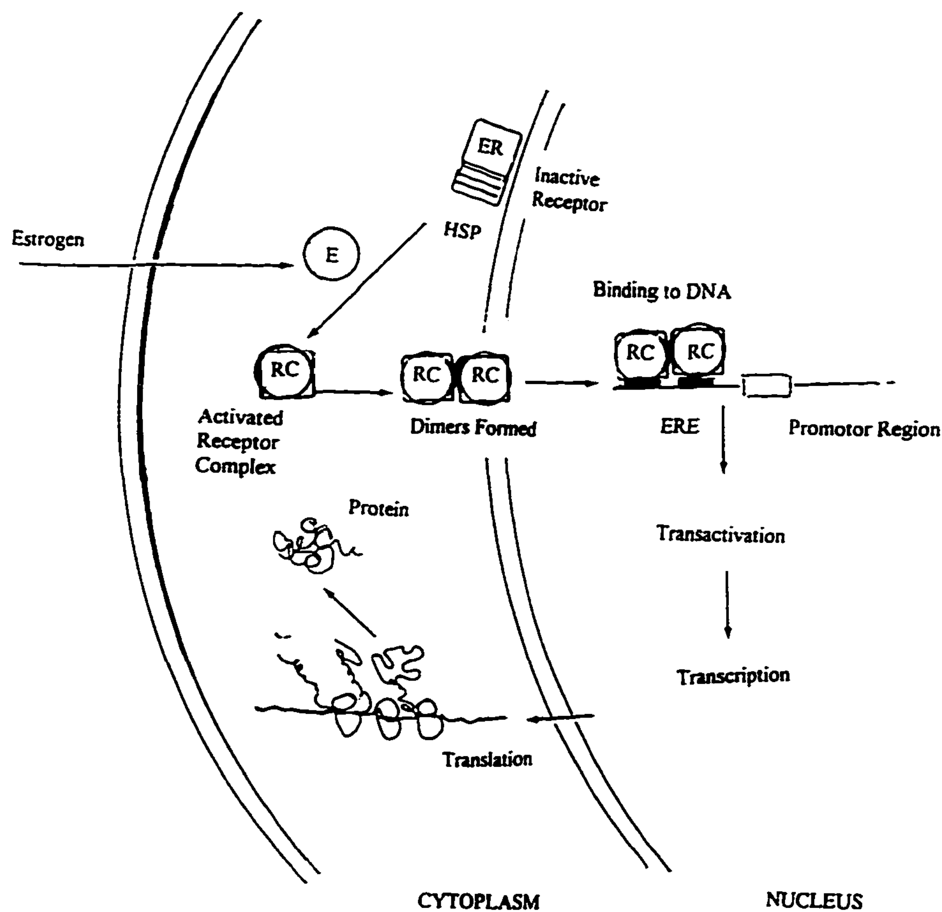

Figure 1. Depiction of the estrogen molecule, its molecular derivation and relationship to other steroid hormones. Note that both major components of estrogen, estrone and estradiol are shown.

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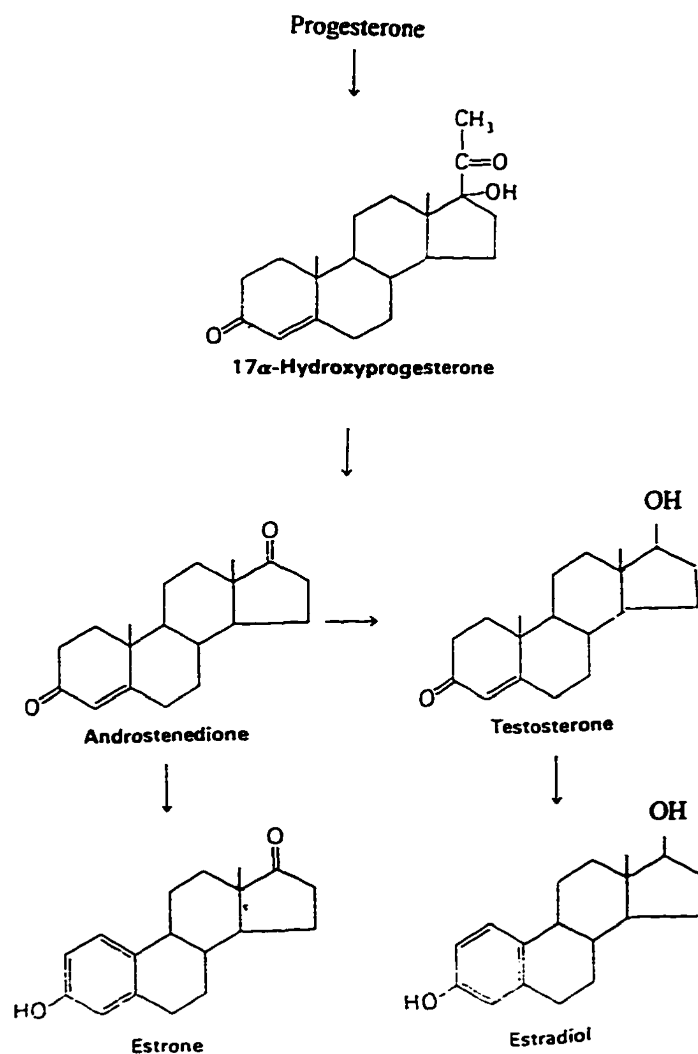

Figure 2. Schematic presentation of the estrogen receptor and how it is believed to act. The estrogen molecule (E) passes through the plasma membrane and binds to the inactive estrogen receptor (ER) which is positioned in the outer nuclear membrane. In its inactivated state the ER is bound by a heat shock protein (HSP). When the receptor is bound by the estrogen molecule, the heat shock protein drops off and the bound estrogen receptor binds to another ER complex (RC) and passes through the nuclear envelope as a dimer where it attaches to a site on the gene known as the estrogen response element (ERE). At this point the gene is activated and transcription commences. The resulting mRNA moves to the cytosol where translation occurs.

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GOALS OF THE CURRENT STUDY

The current research had two main goals. The first goal was to settle the controversial issue of whether all or only a subpopulation of DRG neurons express the estrogen receptor gene. To this end, *in situ* hybridization with an anti-sense RNA probe to the rat ER was used to localize ER mRNA in sections of lumbar DRGs.

The second, main objective of the current study was to analyze the interrelationships among long-term estrogen administration, trkA gene expression, PPT gene expression and warm thermal withdrawal latencies. Specifically, the hypothesis tested by the current study was that estrogen upregulates trkA and by doing so, increases the sensitivity of NGF-receptive neurons to the growth factor. Furthermore, if long term estrogen treatment increases the sensitivity of DRG neurons to NGF, the known NGF-dependent genes, PPT and 68kD neurofilament should, consequently, be upregulated.

To test this hypothesis, the effects of long-term estrogen treatment on high affinity NGF receptor (trkA) mRNA levels was examined using quantitative *in situ* hybridization with an anti-sense RNA probe to trkA. Additionally, similar methods were used to examine the effects of long-term estrogen on PPT and 68kD neurofilament mRNA levels. Finally, a behavioral correlate of changes in PPT gene expression was examined. The Hargreaves' method to measure withdrawal latencies to warm thermal stimuli in response to long-term estrogen treatment was done with a plantar test apparatus.

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METHODS

Animals and Experimental Groups

Forty-three adult female OVX Sprague-Dawley (Harlan Sprague Dawley) were used for this study. Animals were purchased, maintained and treated according to the Eastern Virginia Medical School Animal Care and Use Committee standards and the *NIH Guide for the Care and Use of Laboratory Animals* (approved protocols are on file in the Office of Research). The rats, weighing 200-300 grams at the commencement of the study, were randomly assigned to three groups two weeks post-ovariectomy.

Rats in the first group were ovariectomized with no estrogen replacement. This group is referred to as the OVX group. Bilateral ovariectomies were done at the Harlan facilities. Those in the second group were ovariectomized and received daily subcutaneous estrogen (Premarin, Wyeth-Ayerst) injections at a dose (0.022mg/kg) calculated to result in physiological serum estrogen titers (Washburn, et al., 1993) This group is referred to as 1X, the low dose group. The rats in the third group were ovariectomized and given replacement estrogen subcutaneously at 10 times the daily dose (0.22mg/kg) given to Group 2. This group is referred to as 10X, the high dose group.

Normal, age-matched female rats provided baseline uterine weights for assessment of the effects of estrogen treatments (see Figure 3 in Results). During the length of the study, all animals were housed in community cages with 3-4 animals per cage at the EVMS animal care facility. All subjects were given unlimited standard rat chow and water. The day-night cycle was maintained at 12 hours each of light and dark. Animals in the three groups were killed after either 56 or 90 days of estrogen treatment.

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Warm Thermal Perception Testing

The Hargreaves' method for testing warm thermal perception used the Plantar Test Apparatus (Ugo Basile Biological Research Apparatus, Co.). This apparatus used a light (heat) source capable of emitting a focused light beam. The source was positioned under the hind paw of the rat subject which was confined in an elevated plexiglas compartment. The beam was manually started and when the beam was interrupted by foot withdrawal the trial was automatically terminated. The apparatus' timing device measured the trials in tenths of seconds. A detailed explanation of this apparatus is reported by Hargreaves (1988). Subjects assigned the 90 day survival time were tested daily for seven days beginning at day 80. The order of testing was chosen blindly and subjects were identified after their trial.

Tissue Harvest and Preparation

After 56 days, three rats from each of the three groups were euthanized. After 90 days the remaining 34 rats were killed. After receiving a lethal overdose of a mixture of ketamine and xylazine, (160 mg/Kg ketamine, 16 mg/Kg xylazine) the animals were perfused through the ascending aorta with physiological saline followed by 10% phosphate-buffered formalin. The dorsal root ganglia were removed bilaterally from L4-L6 and collected into fresh 10% buffered formalin. Uteri from the 90-day animals and age-matched normal females were harvested and weighed. The DRGs were embedded in low melting point paraffin with special precautions taken to prevent RNAse contamination. Following embedding, 5µm sections were cut on an American Optical paraffin microtome and placed on Fisherbrand Superfrost slides.

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Probe Synthesis and *in situ* Hybridization

The ER cDNA used for antisense and sense RNA probe synthesis was a 560 base pair fragment from the N-terminus incuding a portion of the binding pocket unique to the estrogen receptor. It was a gift from Dr. Paul Shugrue (Wyeth-Ayerst) and was used with permission of the originator, Dr. M. Muramatsu. The sequence was described by Koike and colleagues (1987).

The trkA cDNA was obtained from Genentech. It was a 545 base pair fragment taken from the extracellular domain. The PPT cDNA was a 560 base pair fragment identified as beta PPT. The DNA fragment used was from a gene region which is uniquely a precursor to substance P (Krause, et al, 1987). It was obtained from Dr. Susan Leeman at Harvard University. The neurofilament cDNA was a 1.2kB fragment cloned from the gene specific for the 68 kD neurofilament subunit (Lewis and Cowan, 1985). It was a gift from Dr. Oswald Steward, University of Virginia, and used with the permission of its originator, Dr. Nick Cowan at New York University.

Detailed technical protocols for probe synthesis and *in situ* hybridization are included in the Appendix. Briefly, sense and antisense RNA probes were radiolabelled with P^{33} UTP using a Promega probe synthesis kit (Promega Riboprobe In Vitro Transcription System). Unincorporated isotope was removed by filtering through a TE Midi Select Column (Sigma). After the addition of tRNA, the probes were shortened to approximately 150 base pairs by alkaline hydrolysis. Probes were precipitated overnight, then pelleted, resuspended and counted with a scintillation counter to determine radionucleotide incorporation. Protocols for radioisotope usage have been

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approved by the EVMS Radiation Safety Committee and are on file in the Office of Research.

Tissues were examined by *in situ* hybridization techniques to localize mRNA for rat estrogen receptor. Quantitative *in situ* hybridization was used to localize and quantify trkA, the high affinity NGF receptor, preprotachykinin (PPT; the substance P precursor) and 68kD neurofilament mRNAs.

Sections were given a pretreatment, hybridized, and post-hybridized. In the pretreatment, the sections were deparaffinized with xylene and rehydrated in graded alcohols. The sections were then permeablized in a 0.2% HCl bath and incubated in proteinase K at 37°C. The sections were fixed in formaldehyde then acetylated with 0.1M triethanolamine with 0.25% acetic anhydride. After rinsing, sections were dehydrated in graded alcohols, delipidated with chloroform and dried under vacuum.

During hybridization, tissue sections were treated with sense and antisense probes which were added to the hybridization solution consisting of 50% formamide, .3M NaCl, 20mM Tris-HCl, EDTA , Denhardt's solution, salmon sperm DNA, Torules Yeast RNA, DEPC treated water and dextran sulfate pipetted onto the slides and incubated overnight in a 60°C humid chamber. In the post-hybridization portion of the procedure, slides were washed, dehydrated and Rnase treated. Slides were again washed and dehydrated, then exposed to Hyperfilm-beta (Amersham, Arlington Heights, Ill) for 2-4 days. Slides were coated with Kodak NTB2 nuclear emulsion diluted at a 1:1 ratio with distilled water, and after 6-21 days (for TrkA, neurofilament and ppt) were developed with a Kodak Dektol and Kodak fixer. Slides were stained for microscopic analysis with a hematoxylin and eosin preparation or cresyl violet.

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Quantification of Silver Grains

The *in situ* hybridations were quantified by an Image I (Universal Imaging Corporation) image analysis system according to the method of McNamara (1993). For this study, slides were blindly coded and non-overlapping fields of neurons were counted. Areas with large numbers of axons were not included. At least 10, and up to 40 observations were made for each slide. A minimum of two slides was used for each subject which served as an internal control for consistency. That is, if specific hybridization was detected on one slide but not on another, the case was discarded. Background counts were made from glass adjacent to the tissue sections and subtracted from the field counts. Sense slides were examined for signal.

Statistical Analysis

A mixed model ANOVA statistical analysis was performed on numerical data collected during grain counting from the *in situ* hybridization slides. Analysis of the foot withdrawal data was performed with a seven-by-three repeated measures ANOVA. The choice of the tests were recommended and the analyses performed by Paul Kolm, Ph.D., the biostatistician at EVMS.

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RESULTS

At the time of euthanization, the absence of ovaries was confirmed in all experimental animals. Figure 3 is a histogram comparing mean uterine weights of the three experimental groups with the mean uterine weight of normal cycling females. The distinct differences between the mean weights of OVX versus estrogen-replaced or normal uteri was considered ample proof of the removal of endogenous estrogen by ovariectomy and restoration of circulating estrogen in the treated animals. Upon reviewing these data, Gerald Pepe, Ph.D. (personal communication), an expert on estrogen and estrogen treatment, suggested that no further assay was needed as verification of estrogen status.

