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A DEVELOPMENTAL STUDY OF THE NMDA RECEPTOR

by

KATHERINE M. FLYNN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

A DEVELOPMENTAL STUDY OF THE NMDA RECEPTOR

by

Katherine M. Flynn

Adviser: Professor Martin P. Schreibman

The N-methyl-*D*-aspartate type of glutamate receptor (NMDAR) is involved in the regulation of many neural functions, including neuroendocrine modulation of the vertebrate brain-pituitary-gonad axis. We have used a freshwater teleost, the platyfish (*Xiphophorus maculatus)*, to document age- and gender-specific variations in NMDAR properties. We first localized immunoreactive (ir-) NMDAR in male and female platyfish brain at four stages of the lifespan. Distribution was limited to cells of the nucleus olfactoretinalis (NOR), a gonadotropin releasing hormone-containing nucleus. The number of ir-NMDAR cells was increased in pubescent and mature females when compared to immature and senescent animals. In males, there was no significant change in ir-NMDAR expression in the NOR at any time in their lifespan. The affinity of the antagonist MK-801 for the NMDA receptor, an indicator of subunit composition, was increased only in pubescent females. Maximum binding of MK-801 to NMDAR also varied with female development, reaching a significant maximum in mature females. In

males, both maximum MK-801 binding and MK-801 affinity remained unchanged throughout development. Chronic injections of MK-801 to immature male and female platyfish resulted in a dose-dependent decrease in both the number of females in puberty and in their average size. Male puberty and growth were unaffected. Radioimmunoassay indicated an increased GnRH content in brain extracts from MK-801 treated females and no effect on GnRH content in treated males. Histological analysis showed decreased size of the gonadotropic zone in the anterior pituitary gland of treated females. There was no evidence that MK-801 directly influences the gonads. These studies are the first evidence of a sexually dimorphic relationship between the timing of NMDA receptor dynamics and neuroendocrine activity in the brain-pituitary gonad axis.

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List of Abbreviations

ANOVA - one way analysis of variance

B_{max} - binding maximum

Bsp - specific binding

Bt - total binding

CNS - central nervous system

cpm - counts per minute

CRH - corticotropin releasing hormone

DAB - 3,3'-diaminobenzidine tetrahydrochloride

EM - early maturer

GH - growth hormone

GnRH - gonadotropin releasing hormone

GtH - gonadotropic hormone(s)

ICC - immunocytochemistry

imm - immature

K_d - dissociation constant

LH - luteinizing hormone

LM - late maturer

mat - mature

MK-801 - dizocilpine maleate

NLT - nucleus lateralis tuberis

NMDA - N-methyl-D-aspartate

NMDAR - N-methyl-D-aspartate receptor

NMDA R1 - R1 subunit of the NMDA receptor

NMDA R2 - R2 subunit of the NMDA receptor

NOR - nucleus olfactoretinalis

NPO - nucleus preopticus

NPP - nucleus preopticus periventricularis

pube - pubescent

R1 - R1 subunit of the NMDA receptor

R2 - R2 subunit of the NMDA receptor

RIA - radioimmunoassay

RPD - rostral pars distalis of the pituitary gland

SD - standard deviation

sen - senescent

INTRODUCTION

I. A Brief History of Neuroendocrine Physiology

In the early 1930s, the pituitary gland was called the "master gland," as it was believed to orchestrate all systemic endocrine functions. While portal vessels linking the pituitary and brain were also discovered in the 1930s, the idea of brain regulation over the gland was not established until blood flow from the brain to the pituitary gland was demonstrated. In the mid 1930s, Houssay and colleagues demonstrated this phenomenon in the toad, and in the late 1940s unidirectional blood flow from hypothalamus to pituitary was confirmed in the rat by Green and Harris [(Sawyer, 1988) for review].

The demonstration of neural regulation over the pituitary gland was also established in the 1940's in a series of experiments by G.W. Harris (1961). In several elegant studies he demonstrated that severing the connection between the brain and the pituitary resulted in a loss of pituitary function, and that regeneration of the portal blood vessels linking the brain to the pituitary reestablished the function of the gland. This work set the stage for the view that one brain function is as a neuroendocrine organ, releasing chemical modulators which travel through the bloodstream to regulate the functions of specific target tissue. The significance of neuroendocrine regulation is exemplified by the remarkable similarities found among the neuroendocrine systems in virtually all multicellular animals.

The neurosecretory cells of the brain are modified neurons, with physiological and morphological characteristics of both neurons and endocrine cells. Most neurosecretory cells make and release modulators that are peptides, though there are examples of other chemical classes (e.g., dopamine). Both neurons and neuroendocrine cells are polar, with anatomically distinct sites of synthesis and release of peptide modulators; synthesis occurs in the cell bodies and release at the axon terminals. We need to credit the pioneering work of the Scharrers and Bargmann for introducing the concept of the neurosecretory cell and its widespread phylogenetic distribution [(Sawyer, 1988; Schreibman, 1986) for review].

The 1950's and 60s saw the isolation and characterization of a number of releasing and inhibiting neurohormones from the hypothalamus [(Sawyer, 1988) for review]. It soon became clear that a primary hypothalamic function is regulation of the pituitary gland. In all vertebrate classes except the cyclostomes (hagfish and lamprey) and the teleosts (bony fishes), the adenohypophysis, or anterior lobe of the pituitary gland, receives its hypothalamic input through the hypothalamo-hypophysial portal blood vessels of the median eminence [(Schreibman, 1986) for review]. In teleosts, there are no portal blood vessels and no median eminence. Instead there is direct innervation of the adenohypophysis by hypothalamic neurons that terminate directly on or near pituitary cells (Kaul and Vollrath, 1974).

Recent evidence indicates the presence of numerous neurotransmitters of several chemical classes including neuropeptide Y, glutamate, and γ-aminobutyric acid (GABA), in mammalian hypothalamus (Brann and Mahesh, 1997; Buijs *et al.*,

1994; Freeman, 1993). In the neuroendocrine areas of several fish species, including platyfish, a similar variety of neurotransmitters, also including FMRF-amide, neurotensin, galanin, and dynorphin have been found (Magliulo-Cepriano *et al.*, 1993; Magliulo-Cepriano and Schreibman, 1993) [(Kah *et al.*, 1993; Matsutami *et al.*, 1986) for review]. The primary excitatory neurotransmitters in the neuroendocrine system are however, the amino acids glutamate and aspartate (van den Pol *et al.*, 1990). Both are found throughout the rat hypothalamus, but glutamate is found in much higher concentration (van den Pol, 1991). Glutamate has also been shown to influence production and/or release of several neurohormones including gonadotropin releasing hormone (GnRH) and corticotropin releasing hormone (CRH) [(Brann and Mahesh, 1994; Zanisi *et al.*, 1994) for review].

II. The Neurotransmitter Glutamate

Glutamate, a simple amino acid, is the major excitatory neurotransmitter in the vertebrate central nervous system (CNS) (Cunningham *et al.*, 1994). There are several areas of glutamate localization or glutamate "pools" in CNS cells including a neurotransmitter pool, a metabolic pool, and a GABA precursor pool in neurons; and a glial pool in astrocytes (Erecinska and Silver, 1990). Regardless of location, glutamate is primarily synthesized from the precursor glutamine, by the enzyme glutaminase. Glial cells are critical in determining glutamate concentrations as they are the only source of glutamine (Martinez-Hernandez *et al.*, 1977).