Estrogen Receptor mRNA Localization in Lumbar DRG

Micrographs showing the localization of ER mRNA within the DRG of an OVX animal from Group 1 by *in situ* hybridization with the ER RNA probe are presented in Figure 4. The darkfield micrograph clearly reveals detectable ER mRNA in a subpopulation of predominantly smaller DRG neurons. The higher magnification brightfield micrograph shows mRNA localization in smaller neurons (arrow). Larger neurons were generally unlabelled by hybridization with the antisense RNA probe to the rat ER. The sense probe verified the specificity of the antisense probe and revealed no hybridization to the DRG sections (data not shown).

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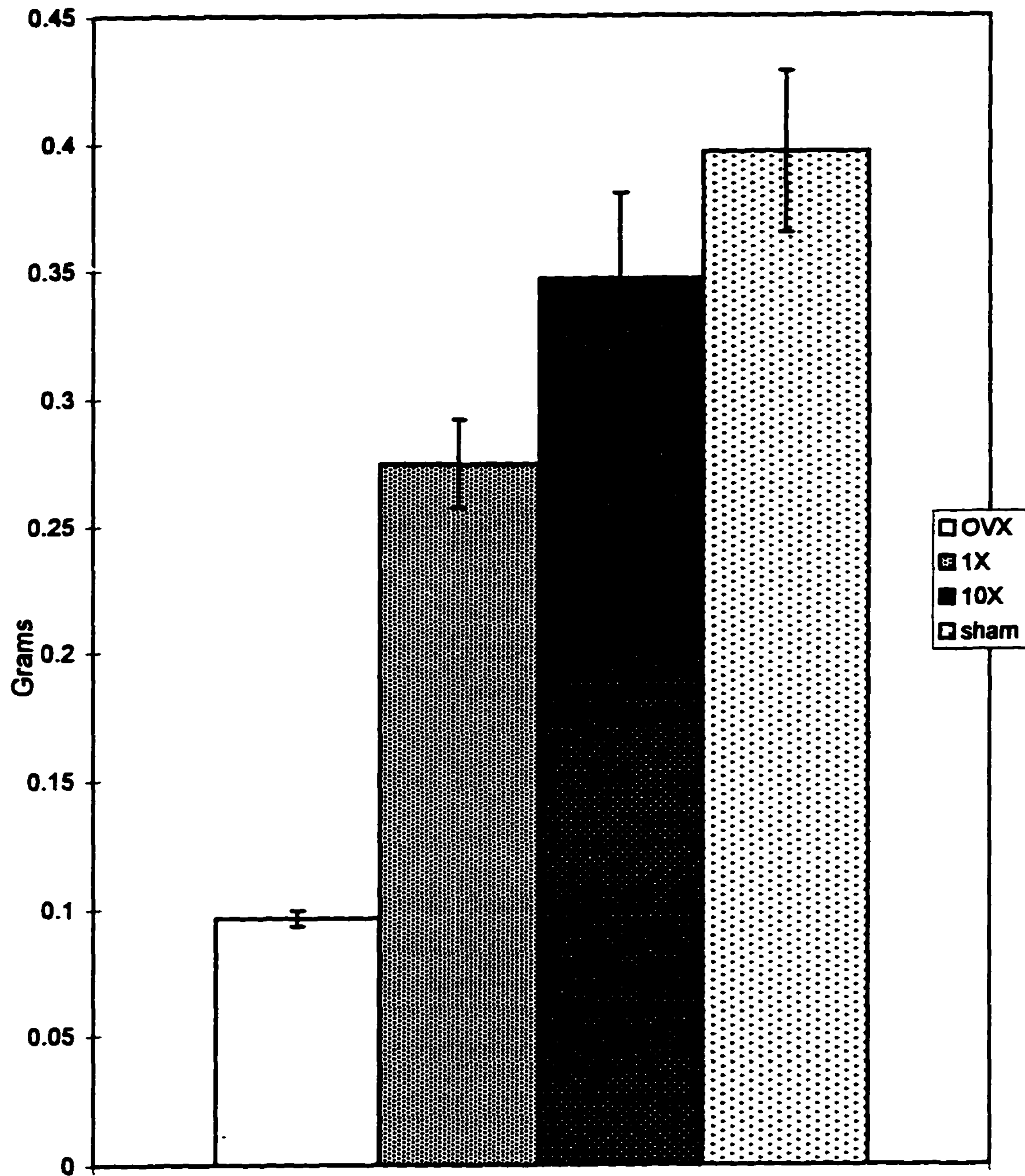

Figure 3. **Bar graph comparison of uterine weights across groups. Low weights confirm a lack of circulating estrogen in the OVX group. Note that replacement groups means and the sham operated group mean were nearly three to five times that of the OVX group.**

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Figure 4. **Darkfield micrograph showing localization of estrogen receptor mRNA in a lumbar DRG by** ***in situ*** **hybridiation** ***(A)*****. The same section in brightfield illumination and at a higher magnification is shown in** ***(B)*****. Bar in darkfield is 100µm. Bar in brightfield is 50µm. The arrows identify the same cluster of neurons in both micrographs.**

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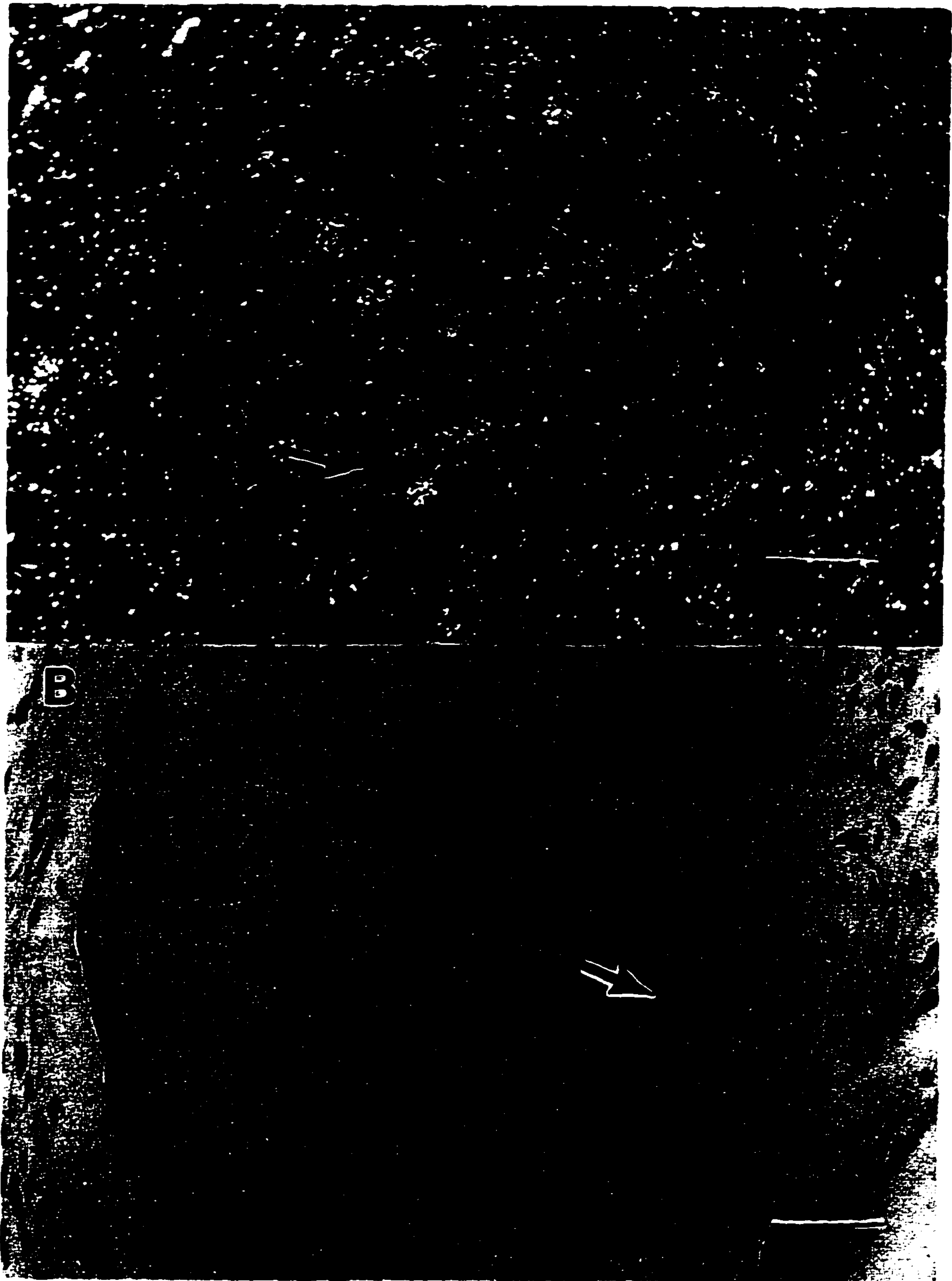

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Effects of Estrogen Treatments on High Affinity Nerve Growth Factor Receptor (trkA) mRNA Levels

Results of the *in situ* hybridization studies using the RNA probe for the high affinty nerve growth factor, trkA, are presented in Figures 5-9. Figure 5 compares the hybridization of the probe to DRG sections from the three groups after 56 days of daily estrogen treatment in the 1X and 10X groups. Figures 5A and 5B are darkfield and brightfield micrographs of a DRG section from an OVX animal. The trkA mRNA was observed mostly, but not exclusively, in small diameter DRG neurons. Micrographs C and D (1X treatment) and E and F (10X treatment) reduces the hybridization of the trkA probe to the tissue indicating reductions in the amounts of trkA mRNA in these tissues.

Two separate *in situ* hybridization experiments were done. Quantitative comparisons of results were performed on tissues hybridized at the same time under identical conditions. Results of the quantification and statistical analyses of trkA experiment 1 are shown in Figure 6. After 56 days of estrogen replacement the mean trkA steady state level of the OVX group was 2472.47 grains per region area (gr/rA) with a standard deviation of 1746.57 gr/rA. The mean trkA steady state level for the 1X group was 1629.9 gr/rA with a standard deviation of 826.9 gr/rA while the mean level for the 10X group was 2084.42 gr/rA with a standard deviation of 1318.22 gr/rA. Statistical analyses revealed that the mean trkA for the OVX was significantly greater than that of the 1X group ($p = .0115$). The statistical difference difference between the 1X group and the 10X group was not significant. The treatment group effect was significant ($p = .0159$).

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Figure 7 is a histogram showing the results of the quantitative analyses of the hybridization of the trkA probe to the DRGs in *in situ* hybridization experiment 2. This experiment was performed in a manner identical to experiment 1, but on DRG sections from different animals from the three groups. The mean steady state mRNA level for the OVX group was 5320.75 gr/rA with a standard deviation of 3928.98 gr/rA. The mean steady state trkA mRNA level for the 1X group was 3654.26 gr/rA with a standard deviation of 1844.74 gr/rA, while that for the 10X group was 3846.8 gr/rA with a standard deviation of 2364.17 gr/rA. Statistical analyses revealed that the mean steady state trkA mRNA level of the OVX group was significantly greater than the mean of the 1X group (p=.0273). The differences between the OVX and the 10X group was nearly significant (p= .0583).

Figure 8 consists of micrographs showing the results of hybridization of DRG sections from the three groups with the trkA RNA probe after 90 days of estrogen treatment. The qualitative results of the longer-term treatment are similar to those of the 56-day treatment, i.e. estrogen treatments reduce the amounts of trkA mRNA in predominantly smaller DRG neurons.

The results of quantification of silver grains indicating trkA mRNA in the DRG sections from the three groups after 90-day of estrogen treatments are presented in Figure 9. The mean steady state trkA mRNA level for the OVX group was 661.56 gr/rA with a standard deviation of 429.41 gr/rA. That for the 1X group was 424.86 gr/rA with a standard deviation of 272.04 gr/rA, while the mean steady state trkA mRNA level for the 10X group was 424.86 with a standard deviation of 201.39. In the case of 90 days of estrogen treatments, statistical analyses showed that the mean steady

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state trkA mRNA level for the OVX group was significantly greater than those from either estrogen replacement group ($p < .0001$).

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Figure 5. **Comparison of results of *in situ* hybridization of DRG sections with an anti-sense probe to trkA mRNA after 56 days of estrogen replacement. Micrographs *A, C and E* are shown in darkfield. Micrographs *B, D and E* are shown in brightfield, at a higher magnification. Sections *A* and *B* are from an OVX animal. Sections *C and D* are from a 1X animal. Sections *E* and *F* are from a 10X animal. All darkfield micrographs are at the same magnification (Bar=100µm). Brightfield micrographs are at the same magnification (Bar=50µm).**