In keeping with its widespread distribution, glutamate modulates a number

of diverse CNS functions in several species. In goldfish, rats, and humans glutamate involvement in learning and memory is well documented (Davis and Klinger, 1995; Wozniak *et al.,* 1990) [(Baron *et al.,* 1996) for review]. Glutamate activity is also implicated in several neurological and psychiatric disorders including Alzheimer's disease, epilepsy, and schizophrenia [(Greenamyre and Young, 1989; Dingledine *et al.,* 1990; Banerjee *et al.,* 1995) respectively]. Glutamate involvement in the brain-pituitary-gonad (BPG) axis is also well established in several classes of vertebrates (Ebling and Cronin, 1998; Flett *et al.,* 1994; Johnson *et al.,* 1996). A hypothalamus-dependent increase in serum levels of gonadotropic hormone (GtH) following glutamate stimulation has been repeatedly demonstrated in mammals [(Brann and Mahesh, 1994; Zanisi *et al.,* 1994) for review]. In the rat, immunoreactive (ir-) glutamate has been found in axonal boutons of several GnRH-containing nuclei of the hypothalamus including the supraoptic, suprachiasmatic, and paraventricular nuclei (van den Pol, 1991). In immature rainbow trout, increased plasma levels of pituitary GtH followed glutamate administration (Flett *et al.,* 1994).

Suspected of contributing to its great diversity are the several discrete receptor types that glutamate interacts with. The receptors are divided into two broad classes, metabotropic and ionotropic. The metabotropic glutamate receptors are coupled to G proteins and they act by modulating intracellular second messengers like inositol phosphate and adenylate cyclase. The ionotropic receptors contain integral, cation-specific ion channels and exist in both open and closed conformations. Three ionotropic receptors are currently recognized. They are

named for their selective exogenous ligands, the NMDA (N-methyl-D-aspartate), AMPA/quisqualate (alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionate/quisqualate), and kainate (kainic acid) receptors.

III. The NMDA Receptor

The NMDA receptor is a large transmembrane protein with a molecular mass of over 100,000 daltons. The physiologically active receptor is a heterodimer, made of two different subunits, NMDA R1 (R1) and NMDA R2 (R2). R1 is the product of a single gene, but it exists in many variations as a result of differential mRNA splicing (Hollmann *et al.,* 1993). R2 is known to exist in four forms, R2A - R2D, each the product of a different gene (Anson *et al.,* 1998). The obligatory subunit is R1; it has both pharmacological and electrophysiological activity when expressed without R2, but R2 has no activity if expressed without R1 (Moriyoshi *et al.,* 1991).

Receptors composed of the different splice variants of R1 have different sensitivities to agonists and antagonists, and combinations of these variants with the different R2 isomers also produce receptor complexes with differing sensitivities (Hollmann *et al.,* 1993). This suggests that an extensive and heterogeneous population of NMDA receptors can exist in the brain, formed by different combinations of R1 and R2. The physiological significance of isoform composition of NMDA receptors is not yet known.

The NMDA receptor has at least five pharmacologically distinct binding sites.

There is a site for the ligand glutamate, a modulatory site where the amino acid glycine binds, a site in the ion channel where exogenous blockers like phencyclidine, MK-801, and other related compounds bind, a voltage dependent Mg^{2+} binding site, and a site for modulation by Zn^{2+} (Cunningham *et al.,* 1994). There are several conditions required for the change in R1 and R2 conformation that causes ion channel opening. They include binding of the glutamate ligand or neurotransmitter and prior depolarization of the postsynaptic cell membrane (Michaelis, 1996).

The NMDA ion channel is permeable to Ca^{2+}, K^{+}, and Na^{+} (Figure 1), but Ca^{2+} is by far the most permeable cation as evidenced by the increased Ca^{2+} current following NMDA receptor stimulation (Cunningham *et al.,* 1994). This increase in intracellular Ca^{2+} stimulates calmodulin, which in turn can stimulate the production of nitric oxide (Bresink *et al.,* 1995). This process is thought to be responsible for glutamate-mediated neurotoxicity (ibid.). Interestingly, glutamate-mediated communication also follows Ca2+ entry through the ion channel (Cunningham *et al.,* 1994).

Several reports suggest that the pulsatile GnRH secretion that initiates puberty, maintenance of reproductive capability, and eventual age-related reproductive demise is influenced, and perhaps regulated, by NMDA receptors [(Brann and Mahesh, 1994; Brann and Mahesh, 1997) for review]. Evidence in fishes, birds, amphibians, and mammals suggests that glutamate acts through the NMDA receptor to modulate neural regulation of the BPG axis (Figure 2) (Aamodt *et al.,* 1995; Cline *et al.,* 1994; Flynn *et al.,* 1997; Gore *et al.,* 1996;

Johnson *et al.,* 1996; Petralia *et al.,* 1994). The R1 subunit of the NMDA receptor protein has been localized in cell bodies of several hypothalamic nuclei in fish, rats, and humans (Flynn *et al.,* 1997; Johnson *et al.,* 1996; Petralia *et al.,* 1994). In platyfish, zebra finches, and rats, NMDA receptor properties have been shown to change at sexual maturity (Aamodt *et al.,* 1995; Flynn *et al.,* 1996; Flynn *et al.,* 1998; Gore *et al.,* 1996). NMDA injections to sexually regressed Syrian hamsters will activate their reproductive systems, as measured by gonad size and histology, and by testosterone secretion (Urbanski *et al.,* 1993). In addition, NMDA receptor stimulation with the highly specific ligand N-methyl-D,L-aspartate (NMA) has been shown to increase pulsatile GnRH secretion, *in vitro*, from mammalian hypothalamic explants (Bourguignon *et al.,* 1993). In maturing female rats, an increased expression of kainate receptor mRNA has also recently been demonstrated (Eyigor and Jennes, 1997). By far, most studies on NMDA regulation of the reproductive system have been done in rats.

There is an overall lack of information on the biology of NMDA receptors in non-mammals, and this prevents a comparative view of NMDA interaction with the brain-pituitary-gonad axis. In several species of fishes, NMDA receptors have been localized in GnRH-containing neuroendocrine brain areas (Bottai *et al.,* 1997; Flett *et al.,* 1994; Flynn *et al.,* 1997). In the only study on the relationship between development and NMDA function in a non-mammal, a combined study of male and female platyfish in our laboratory recently demonstrated an increase in R1 protein

localization in GnRH-containing cells at puberty, and a decrease at senescence (Flynn *et al.*, 1997).

IV. The Platyfish Model

a. Animals

The platyfish, *Xiphophorus maculatus*, is a small freshwater teleost native to southern Mexico. Platyfish are excellent laboratory animals for a number of reasons including the following:

- they have been studied extensively for over 60 years,
- they have a relatively short average lifespan of 2.5 years,
- they breed continuously in captivity throughout the year,
- they have 20 to 40 offspring per brood,
- they may produce multiple broods from a single insemination,
- they may have new broods as often as every 28 days,
- their stage of maturity may be determined by external observation, and
- their age at sexual maturation is under genetic control.

Furthermore, platyfish and other members of the genus *Xiphophorus* have a reproductive system which resembles that of higher vertebrates, including mammals, in the following ways:

- males and females have a sexually dimorphic body structure,
- males inseminate females by copulation,

- gestation occurs within the female,
- the female gives birth to free living young,

and

- the components and interactions of the BPG axis are similar.

The platyfish is particularly well suited to studying age- and gender-related events in the reproductive system. In fact, extensive longitudinal studies have established a virtual calendar of neuroendocrine events in platyfish. As in mammals, maturational changes in platyfish reproductive development depend on stepwise developmental events in GnRH centers in the brain and GtH-producing cells in the pituitary gland, culminating in an adult gonad capable of producing steroid hormones and functional gametes (Halpern-Sebold and Schreibman, 1983; Schreibman and Margolis-Nunno, 1987). Our laboratory has shown that in both males and females three brain nuclei, and then the pituitary gland, display the various forms of the GnRH decapeptide in a sequential manner as development proceeds (ibid.).