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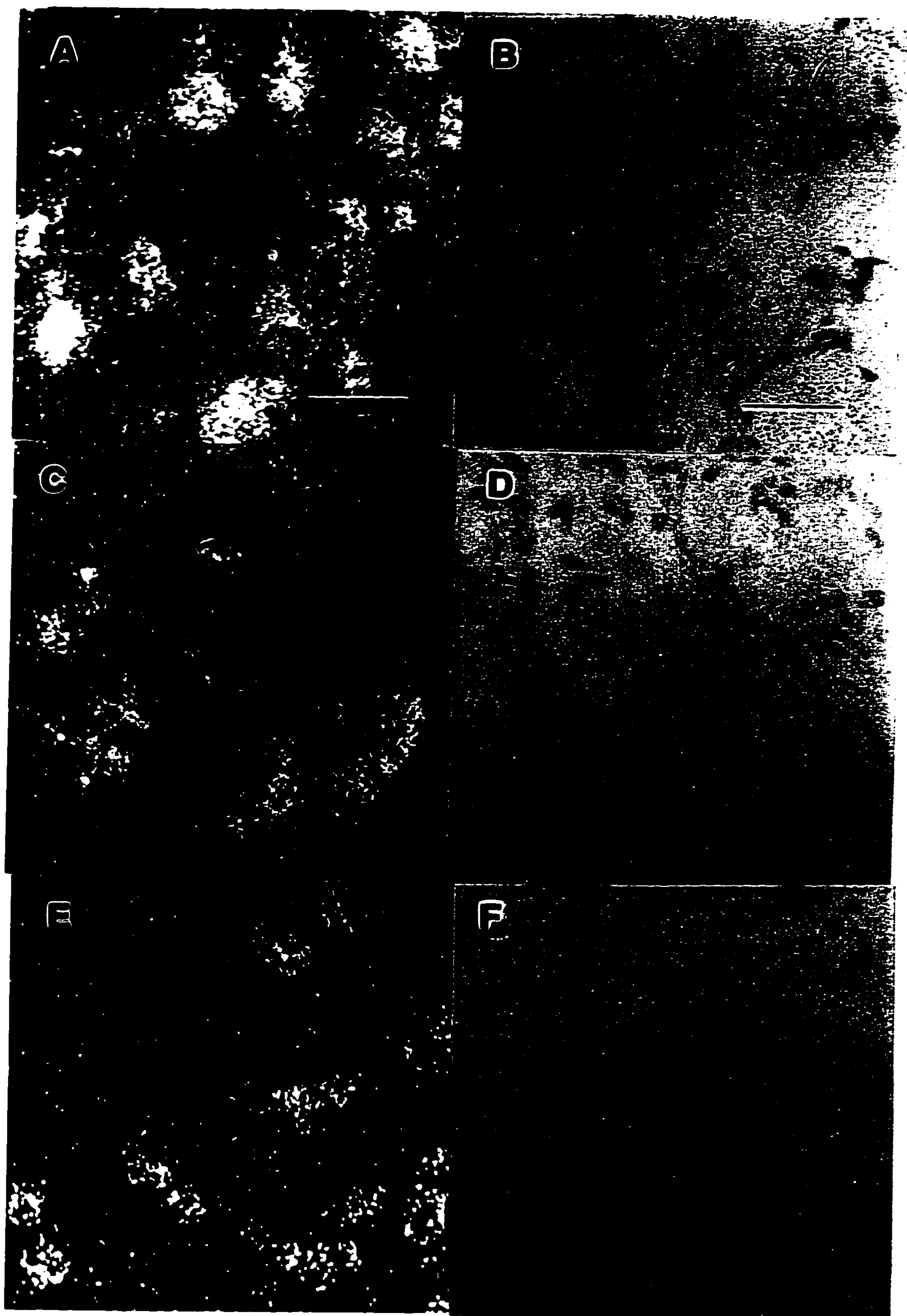

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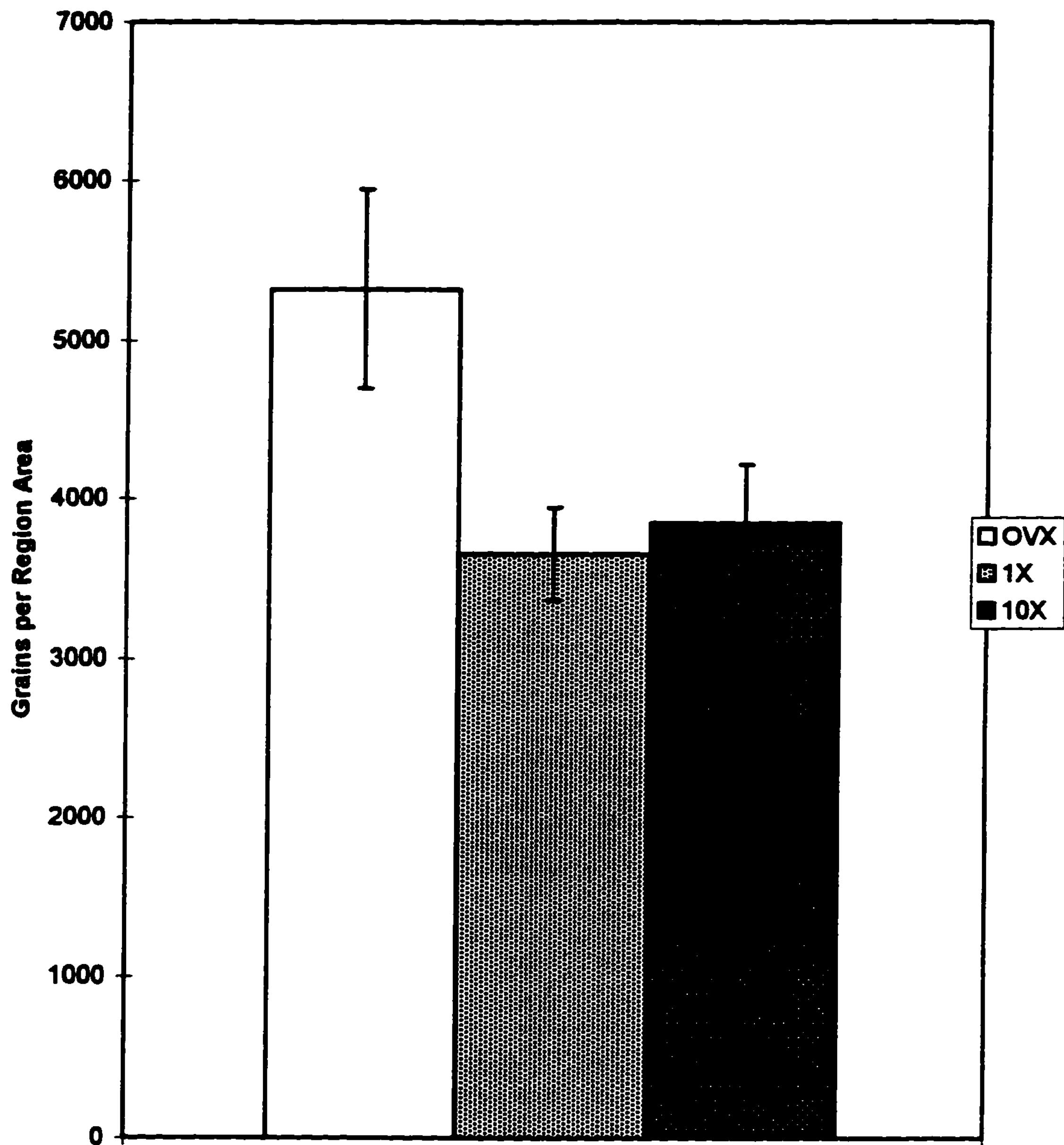

Figure 6. **Histogram comparing steady state trkA mRNA levels in the DRG in OVX and estrogen treated groups after 56 days of treatment. (experiment 1)**

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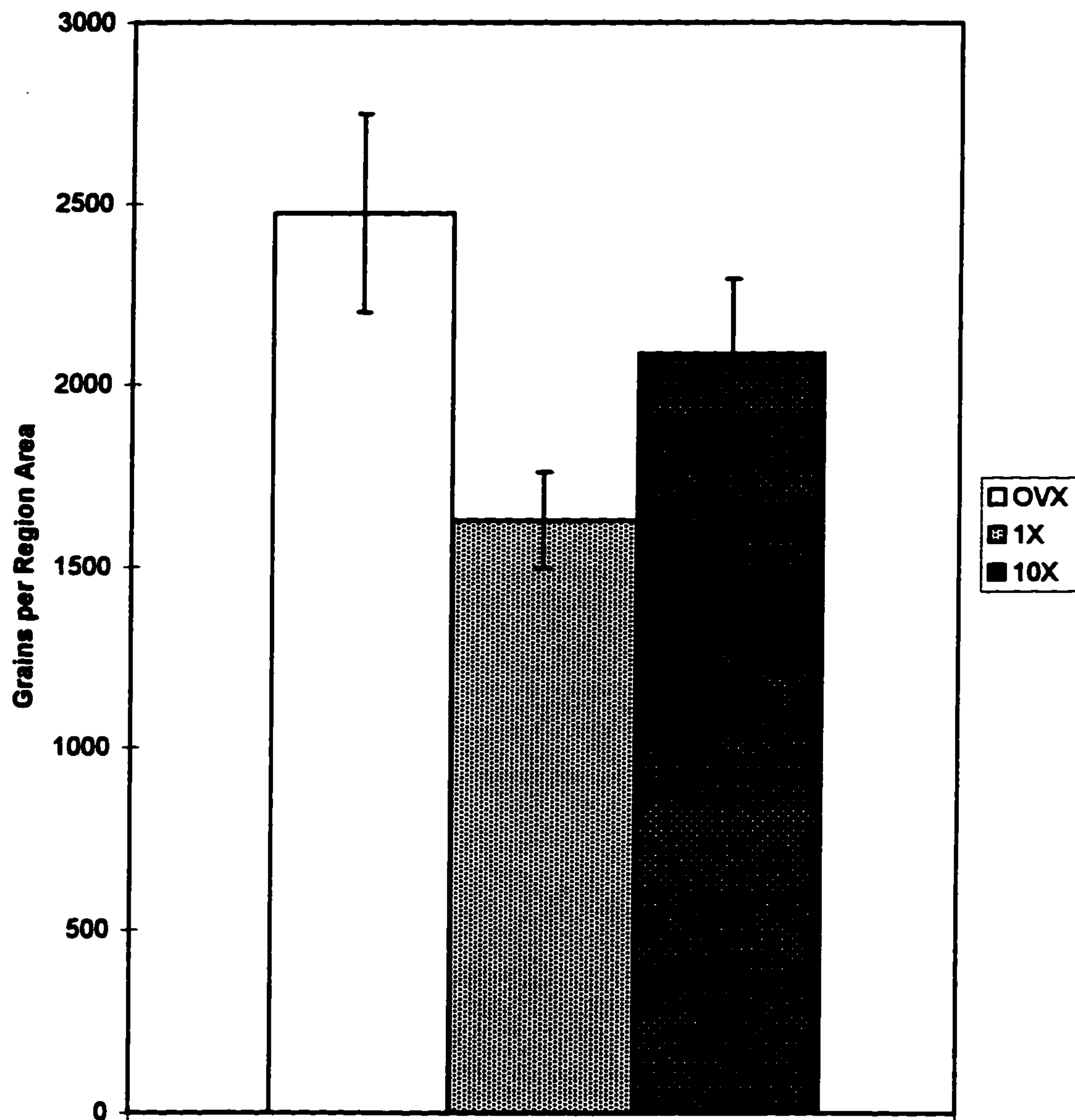

Figure 7. Histogram comparing steady state trkA mRNA levels in DRG by *in situ* hybridization after 56 days of estrogen replacement. (experiment 2) Subjects used for this experiment are from the same pool of subjects within each group, but are not identical to those used in experiment 1.

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Figure 8. **Comparison of results of *in situ* hybridization of DRG sections with anti-sense probe to trkA mRNA after 90 days of estrogen replacement. Micrographs *A*, *C* and *E* are shown in darkfield. Corresponding sections are shown at a higher magnification in brightfield in *B*, *D* and *F*. Sections shown in *A* and *B* are from an OVX animal. Sections shown in *C* and *D* are from a 1X animal. Sections shown in *E* and *F* are from a 10X animal. Darkfield micrographs are at the same magnification (Bar=100 µm). Brightfield micrographs are at the same magnification (Bar=50 µm). Arrows indicate the same neurons in darkfield and brightfield.**

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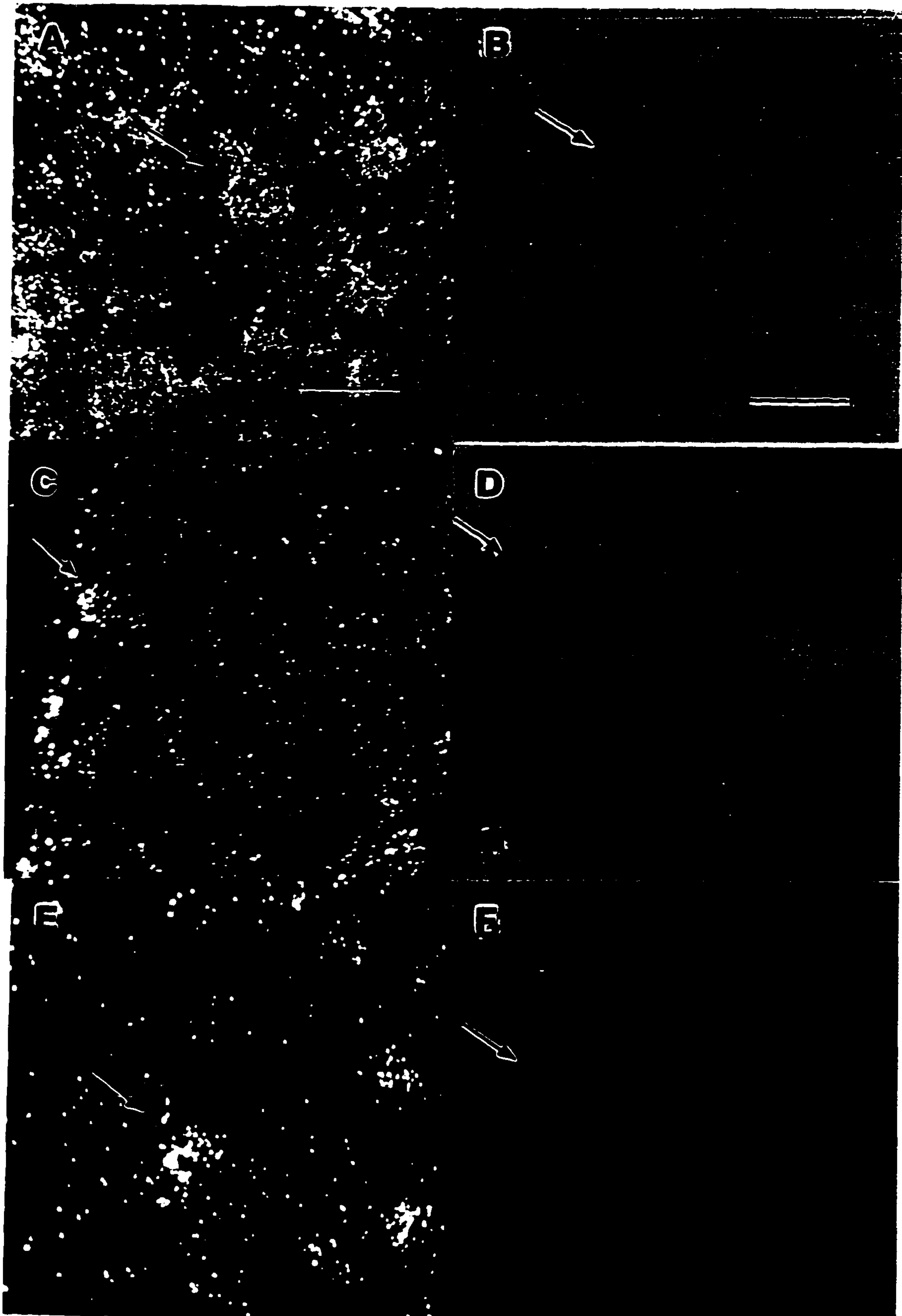