The nucleus olfactoretinalis (NOR), located bilaterally at the base of the telencephalon, is the first brain area to contain GnRH. This is a relatively small nucleus of fixed size, 80 cell bodies on each side of the brain. GnRH localization follows in the nucleus preopticus periventricularis (NPP), located just anterior to the optic chiasm, and finally in the nucleus lateralis tuberis (NLT), located just dorsal to the pituitary gland (Figure 4A). In platyfish, the timing of GnRH expression in these three nuclei, and hence the timing of reproductive development, is a genetically

regulated process that may occur at widely disparate ages ranging from 8-9 weeks to 20-24 months. Nonetheless it is an identical process in all animals (Schreibman and Kallman, 1978).

In the platyfish NOR, at least two forms of the GnRH decapeptide are expressed when animals are just several weeks old (Halpern-Sebold *et al.,* 1986; Magliulo-Cepriano *et al.,* 1994). The two more caudal GnRH-containing neuroendocrine nuclei, the NPP and NLT, do not display any form of GnRH peptide until shortly before puberty. In early maturers this can occur as early as 5 weeks of age, while for late maturers it may not occur until as late as 14 months of age. The appearance of GnRH in the NPP and NLT correlates with developmental stage and not with chronological age, thus supporting the idea that the orderly appearance of GnRH in the three brain centers initiates the developmental changes in the reproductive system.

b. Genetics

In platyfish, age at sexual maturation, or puberty, is genetically controlled (Kallman *et al.,* 1973; Kallman and Borkoski, 1978). A sex-linked gene, P, determines the age at which maturity occurs. At least 9 P alleles have been identified in laboratory stocks (Kallman, 1984; Schreibman *et al.,* 1994) and, depending upon genotype, puberty will occur at a predictable time which varies from 8 to 104 weeks of age. The P alleles are closely linked to pigment genes which serve as phenotypic markers for indicating age at maturity early in life. The process

of sexual maturation is similar in fish with varying genotypes raised under identical conditions despite that the event may be several weeks, months, or even a year apart. The maturational changes that occur begin with neuroendocrine events and culminate in physically mature adults capable of reproduction.

The mating of our platyfish is carefully planned and executed to produce offspring of specific genotype and phenotype. We routinely breed the following animals, which we define as early maturers (EMs):

Phenotype	**Genotype**	**P Alleles**	**Age at Puberty**
spot sided (male)	X-Sp Y-Sr	$\underline{P^1P^2}$	14 weeks
spot sided (female)	X-Sp X-Sp	$\underline{P^1P^1}$	11 weeks

We routinely breed late maturers (LMs) as well:

Phenotype	**Genotype**	**P Alleles**	**Age at Puberty**
black band (male)	X-N Y-Sr	$\underline{P^5P^2}$	30 weeks
black band (female)	X-N X-N	$\underline{P^5P^5}$	34-104 weeks

We also produce broods that contain both EMs and LMs by performing the following cross:

	X-Sp Y-Sr	x	X-N X-N
Phenotype	spot sided (male)		black band (female)
P Alleles	P^1P^2		P^5P^5
Age at Puberty	14 weeks		34-104 weeks

The offspring of this cross will begin to mature at widely separated ages, and this can be predicted shortly after birth because the P allele is linked to body pigmentation patterns. These broods are invaluable to studies of reproductive system development and the BPG axis because they provide a mechanism for removing the effects of chronological age on physiological development.

c. Evaluating Stage of Development

Platyfish development may be monitored by noting the metamorphosis of the male anal fin into the gonopodium, a highly differentiated organ specialized for transmitting sperm into the female (Figure 3). The anal fin of male *Xiphophorus* undergoes an androgen-dependent metamorphosis that can be separated into six distinct stages which have been correlated to the onset of puberty and to stages of gonad development (Kallman, 1975). The development of the gonads is directly related to the appearance of GnRH in the pituitary gland and the concomitant

proliferation of pituitary gonadotropes and their secretion of gonadotropin. Gonopodium development, therefore, is an indirect, but reliable and non-invasive method of staging male platyfish according to their BPG axis development. The development of females may be estimated by monitoring their male broodmates of similar P allele genotype.

The average platyfish lifespan is 2.5 years. Little change in body structure occurs in aged animals of either sex. In senescent males, reproductive system function is maintained even in animals as old as 4.5 years as shown by the presence of ir-GtH and ir-GnRH in the pituitary gland and by continued sperm production in the testes (Schreibman *et al.,* 1983). Similar extensive longitudinal studies of age-related changes in the brain and pituitary of female platyfish have not been done. Senescent changes however, do occur in the gonads of both aged males and females. In both sexes there is atresia, increased connective tissue production, and formation of melanin deposits in the serosa surrounding the gonads (Schreibman *et al.,* 1991). As in several other vertebrate species, however, the age-related changes are far more extensive in females than they are in males.

V. Regulation of Development

In organisms ranging from snails (Janse *et al.,* 1996) to humans (Bjorntorp, 1995), hormonal, neurotransmitter, and histological changes have been shown to occur in the reproductive system with increasing age. When we discuss "aging" and "age-related change" we refer not only to senescent or "old" animals, but to the

entire life continuum, beginning at birth (and perhaps earlier) and ending with death. A popular theory of aging regulation suggests the existence of a "clock" in the hypothalamus where age-related changes in neurotransmitter function occur that lead to developmental changes and ultimately to the aging of the organism (Timiras, 1989). As the brain's neurotransmitter systems age they are likely to initiate age-related changes in other (and perhaps all) physiological systems.

The complexity of age-related neurotransmitter function is daunting. There are literally dozens of neurotransmitters which regulate a multiplicity of activities, and many of them have multiple receptors which exist in multiple forms. Numerous age-related changes have been shown to occur in neurotransmitter, neurohormone, and hormone levels in both mammals (Timiras, 1989; Vom Saal and Finch, 1988) and fish (Schreibman *et al.,* 1987; Schreibman *et al.,* 1991), and may be a mechanism for the genetic control of major life cycle events. In platyfish, both neurotransmitter localization in neuroendocrine brain nuclei and in the pituitary gland, and steroid hormone and gamete production by the gonads, have been shown to vary with age [(Schreibman *et al.,* 1991) for review]. In humans, concentrations of several hormones including growth hormone and the sex steroids, decrease before characteristic aging changes begin (Bjorntorp, 1995). In the rat hypothalamus and striatum, decreased glutamate release occurs with increasing age (Exposito *et al.,* 1995; Porras and Mora, 1995). As mice age, binding properties of the NMDA receptor change in both cortex and hippocampus (Magnusson, 1995).

Gender-specific changes in NMDA activity during mammalian development

and aging are implied by the literature but have not been thoroughly investigated. In female rats, an increased expression of NMDA R1 protein and mRNA levels at puberty indicates the possibility of an increase in receptor stimulation (Gore *et al.*, 1996). No comparable study has been done in males. In mature but sexually regressed male hamsters, an increased production of testosterone follows NMDA receptor stimulation (Urbanski *et al.*, 1993). In immature male rats, however, a decrease in response to NMDA stimulation has been reported (Otoya *et al.*, 1996). Neither of these groups has examined NMDA activity in females. In hypothalamic explants from senescent rats of both sexes, NMDA injections stimulate less GnRH and luteinizing hormone (LH) release than in mature animals (Arias *et al.*, 1996; Sortino *et al.*, 1996).

These studies suggest both gender- and age-related differences in NMDA function in the BPG axis. However, it is difficult to draw general conclusions about NMDA activity from studies that employ different techniques, different animal systems, and different stages in the lifespan. Our study addresses these issues by using the same techniques to examine NMDA properties throughout the lifespan of both males and females in a single organism.