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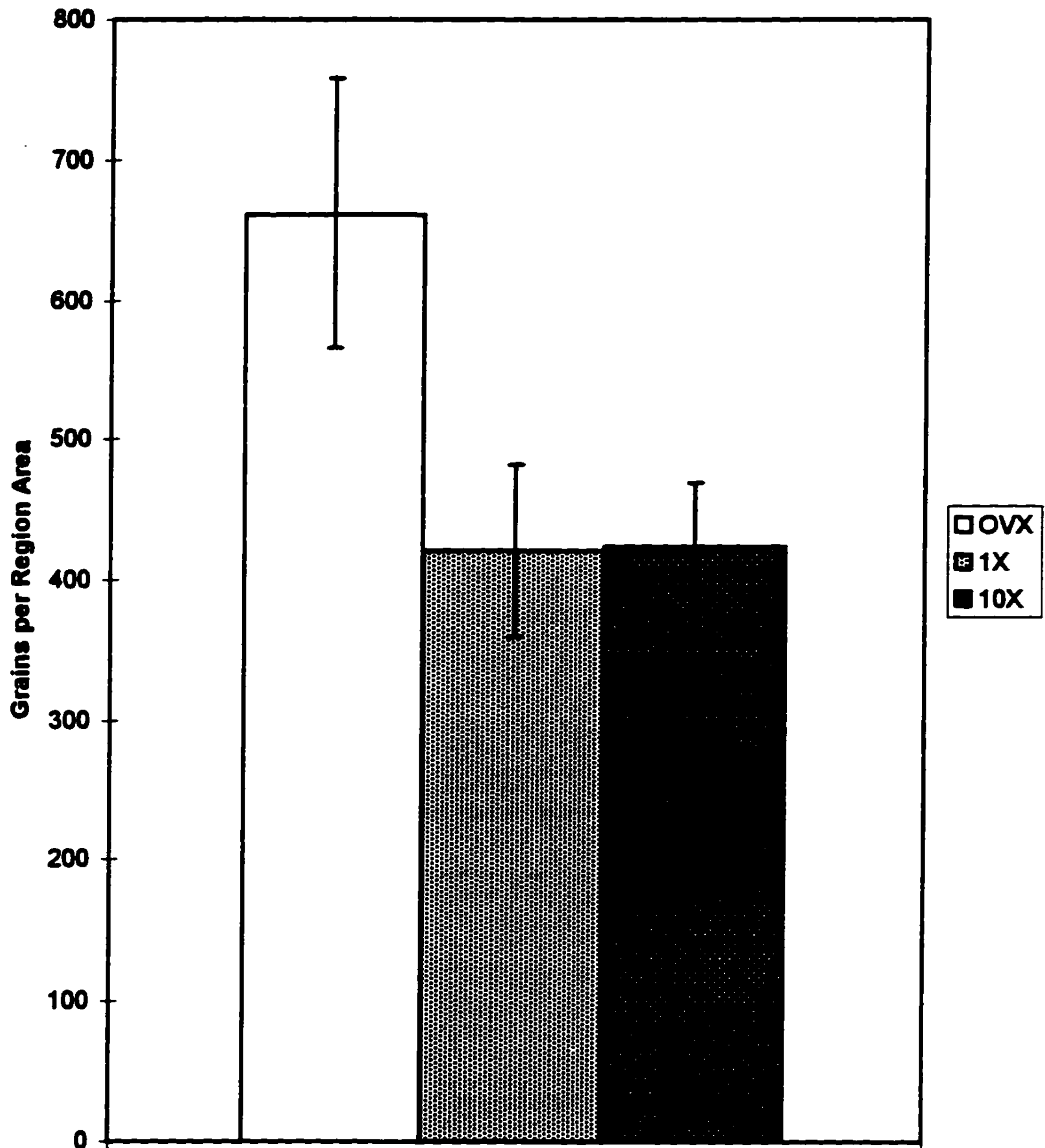

Figure 9. Histogram comparing trkA mRNA levels in OVX and estrogen treated groups after 90 days. Means in the replacement groups are significantly lower from that of the OVX group ($p < 0.0001$ for both comparisons).

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Effects of Estrogen Treatments on Preprotachykinin (PPT) mRNA Levels

Results of the *in situ* hybridization experiments using an RNA probe for the PPT gene are presented in figures 10-13. Figure 10 is a comparison of micrographs of DRG sections hybridized with the PPT probe from the three treatment groups after 56 days of treatments. The PPT mRNA, as would be expected from previous studies showing localization of substance P peptide, was found mainly in smaller DRG neurons. The estrogen treatments appeared to reduce the amount of mRNA in DRG sections from the treated groups.

A histogram comparing mean steady state PPT mRNA levels across the three groups after 56 days of estrogen treatments is shown in Figure 11. The mean steady state level PPT mRNA level for the OVX group (1521 gr/rA) was greater than either the mean steady state PPT mRNA levels for the 1X treatment group (901 gr/rA) or the 10X treatment group (836 gr/rA).

After 90 days of treatments, PPT steady state mRNA levels were decreased by estrogen replacement therapies, as was observed after 56 days of estrogen treatments. These data are shown in the comparison of micrographs in Figure 12. As was seen after 56 days of treatment, there is an apparent decrease in the numbers of silver grains indicative of PPT mRNA localization over the DRG sections from the estrogen treated animals.

A histogram depicting quantification of these differences is shown in Figure 13. Here, the mean steady state PPT mRNA level for the OVX group was 661.56 gr/rA with a standard deviation of 429.41 gr/rA. That for the 1X group was 421.33 gr/rA with a standard deviation of 272.04 gr/rA, while the mean steady state PPT mRNA level for the

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10X group after 90 days of estrogen treatment was 424.86 gr/rA with a standard deviation of 201.39 gr/rA. The mean steady state PPT mRNA levels for both estrogen replacement groups were significantly lower than the that for the OVX group ($p < .0001$).

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Figure 10. **Comparison of results of *in situ* hybridization of DRG sections with anti-sense probe to preprotachykinin (PPT) after 56 days of estrogen replacement. Micrographs *A, C* and *E* are shown in darkfield, and micrographs *B, D,* and *F* are shown in brightfield, at a higher magnification. Sections shown in *A* and *B* are of an OVX animal. Sections shown in *C* and *D* are from a 1X animal. Sections shown in *E* and *F* are from a 10X animal. Micrographs *A, C* and *E* are at the same magnification (Bar=100 µm). Micrographs *B, D* and *F* are at the same magnification (Bar=50 µm).**

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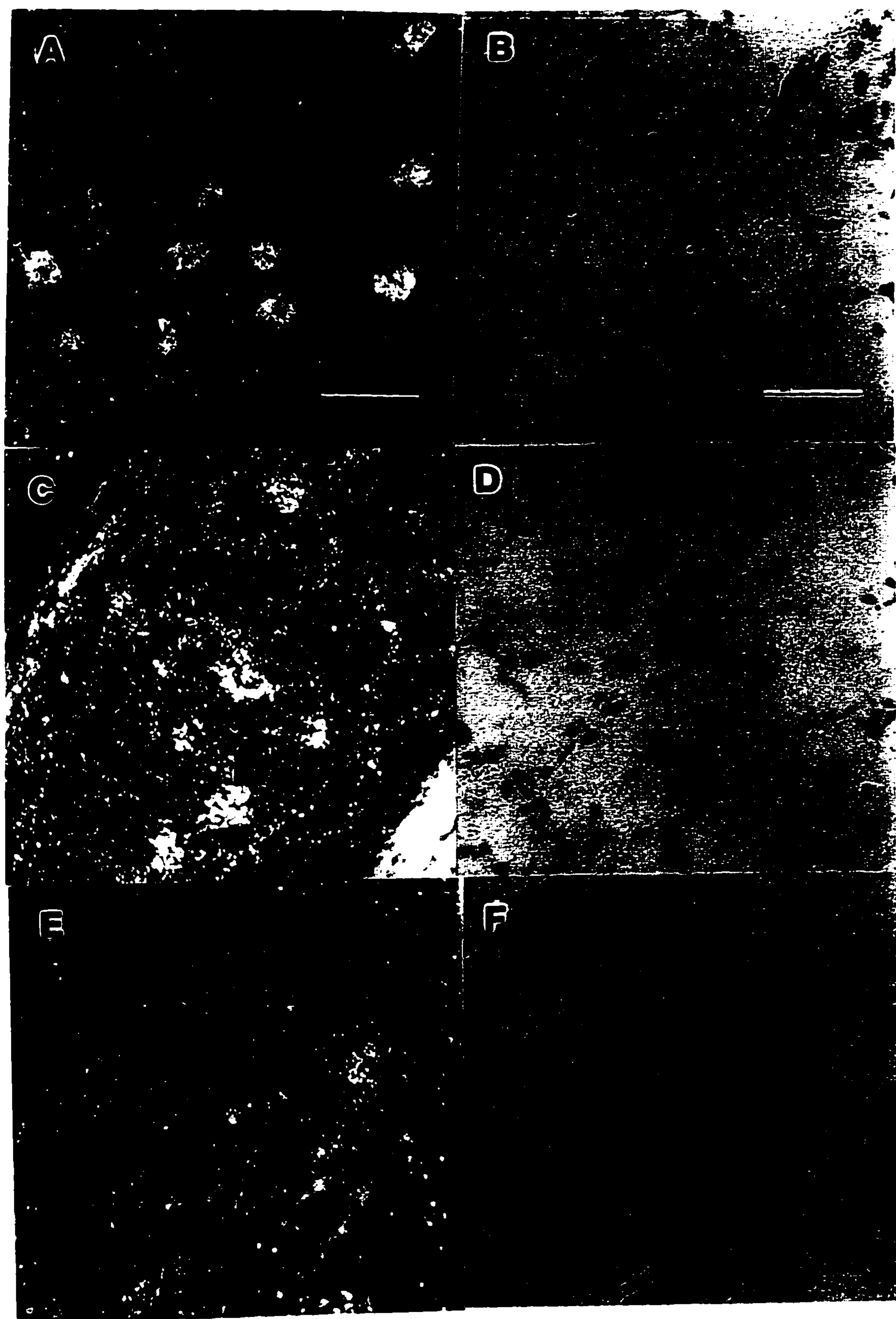

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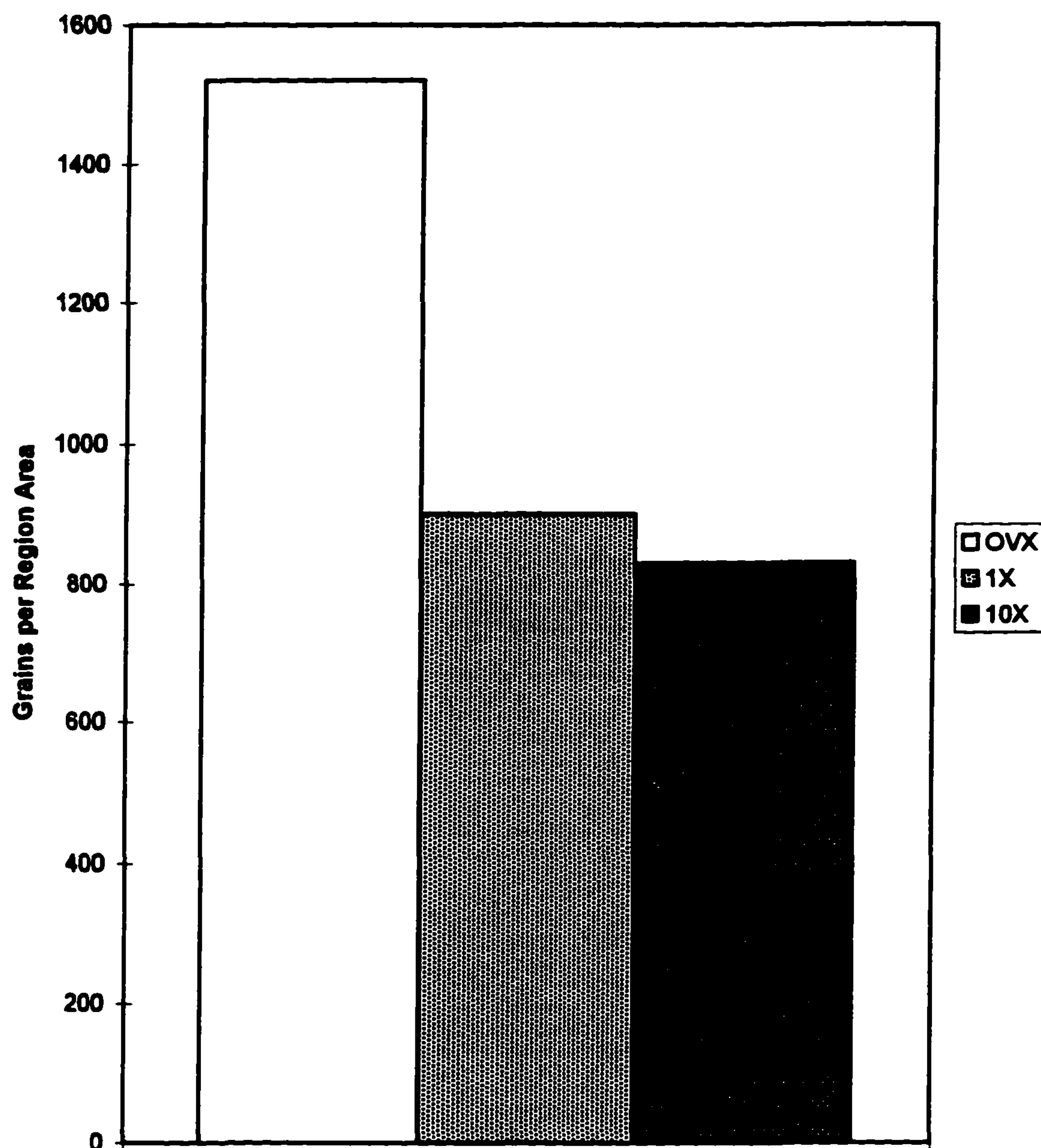

Figure 11. **Histogram comparing of PPT mRNA levels in OVX and estrogen treated groups after 56 days.**

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Figure 12. **Comparison of results of an *in situ* hybridization with an antisense probe to PPT after 90 days of estrogen replacement. Sections *A, C* and *E* are darkfield micrographs. Sections *B, D* and *F* are higher magnification brightfield micrographs. Sections *A* and *B* are from an OVX animal. Sections *B* and *C* are from an animal receiving 1X replacement estrogen. Sections *D* and *F* are from an animal receiving 10X replacement estrogen. Micrographs *A, C* and *E* are at the same magnification (Bar=100 μm). Micrographs *B, D* and *F* are at the same magnification (Bar=50 μm).**

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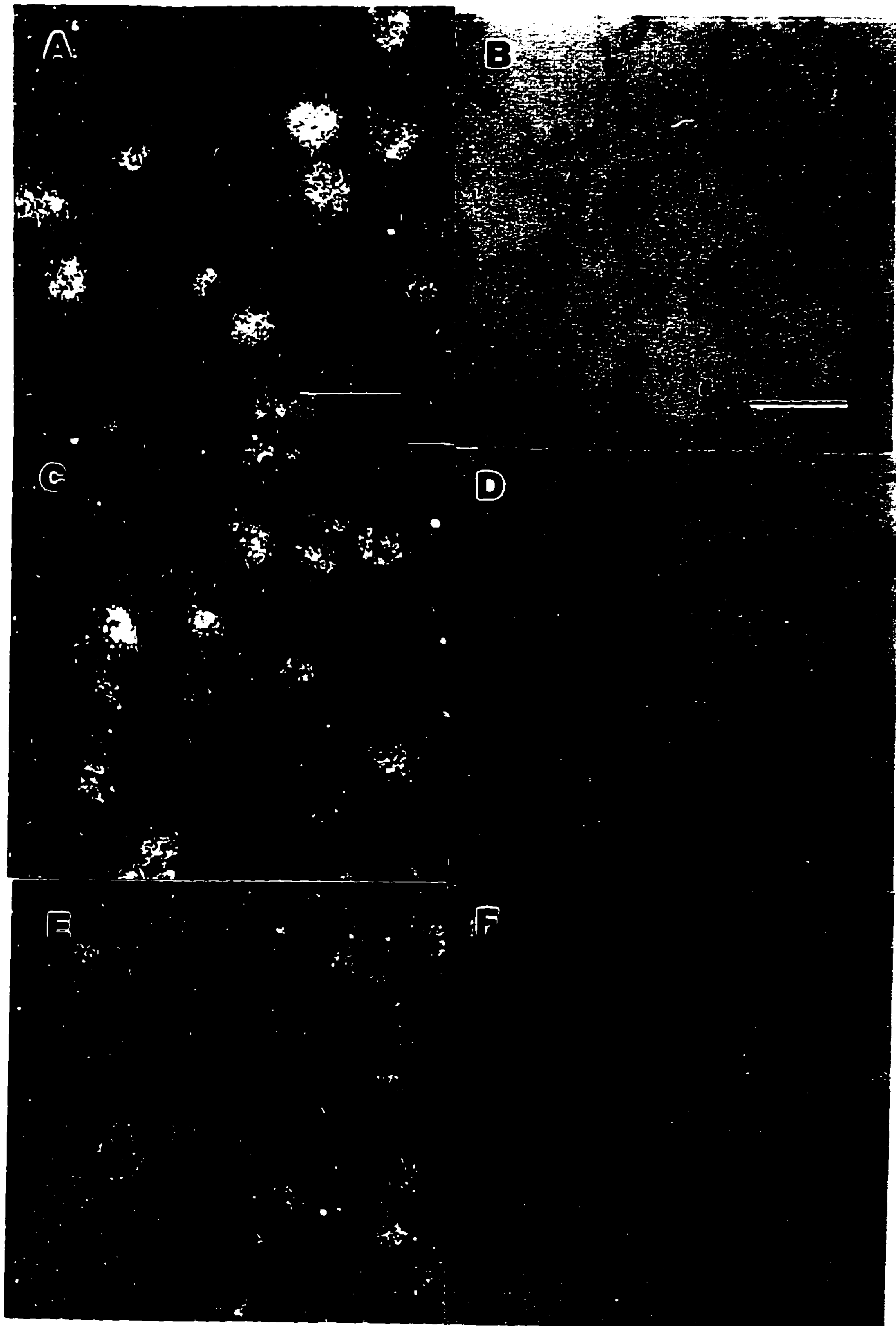

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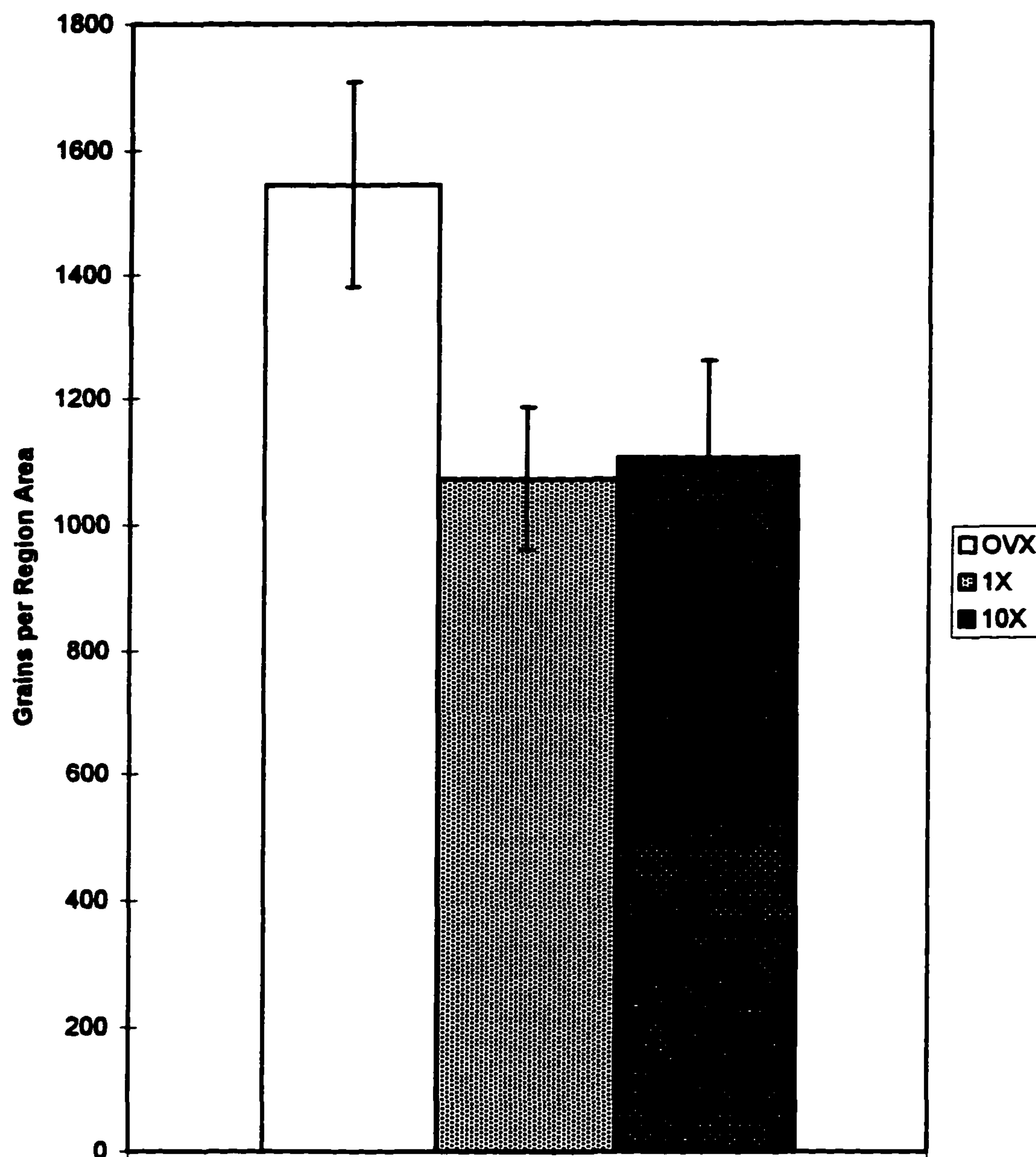

Figure 13. **Histogram comparing PPT mRNA levels in OVX and estrogen treated groups after 90 days. Means for replacement groups are significantly lower than the OVX group (p <0.0001 for both comparisons).**

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Effects of Estrogen Treatments on Warm Thermal Withdrawal Latencies

Results of the thermal withdrawal test indicate a trend of reduced thermal sensitivity when estrogen replaced groups were compared to the OVX animals. Results of the analyses of warm thermal withdrawal latencies as measured with the warm plantar withdrawal testing apparatus are presented in Figure 14. These data were analyzed by a three (group) by seven (day) repeated measures analysis of variance (ANOVA). The mean withdrawal latency for the OVX estrogen treatment group was less (meaning a shorter time for foot withdrawal) than that for the 1X estrogen treatment group which was, in turn, less than the mean withdrawal latency for the 10X group. On a daily basis, this phenomenon, though small, is surprisingly consistent. It is true for 18 out of 21 means. However, the repeated measures ANOVA did not find significant differences for the main effect, that is a comparison of means for all subjects between groups. Each group terminated their trials significantly faster with repeated measurements, i.e., there was a day effect ($p=.0014$). The amount of this decline was not significantly different between groups.

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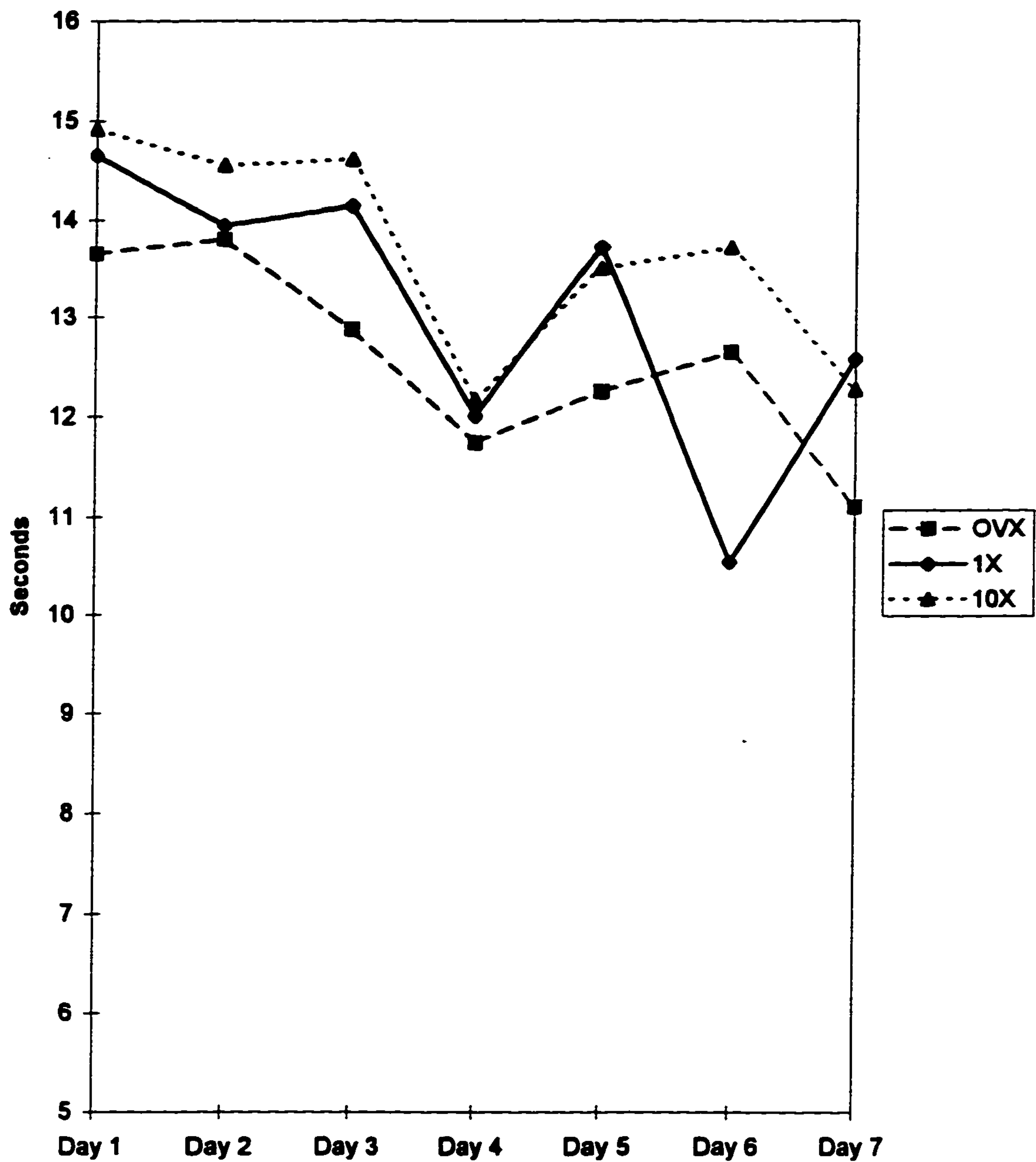

Figure 14. **Comparison of mean withdrawal latencies on seven successive days to a warm thermal stimulus.**

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<u>Effects of Estrogen Treatments on 68kD Neurofilament mRNA Levels</u>

Dramatic qualitative differences in hybridization of the probe to these tissues are demonstrated. The 68kD neurofilament mRNA is most abundant in larger DRG neurons although all DRG neurons express the gene. Estrogen treatments dramatically increase the density of the silver grains over the larger neurons indicating increased steady state 68kD neurofilament mRNA levels. The results of *in situ* hybridizations using an RNA probe to the 68kD neurofilament gene are shown in Figures 15-18. Figure 15 shows micrographs comparing the results of hybridization with the 68kD neurofilament antisense probe to DRG sections from the three groups after 56 days of estrogen treatment

Figures 16 and 17 show quantitative results of these differences and reveal a possible dose-dependent effect of estrogen treatment on 68kD neurofilament gene expression. Similar to the data presented for trkA, experiments 1 and 2 use subjects from the same pool but are not identical. In the first experiment, the mean steady state 68kD mRNA level for the OVX group was 540.10 g/rA. That for the 1X estrogen treatment group was nearly four times greater (2069.85 g/rA), while that for the 10X estrogen treatment group was nearly six times greater (3279.35 g/rA). Statistical analyses revealed that these differences among groups were significant ($p > .0001$).

In the second *in situ* hybridization experiment, the mean steady state 68kD neurofilament mRNA level for the OVX group was 1259.95 g/rA, the mean for the 1X estrogen treatment group was nearly three time greater (3013.8 g/rA) while that 10X estrogen treatment group was nearly five times greater (5132.85 g/rA). Statistical

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analyses of these data, as for the first experiment, differences among groups that are significant (p>.0001).

Figure 18 shows the results of quantification of mean steady state 68kD neurofilament mRNA levels for the 90 day estrogen treatment regimen. These data vary slightly from the results of the 56 day treatment regimen. Following 90 days of estrogen treatments, the mean steady state 68kD neurofilament mRNA levels were nearly three times greater in the treatment groups (1X: 2952.35 g/rA, 10X: 3426 g/rA) as compared to those of the OVX group (1145.8 gr/rA). On the advice of Dr. Kolm, statistical analyses were not performed on these data since it was not possible to obtain grain counts from equal numbers of DRG areas for the 10X group.

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Figure 15. Comparison of results of *in situ* hybridization of DRG sections with anti-sense probe to neurofilament (68 kD) after 56 days of estrogen replacement. Micrographs *A*, *C* and *E* are shown in darkfield, and micrographs *B*, *D* and *F* are shown at a higher magnification with brightfield illumination. Sections shown in *A* and *B* are from OVX animal. Sections shown in *C* and *D* are from an animal receiving 1X replacement estrogen. Sections shown in *E* and *F* are from an animal receiving 10X replacement estrogen. Bars in darkfield micrographs, *A*, *C*, and *E* are 100 µm. Bars in brightfield micrographs *B*, *D*, and *F* are 25 µm.

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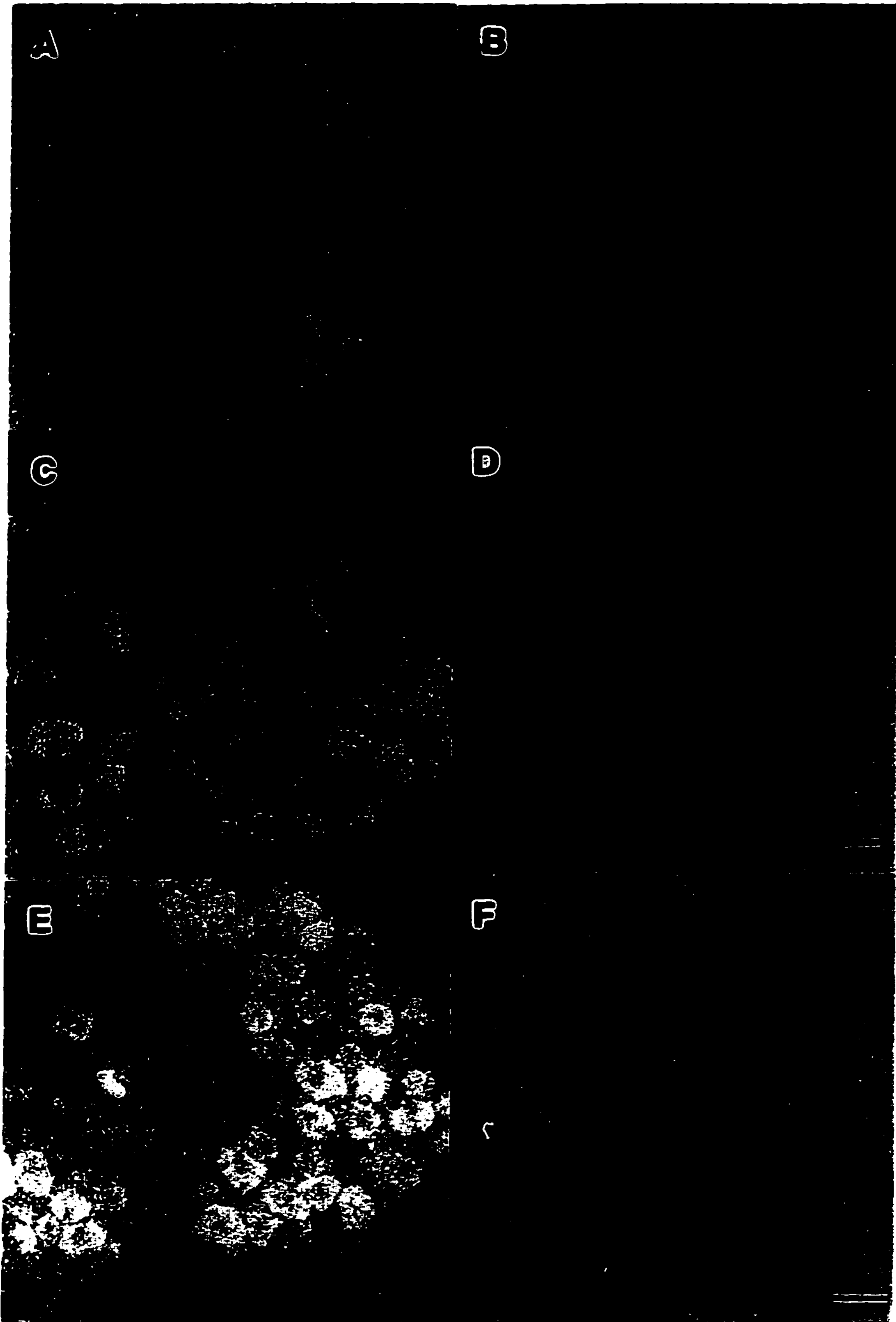

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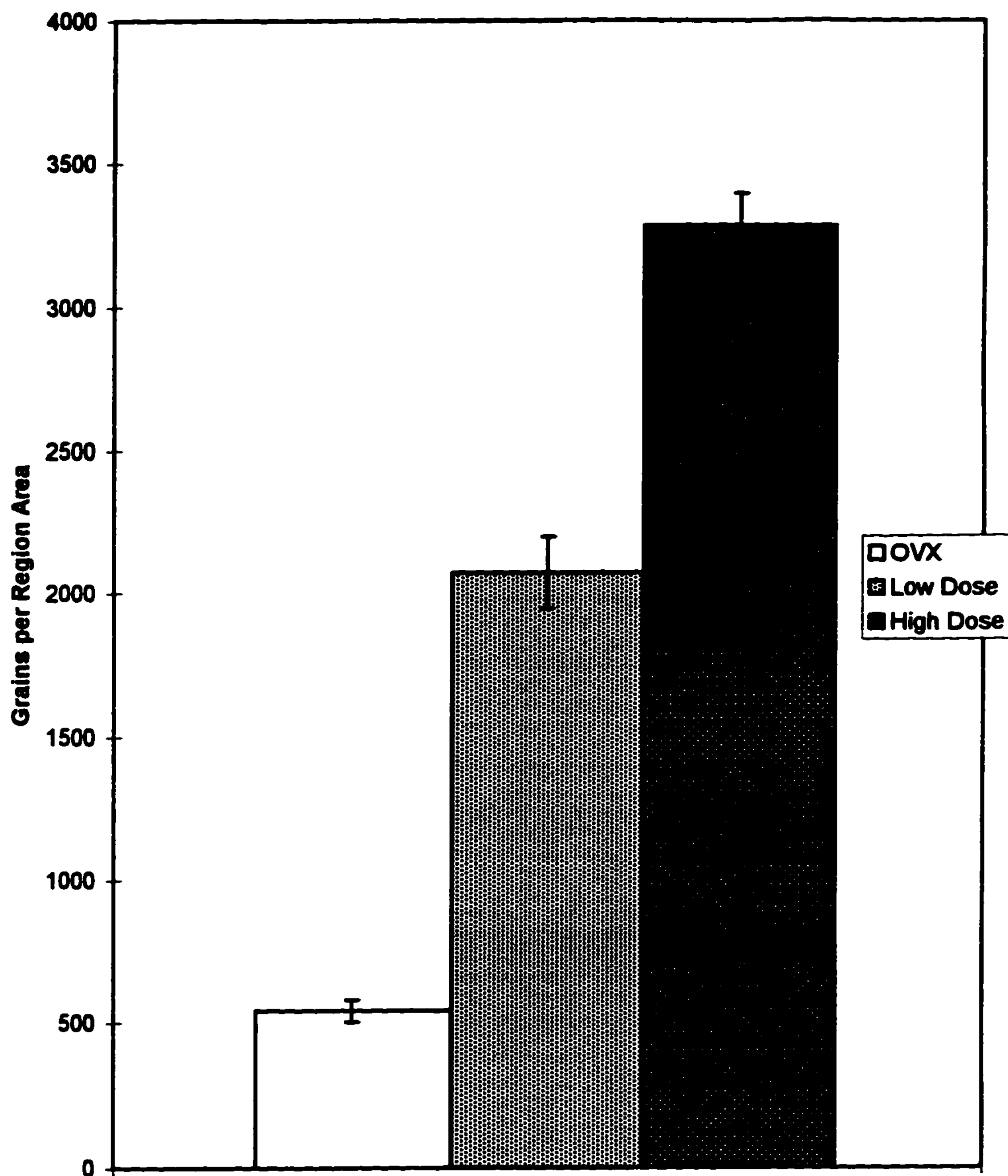

Figure 16. Histogram comparing neurofilament mRNA levels in OVX and estrogen treated groups after 56 days, experiment 1. All means are statistically different from each other ($p < .0001$).

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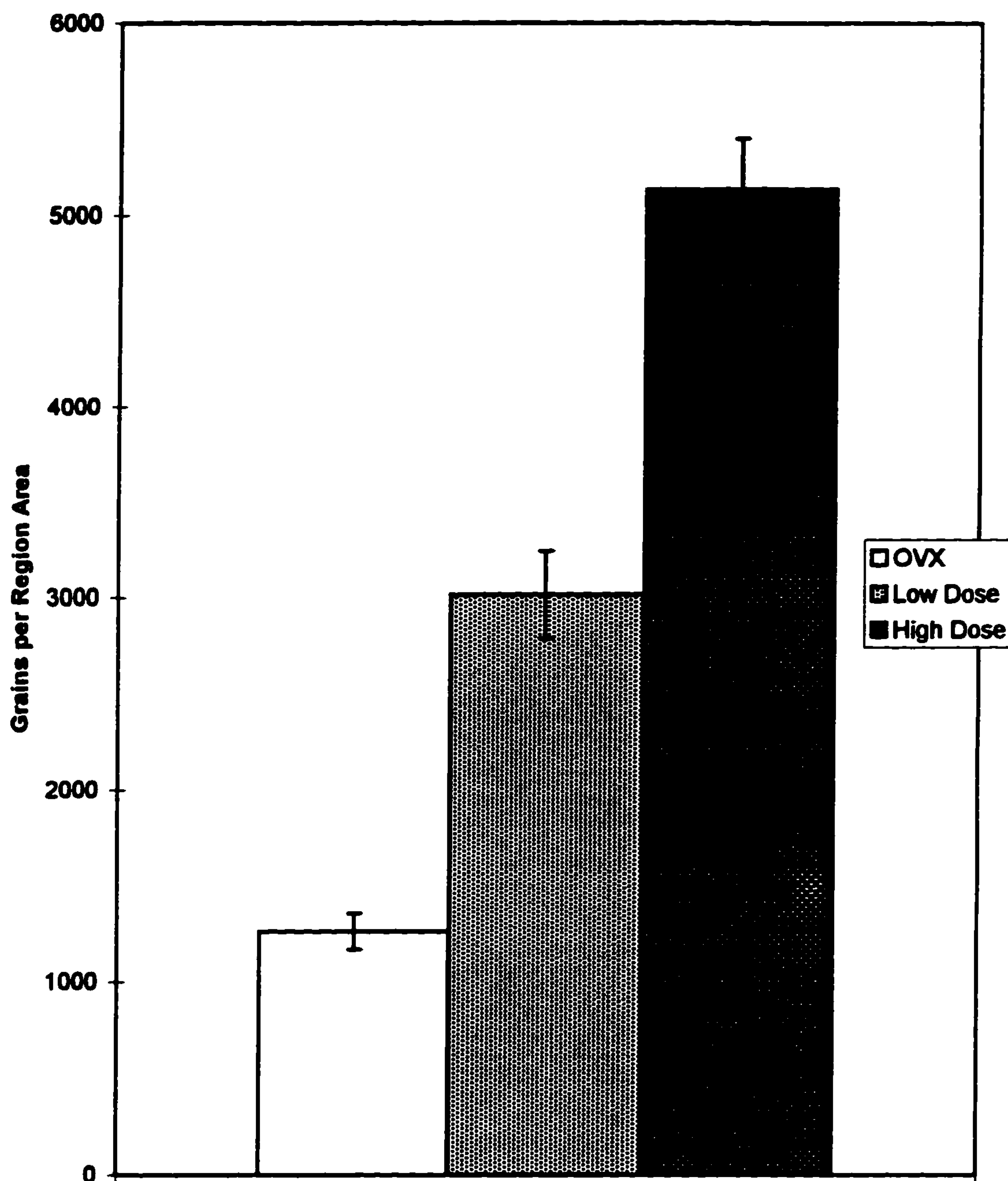

Figure 17. Histogram comparing neurofilament mRNA levels in OVX and estrogen treated groups after 56 days, experiment 2. Sections are from the same pool of subjects as in experiment 1. All means are statistically different from each other ($p < .0001$).

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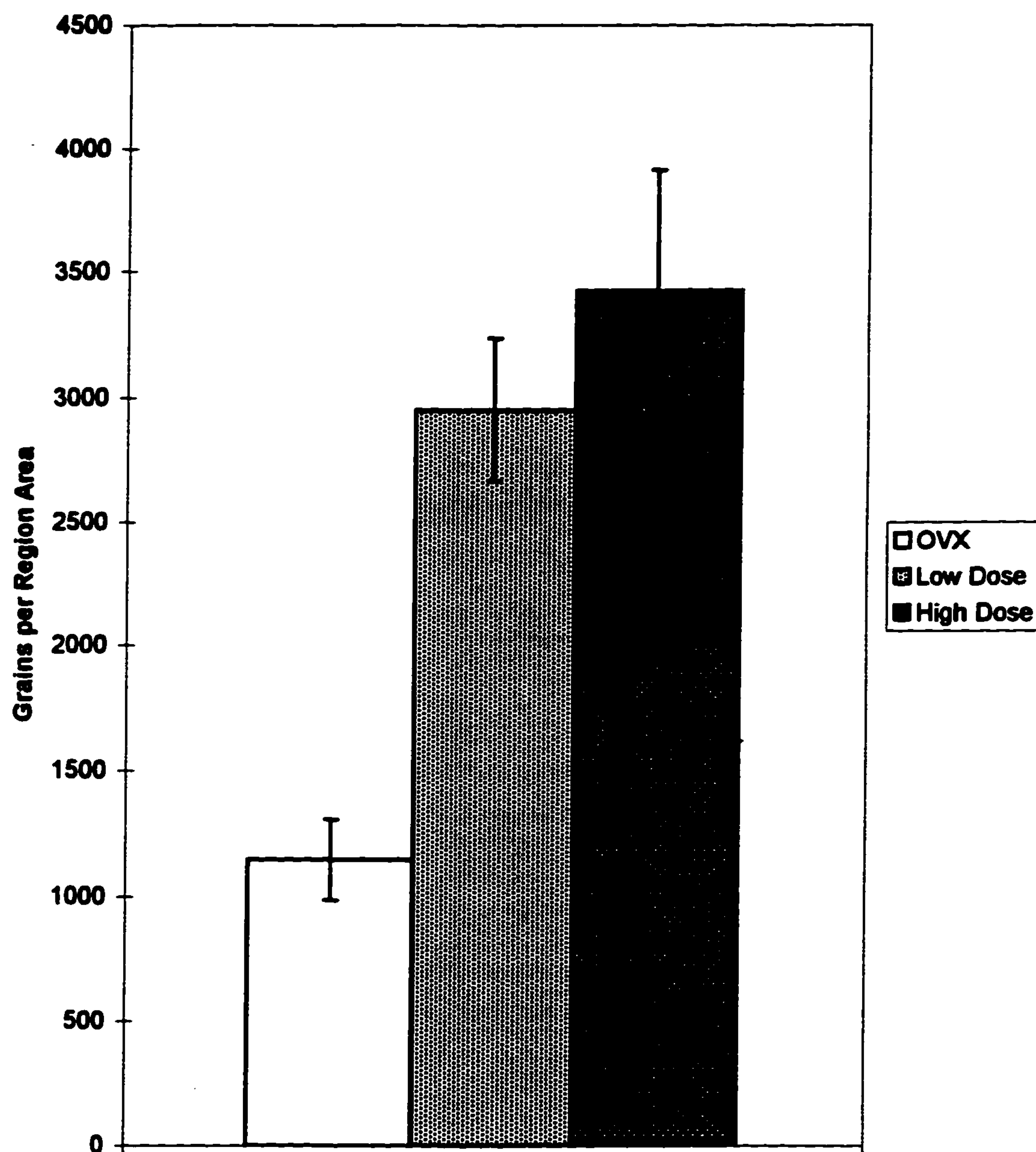

Figure 18. **Histogram comparing neurofilament mRNA levels in OVX and estrogen treated groups after 90 days.**

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DISCUSSION

The current study demonstrates that long-term estrogen replacement in OVX rats has profound effects on a number of important DRG neuronal genes. Long-term estrogen replacement down-regulates the the high affinity NGF receptor, trkA gene, as well as, the PPT gene, which is responsible for the synthesis of the putative nociceptive neuropeptide, substance P. In addition, long-term estrogen administration dramatically increases 68kD neurofilament mRNA levels. These findings are among the first to suggest direct effects of long-term estrogen treatment on primary sensory neuronal gene expression. Such evidence provides initial insight into mechanisms that underlie altered somatosensory function during the reproductive cycle of rats.

More importantly for the millions of women facing loss of estrogen, they suggest potential effects of estrogen replacement therapy on skin sensation. The use of estrogen replacement therapy for these women, to treat the obvious consequences of estrogen loss, such as osteoporosis, is gaining greater acceptance. The implications of the data from the current study for the increasing use of estrogen replacement therapy in menopausal women are obvious. The form, dosage, and time-course of estrogen replacement may be critical and may depend on the specific symptoms and systems being treated. While chronic, long-term estrogen therapy might be beneficial in the treatment of some systems, such as the skeletal or cardiovascular systems, the current data suggest that chronic, long-term estrogen treatment may alter somatosensory function and perhaps, in some instances, particularly nociception, have a deleterious effect.

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Estrogen Receptor Gene Expression in Lumbar DRGs

There has been a controversy in the literature regarding the number and types of DRG neurons that express the ER gene. Sohrabji and her colleagues (1994) reported that ER mRNA was present in all DRG neurons. In contrast to these data, Sarajari and her colleagues (1996) reported that ER gene expression was characteristic of a subpopulation of predominantly smaller DRG neurons.

One goal of the current study was to resolve this controversy by determining whether all or only a subpopulation of DRG neurons express the ER gene. The results of eight separate *in situ* hybridization experiments using probes to localize ER rmRNA within the lumbar DRG support those reports showing ER gene expression in only smaller DRG neurons (Sarajari and Oblinger, 1996). However, the final resolution of this issue may ultimately depend on methods with greater sensitivity for the detection of very low abundance mRNA in tissue sections.

To date, few photomicrographs of ER mRNA have been published which use a radioactive label. It is difficult to compare results on the basis of description alone. Only Simerly (1990) has published photomicrographs showing ER mRNA localization in the hypothalamus using this method. No one has published similar data for the spinal cord and DRG, both regions purported to have neuronal ER expression.

Cell biologists have suggested that ERs are largely recycled, that is, they return to the outer nuclear envelope after they detach from the estrogen response element of the gene (Ing and O'Malley, 1995). If this is the case, the mRNA needed for the production of new receptors may be very low in abundance making it particularly difficult to detect by conventional *in situ* hybridization methods.

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In the current study, our *in situ* hybridization methodology revealed the results of hybridization with radioactive probes to such high abundance mRNAs as 68kD neurofilament, PPT, and trkA within 1-2 weeks of exposure of the labelled slides to the NTB2 emulsion. By contrast, the *in situ* experiments yielding the best results for ER mRNA localization were exposed for 4-5 weeks or longer. Indeed, Oblinger (personal communication) used 16 week exposures to emulsion to reveal ER mRNA localization. Therefore, it remains a possibility that all DRG neurons may express the ER gene, but the technique available to us only allowed visualization of ER mRNA in some smaller neurons, where steady state ER mRNA levels are maximal. Quanitative analysis of *in situ* hybridizations may be ineffective as a method of data analysis for low abundant mRNAs. The report showing ER mRNA localization in all DRG neurons used a digoxygenin labelling methodology (Sohrabji, et al., 1994). There is little reason, however, to believe that that particular approach is more sensitive than one using radioactively labelled RNA as a probe.

Differences in the fragment of the ER gene selected for probe synthesis is another variable that may add to the disparity between studies. Sohrabji and her colleagues (1994) used a 48-base pair oligonucleotide probe constructed at their own university. All other *in situ* hybridization studies of ER expression in nervous tissue use variations of the cDNA probe cloned by Miramatsu (Koike, et al., 1987). Because of the very large degree of homology with other hormone receptors, it is critical to select the fragment for the probe from a portion of the region coded for the receptor binding pocket. Both the Sohrabji group and the Sarajari group appear to accommodate this

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requirement. This region has very little homology with the binding pockets of other hormone receptors.

In exploring the effects of estrogen on nervous tissues, one must also consider potential non-genomic actions of the hormone. The possibility that estrogen may enter DRG neurons by an as yet undescribed, additional route which bypasses the traditional ER is intriguing. In general, non-genomic estrogen effects should be identifiable by their short latencies. These latencies are measured in minutes rather than hours and, as such, were beyond the resolution provided by the current study.

Regulation of NGF Receptor Genes by Estrogen

Clearly many questions remain concerning the relationship between estrogen and the neurotrophin, NGF. In order to begin to understand this relationship, one must begin by analyzing the effects of estrogen on the low-affinity (p75) and high-affinity (trkA) NGF receptors. The current study has established the effects of long-term estrogen treatment on trkA mRNA levels. Others have studied estrogen's effects on the low-affinity NGF receptor, p75 (Gibbs and Pfaff, 1992, Sohrabji et al, 1994 and Toran-Allerand et al, 1992).

Without first considering the effects of estrogen, one finds that there remains some confusion about the interaction between p75 and trkA. The roles of these two receptors in mediating NGF's action are not entirely understood. Emerging evidence indicates that p75, which may, more properly, be referred to as the neurotrophin receptor, influences the sensitivity of DRG neurons to whichever of the tyrosine kinase type of receptors that they co-expressed, e.g. trkA, trkB or trkC (Wright and Snider,

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1995). Thereby, p75 may, in some way, determine the sensitivity of a trkA expressing DRG neuron to NGF or a trkB expressing neuron to brain derived neurotrophic factor (BDNF).

Because high affinity NGF binding and the intracellular effects of NGF on DRG neurons is dependent on trkA expression, the current study focused on the effects of long-term estrogen treatment on trkA gene expression. In the current study, trkA mRNA levels were clearly and significantly downregulated by both 1X and 10X daily estrogen treatments over 90 days. Treatments over the shorter, 56-day time period had similar effects.

These data show that chronic, long-term estrogen treatment decreases the amount of trkA mRNA in lumbar DRG neurons. Such a decrease would be expected to decrease the production of high-affinity NGF receptors by DRG neurons and thereby decrease the sensitivity of these cells to the neurotrophin. This, in turn, should down-regulate NGF-dependent gene expression in the DRG. Further data from this study fulfill this expectation by showing a coincident effect of estrogen treatments on DRG PPT gene expression.

Effects of Estrogen Treatments on PPT Gene Expression and Nociception

As stated earlier, about 40% of DRG neurons bind NGF and express the trkA gene. Of these, about half express the PPT gene. In those 20% of DRG neurons, PPT gene expression (Hamar and Lindsay, 1989) and therefore, substance P levels (Wong and Oblinger, 1992) are regulated by NGF. Since, as discussed above, chronic, long-term

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estrogen treatment decreases DRG trkA mRNA levels, it should have a downstream effect on NGF-regulated genes, such as the PPT gene.

The current data reveal a close relationship between the effects of chronic, long-term estrogen treatment on trkA mRNA levels and the effects on PPT mRNA levels. Both 56 and 90 days of daily estrogen treatment decreased PPT mRNA levels in both replacement groups compared to those of the OVX groups. After 56 days the result is apparent.

Other than means, statistical analysis was not performed for the 56-day group for the following reason. This was because only a small tissue section was available for one animal (please see Methods) and only five data points were collected from one slide. Due to the restrictions of the statistical test, i.e., an entirely balanced format, the decision was made to include data collected at the rate of 10 data points per slide per animal for all other slides rather than discard half of the data. (115 data points were collected rather than 120). However, no portion of this data is inconsistent with that for the 90-day group in which PPT mRNA levels in both replacement groups were decreased in comparison to that of the OVX group. It is important to note that the replacement effect was not dose dependent since significant differences were not found between the two replacement groups.

Evidence from the current study support the results of an earlier, short-term study showing decreased substance P immunoreactivity in lumbar DRG neurons after 21 days of continuous estradiol replacement (Sarajari and Oblinger, 1995). Substance P is involved in the mediation of nociception and is characteristic of small diameter axons from correspondingly small DRG neurons. A reduction in PPT gene expression and

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consequent decrease in substance P peptide levels should increase nociceptive thresholds, i.e. make the neurons less sensitive to painful stimuli. The results of the testing, in the current study, using Hargreaves' method of testing warm thermal withdrawal latencies generally uphold this expectation.

Warm Thermal Plantar Withdrawal Latencies

The OVX rats that received replacement estrogen showed a tendency to be less sensitive to noxious warm thermal stimuli as indicated by the time it took them to withdraw their hindlimb after onset of painful plantar stimulation. Although means for the 10X replacement group were higher than means for the 1X group, which, in turn, were higher than the OVX group the differences between the means were not significant.

The lack of statistically significant differences between treatment groups and the untreated OVX group may be due to inherent limitations in the apparatus and its measuring procedures. The phenomenon of "spontaneous walk-offs", which could occur within seconds of applying the stimulus are difficult to control for and as such, could not be separated from actual stimulus-induced withdrawals. Behavior such as paw licking or rapid jerks removing the foot from the heat source were often present as an indication of a response to noxious warm thermal stimuli. Conversely, a low-latency walk-off time with no apparent reaction to a noxious stimuli was suspicious as producing a false data point, yet lacked clear objective reason to be discarded as false. This variability accounted for a portion of the standard deviation. Since a learning effect was demonstrated, i.e. a quicker withdrawal response in all groups as the study progressed, retests for suspicious data points were also not appropriate.

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A second issue must also be considered in the interpretation of these data. Although the Hargreaves' device is referred to as a warm thermal testing apparatus, it is somewhat of a misnomer. It more appropriately measures withdrawal from noxious warm stimuli. Kenshalo (1979) points out, in his review article, that psychophysical testing, i.e. detecting the presence of a warm stimuli, discriminating temperature changes in warm stimuli and measuring withdrawal times from a noxious warm stimuli all have overlapping components but are distinctly different types of discriminations. When appropriate, controls separating motivational components are used, tests in humans show that elderly females (compared to elderly males and younger males and females) have a reduced ability to sense pain from warm thermal stimuli (Clark and Mehl, 1971).

During the period of peak estrogen levels, the proestrous phase of the normal rat cycle, sensitivity to pain measured by thermal withdrawal (tailflick) was reported to be greatest (Frye, 1992). Although the results of the current study indicate a reduction in sensitivity to noxious warm stimuli with long-term continuously replaced estrogen, one must consider the potentially different impact of estrogen titers that rise and fall within days and an estrogen serum level that remains constant for a extended period. Indeed, in normally cycling animals, trkA mRNA levels are highest at proestrous, the time of peak circulating estrogen (Sohrabji, et al., 1994). From the current data, one would expect PPT mRNA levels to also be high at proestrous. If so, substance P levels would be elevated in the DRGs and pain sensitivity should be heightened as shown by Frye (1992). For some as yet unknown reason, long-term, constant estrogen levels decrease trkA and PPT gene expression and thereby reduce sensitivity to noxious warm stimuli.

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The foot withdrawal times show a longer latency when each groups' first day data is compared to its own seven-day mean. This apparently indicates a learning phenomenon, namely warmth paired with pain yields foot withdrawal to warmth discrimination. The role of the hippocampus in learning and memory is widely accepted. The role of estrogen on hippocampus is now being investigated in many laboratories(Klintsova, et al., 1995, Bettini, et al.,1992, Teyler, et al. 1980). Since the reduction in times within groups was not significantly different, no effects of estrogen on learning are apparent in this study.

Effect of Long-Term Estrogen Treatment on 68kD Neurofilament Gene Expression

Data from the current study show an estrogen-dependent increase in 68kD neurofilament mRNA levels. This response to estrogen appears to involve all or nearly all neurons. If only a subpopulation of DRG neurons express the ER gene, the dramatic effects of estrogen on neurofilament gene expression in all or most DRG is more difficult to explain.

The 56-day group analyzed for neurofilament gene expression showed highly significant results while the data from the 90-day group was not as clear-cut. Replacement groups, Groups I and II and 1 of 2 subjects included in the OVX group produced data equivalent to the 56-day study.

The other subject in the OVX group was clearly different and produced results which were not significant. There are several possible explanations for this finding. It may be an indication of a reversal of the 56-day results such as the biphasic responses reported for estrogen's effect on trkA mRNA levels in the forebrain (Gibbs, 1992). Another

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explanation may be that replacement estrogen responsiveness is innately variable. Authors in two studies (Sohrabji et al., 1994; Kow and Pfaff, 1973) make specific points in their discussions concerning the unexpectedly large variability of their measured dependent variables as of result of the administration of exogenous estrogen. This phenomenon was confirmed by a report at the recent, 1997 AFAR (American Federation of Aging Researech) meeting concerning replacement estrogen in humans (Kowas, personal communication). Either the very small subject number included in the OVX 90-day group could not show significant differences given a moderate amount of intrasubject variability, not atypical to results of other estrogen replacement experiments, or, the dose-dependent upregulation of neurofilament mRNA seen in the 56-day groups is not a feature of longer term treatment with the higher dose. In the case of the acetycholinesterase-estrogen relationship in forebrain, the time-course of a biphasic response is shortened as estrogen doses are increased (Luine, 1985).

Based on the population of the DRG neurons in which neurofilament was upregulated, the current study found that estrogen working through its effect on NGF receptors could not account for the dramatic increase in mRNA levels demonstrated. Virtually all large neurons displayed a climactic increase in the amount of neurofilament mRNA present following long-term estrogen treatment. Since exogenous NGF only restores neurofilament mRNA levels in NGF receptor-expressing neurons, predominantly smaller cells following axotomy (Verge, et al., 1990), estrogen must be working through a mechanism other than one associated with trkA gene expression.

Although it has been demonstrated that regulation of neurofilament by NGF molecules results in a coordinate upregulation of all three neurofilament subunits genes

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in vitro (Lindebaum, 1988), neurofilament subunit gene expression may not always be coordinately upregulated *in vivo*, as suggested by the work of Muma and colleagues (1990).

Typically, however, in the adult DRG, neurofilament subunit gene expression is coordinate and therefore, long-term estrogen treatment would be predicted to upregulate the 145kD and 200kD neurofilament genes. If so, one would predict further that treatment by estrogen would increase intermediate filament synthesis and assembly in DRG neurons. This would increase sensory axon diameters and consequently improve conduction velocities. This anticipated physiological correlate to estrogen treatment could easily be tested if the necessary testing equipment becomes available to the laboratory.

Use of Different Forms of Estrogen

Caution must be taken when comparing research studies that used different forms of estrogen. Premarin (Wyeth-Ayerst), which was the form of estrogen used in the current study, is a commercial formulation widely used in clinical estrogen replacement therapy. It is from natural sources, namely pregnant mare urine (from which its name is taken). It is composed of a mixture of conjugated estrogens. According to Wyeth-Ayerst, the manufacturer, it has five components. The major components are estrone, equilin and 17 α-dihydroequilin. The minor components are 17 α-estradiol equilenin and 17 α-dihydroequilenin. Washburn (1993) tested the components of Premarin. He examined ten forms of estrogen present in Premarin individually. This suggests that the five components not listed by Wyeth-Ayerst are either in such minute quantities that

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reporting their presence in the formulation is not required or that the five additional components tested by Washburn are breakdown metabolites of the five listed components.

According to Premarin's manufacturer, Wyeth-Ayerst, estrogens are produced by the ovaries, which make several kinds of estrogens. They further state that all estrogens have similar properties and therefore much the same usefulness, side effects and risks. While this is true at this time, it does not say that there are "no differences", only that they have not been well investigated. Two studies (Washburn, et al., 1993 and Mermelstein, 1996) have looked specifically for and have found differences in the relative effects of the various forms of estrogen in different estrogen-responsive tissues.

Differential effects of the individual Premarin components may allow benefits of estrogen to be administered to women otherwise at risk for breast or endometrial cancer. During the normal aging process, the composition of the circulating estrogen changes from predominantly estradiol to estrone (Erickson and Scheiber, 1995). This might suggest that the collateral benefits of estrogen to non-reproductive organs and tissues are maintained more by estrone than by estradiol.

Is estrogen a nervous tissue growth factor and does it interact with growth factors other than NGF? It is known that estrogen promotes the growth of some tumors in breast and endometrium and therefore, acts as a growth factor in these tissues. One study has indicated a possible connection with insulin-like growth factor (IGF) in the nervous system (Toran-Allerand et al, 1988). In that study, explants of hypothalamus and forebrain were used. The data from the study clearly suggested that estrogen may act synergistically with growth factors other than NGF, such as IGF. This may be true

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for the DRG where neurons are known to be responsive to members of the insulin gene family.

Resolution of Apparently Conflicting Results

How can results (Sohrabji, et al, 1994) demonstrating an upregulation in trkA in the DRG after estrogen administration be reconciled with the results of the current study? The time course and dosage are apparently critical. Demonstration of a biphasic response to estrogen in the CNS first emerged with the the interaction of estrogen and choline acetyl transferase (ChAT) in basal forebrain. Not only was the measured effect reversed after long-term exposure, but the time course of this reversal was dose dependent (Liune, 1985).

The emerging story on the low affinity NGF receptor has given us additional clues. Recent data examining the effect of extended administration of estrogen on p75 in basal forebrain neurons parallels the findings in the current study. This study (Gibbs and Pfaff, 1992) found that after two weeks of estrogen replacement in rat the activity of p75 was reversed compared to that found at earlier times .

A consistent story is emerging which seems to reconcile the apparently contradictory results of previous and current studies. Firstly, trkA and p75 act inversely at each group of time points (short term vs. long term) and conversely from short term to long term. Our report examined only trkA, but the combined data of several labs now makes sense.

Short-term exogenous estrogen replacement (approximating the timing of the normal rat estrus cycle) as well as endogenous high levels of estrogen (proestrous levels)

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in normally cycling rats result in an upregulation of trkA and a downregulation of p75 in the basal forebrain and lumbar DRGs (Gibbs, 1992, Toran-Allerand, 1992 and Sohrabji, 1994). In contrast, long term, continuous estrogen replacement results in a down-regulation of trkA (Gibbs and Pfaff, 1992) in forebrain and in DRG (current study) and an upregulation of p75 in forebrain (Gibbs, 1992).

An analysis of known data concerning substance P, PPT and the resultant response of withdrawal to noxious warm stimuli follows logically. Over the short term an upregulation of trkA during proestrous would expectedly yield an upregulation of substance P resulting in greater sensitivity to noxious warm stimuli (Frye, 1988) during normal cycling. Conversely, over the long-term a down-regulation of trkA would be expected to yield a down-regulation of substance P in DRG (Sarajari and Oblinger, 1995) and PPT in DRG (current study) as well as diminished sensitivity to noxious warm stimuli (current study).

The results show that the DRG cell populations containing ER, trkA and PPT mRNAs are overlapping. Many small DRG neurons contain ER, PPT (substance P) and trkA. In the current study neither the regulation of PPT nor the regulation of trkA mRNA levels by estrogen was dose-dependent.

The cell population and the dynamics involving NGF and neurofilament (Hoffman, 1984, 1985, and 1987) and estrogen, NGF (receptor) and neurofilament in this study are very different from the cell population and dynamics involved with NGF (receptor) estrogen and PPT. Neurofilament mRNA levels after long-term (56-day) estrogen replacement are increased and are dose dependent. Whether the time course is merely different from that seen for PPT and trkA, and that an eventual down-regulation

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for neurofilament occurs with continuous replacement is uncertain. Although the mean mRNA level for the 90-day 10X is much higher than that for either the 1X or the OVX group, the mean disguises the fact that the animals within this group reflect very different means (variability) and hence the 10X group is not statistically different from the 1X group. Whether this reflects a deterioration of the phenomenon observed at 56 days or not cannot be determined at this point.

Alterations in sensory function resulting from changes in estrogen levels are largely unexplored. A few scattered papers indicate intriguing possibilities in several sensory modalities. Hearing might be affected by estrogen. ERs have been found recently in the cochlear nucleus (Simerly, 1990) and estrogen levels have been reported to affect . In the auditory system, one study (Coleman, et al., 1994) has reported differences based on estrogen status. The results of this study provide a significant and positive result which has many ramifications and opens the possibilities for broadened investigations on the effects of estrogen on sensation. Finally, the hypothesis can be raised, does estrogen affect sensation in males, and how do other hormones affect sensation? It is entirely unknown whether males have estrogen receptors on sensory cells. Whether or not parallel processes occur in males with different hormones is also unknown. What is known is that males convert testosterone to estrogen with the enzyme aromatase in certain basal forebrain nuclei, i.e. a mechanism exists in the nervous system of males which makes testosterone conversion to estrogen a possibility. Also, males show an increase in cutaneous receptive field after the administration of exogenous estrogen (Kow and Pfaff, 1973). Considering the highly homologous nature of all

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steroid hormones and their receptors, it is not unlikely that some non-specific binding may occur between hormones and their ligands.

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CONCLUSIONS

Traditionally, the effects of estrogen in brain have been characterized as effects associated with reproduction. The role of the hypothalamus as a regulator of the reproductive cycle is well documented. Yet, perhaps it is our definition of reproductive behavior which needs to be re-examined. In thė animal studies in which an increase in skin sensitivity was shown to occur as a result of estrogen, behavior indicating the female's acceptance of the male can be identified as the probable link to reproduction. Nesting behavior, a secondary reproductive function was documented in the canary brood patch study. In the current Physicians Desk Reference's review on Premarin, it states that one of the effects of this hormone replacement is a dramatic increase in the number of reports of sexual fantasy. The possible number of synapses which are involved in this neural activity are imponderable, yet somehow occur as a result of estrogen replacement. When we consider that the role of sex is, of course, directly responsible for the survival of the species, it makes sense that the molecule of estrogen is probably as old as sexual reproduction itself and that its role within each species has evolved as well. What we define as sexual behavior may be broadened to include many behaviors secondary to the reproductive cycle, per se.

During the normal process of aging the primary reproductive function for females ceases. The point is, of course, that the role of estrogen in the normal aging body has many overlapping functions with that of secondary sexual and reproductive behavior. For example, skin sensitivity, which is important in mating and nesting behavior, is important during all ages of one's life.

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The somatosensory sensations include vibration sense, two-point touch discrimination, pain and temperature as well as proprioception. These sensations make use of different subsets of receptors and are subserved by different groups of DRG neurons. The experiments in this paper have begun to sort out how long-term continuous estrogen replacement affects these DRG neurons and the sensations which they carry.

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APPENDICES

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Protocol for Riboprobe Synthesis

Dry down even aliquots of 33P in speed-vac for 30 minutes.

Set up 10 ul transcription rxns (n + 1):

X1	X____
2 ul transcription buffer	______
1 ul DTT	______
1 ul Rnasin	______
0.5 ul rATP	______
0.5 ul rCTP	______
0.5 ul rGTP	______
1.5 ul sH2O	______

Add 7 ul transcription rxn to dried down isotope and vortex.

Add 2 ul of (2ug/ul) DNA template (sense and antisense of each).

Add 1 ul of appropriate polymerase enzyme to appropriate tube:

DNA	Polymerase Enzyme
______	______
______	______
______	______
______	______
______	______
______	______

Incubate tubes for 1 hour at 37°C.

Tough rxn mixture with microtip and rinse in 5 ul of DEPC H2O. Spot 2 ul on chromotography strip and allow to air dry. Place spotted end in 1M KH2PO4 and allow buffer to run to top of strip.

Air dry strip and expose to film for 30 minutes.

Stop transcription rxn by adding 1 ul Dnase to each tube and incubate for 15 min at 37°C.

Add 30 ul DEPC H2O to bring to final volume of 40 ul per tube.

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Protocol for *in situ* Hybridization

Deparaffinate:	Xylene	5 min	3X
	100 % ETOH	3 min	2X
	95% ETOH	3 min	1X
	70% ETOH	3 min	1X
	50% ETOH	3 min	1X

PRE-HYBRIDIZATION

1. Incubate sections in 0.02M HCL 10 min at RT. (Partially solubilizes the highly cross-linked acidic nuclear proteins, enabling easier access to the probe.)
(300 ul HCL in 200 mls DEPC H2O)

2. Wash in 1X PBS 5 min, 2X.
(20 ml 10X PBS in 180 ml DEPC H2O)

3. Wash in 1X PBS that is 0.01% Triton X-100 90 sec.
(20 ul in 200 ml 1X PBS)

4. Rinse briefly in DEPC H2O.

5. Treat with 2X SSC at 70°C 30 min. (Loosens lipid membrane proteins.)
(20 ml 20X SSC in 180 ml DEPC H2O)

6. Proteinase K treat at 37°C 30 min. (Degrades many of the cross-linked proteins.)
(20 ml 1M Tris, 20 ml 0.5M EDTA in 160 ml DEPC H2O. Add 100-200 ul of 10 mg/ml Proteinase K just prior to adding slides.)

7. Rinse briefly in DEPC H2O.

8. Fix in 4% formaldehyde or paraformaldehyde, 1X PBS 10 min. (Helps to prevent loss of nucleic acid sequences during remaining steps.)
(180 ml 4% formaldehyde plus 20 ml 1X PBS)

9. Rinse briefly in 1X PBS.

10. Acetylate 10 min while shaking 0.1M TEA with acetic anhydride added just before slides. (Reduces level of endogenous alkaline phosphatase in the tissue.)
(20 ml 1M TEA in 180 ml DEPC H2O plus 500 ul acetic anhydride added just prior to adding slides.)

11. Rinse in 2X SSC 1 min.

12. Serially dehydrate in ETOH:

70%	1-5 min
80%	1 min
95%	1 min
100%	1 min

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VITA

Sheila Anne Scoville was born in Grand Rapids, Minnesota on March 1, 1948. The author received a Bachelor's of Science from the University of Wisconsin at La Crosse in 1969, and a Master's of Science from The Florida State University, Tallahassee, Florida, in 1972.

At Florida State she was awarded a National Science Foundation Fellowship. For the next ten years she was employed by the Department of Neurosurgery at the University of Virginia, Charlottesville, Va. Two of these years of employment were spent in the Bahamas and Puerto Rico working on a project on the evolution of primitive vertebrate brains, especially sharks.

She married E. Martin Schara in 1981. They have a daughter, Brooke. At this time she became active in horticulture. She managed one her husband's businesses which was a chain of retail greenhouses.

In 1991 she began working at the Eastern Virginia School of Medicine in the Department of Neurosurgery where she conducted research jointly with the Department of Anatomy. She is a member of the Sigma Xi Scientific Research Society.

The author has received a tuition stipend from the Eastern Virginia School of Medicine and the Old Dominion University from 1993-1997. Additionally she received a scholarship award for from the Glenn Foundation of the American Federation of Aging Research, AFAR.

The following is list of publications selected from a total of 12 publications.

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