VI. Objectives

It is clear that the neuroendocrine control of reproduction in both teleosts and mammals is multifactorial and highly complex. The unraveling of the myriad interactions that result in the development, functioning, and decline of the

reproductive system has been the object of decades of scientific study and will, no doubt, continue to be so for many more. This report seeks to expand our knowledge by addressing the following specific questions:

- What is the distribution of the R1 subunit of the NMDA receptor in the platyfish?
- Is this distribution sexually dimorphic? Does it change during the lifespan?
- What are the binding properties (binding maximum and dissociation constant) of the NMDA receptor in the platyfish?
- Are these properties sexually dimorphic? Do they change during the lifespan?
- What are the effects on the BPG axis of chronic NMDA receptor antagonism?
- Are these effects sexually dimorphic?
- How does this information contribute to our understanding of the genetic and neuroendocrine control of reproductive system development, function, and decline in the platyfish?

MATERIALS AND METHODS

I. Animals

Male and female platyfish (*Xiphophorus maculatus*), derived from genetically defined JP 163 stocks which originated at the Genetics Laboratory of the Osborn Laboratories of Marine Sciences at the New York Aquarium for Wildlife Conservation, have been maintained in our laboratories since the early 1970s and inbred for up to 80 generations by brother and sister matings. For this study, all animals were kept at a ratio of one fish per gallon in aquaria containing plants, gravel, snails, and aged tap water at 27° C. They received 16 hours of artificial light per day and were fed a beef liver-cereal paste or live brine shrimp nauplii, supplemented by dried flake food three times per day. Animals were either of the early maturing (EM) phenotype, which begin puberty at about 8 weeks of age and are completely mature at 3-4 months, or of the late maturing (LM) phenotype, which enter puberty at about 9 months of age and are completely mature at 12-15 months (Schreibman and Kallman, 1977; Schreibman and Kallman, 1978). Maturation was identified by monitoring the metamorphosis of the male anal fin into the specialized gonopodium (see Introduction).

Animals were staged at autopsy according to gonadal development and anal fin metamorphosis (Table 1). Immature animals were defined as those with small, transparent, and undifferentiated gonads and no metamorphosis of the anal fin in

males. Pubescent animals were defined as those who had begun, but not completed, reproductive maturation. Females would have a mixture of mature, large yolky oocytes and immature, small white oocytes, and males would have enlarged testes and a gonopodium at stage 2 or 3 (Figure 3). Mature animals were defined as those with large and fully mature gonads. Female ovaries contained all yolky oocytes and males had large, translucent, sperm-containing testes and a fully differentiated, stage 6 gonopodium. Senescent animals were defined as those at least 2 years old. Females had ovaries with a mixture of large yolky and small atretic oocytes and males had mature, translucent testes. Both sexes had age-related melanin deposition on the peritoneum surrounding the gonads and some gonad atresia.

II. Methods of Analysis

a. Histology and Immunocytochemistry

Seventy five male and female platyfish, comprised of EMs and LMs, were sacrificed by decapitation at one of the following developmental stages/ages; immature (n=23) were between 1 and 6 months old, pubescent (n=16) were between 4 and 5 months old, mature (n=22) were between 3.5 and 10 months old, and senescent (n=14) were between 24 and 36 months old. Heads were immediately fixed in Bouin's solution under vacuum, decalcified (S/P Decalcifying Solution; Baxter, McGaw Park, IL), dehydrated in a graded ethanol and butanol (Zirkle) series, and embedded in Polyfin (Triangle Biomedical Supplies, Durham, NC). Five micrometer thick, serial, sagittal sections were mounted on gelatin coated slides.

Every fifth section was stained with Masson's trichrome for cytological evaluation; the remaining sections were used for immunocytochemical (ICC) analysis with the avidin-biotin method (Vectastain-Elite; Vector Laboratories, Burlingame, CA) as modified for our material (Margolis-Kazan and Schreibman, 1981; Margolis-Kazan *et al.*, 1981). Sites of antigen localization were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO) in Tris buffer with 0.0125% H_2O_2 at pH 7.4. Antisera to a synthetic peptide corresponding to the C terminus of the R1 subunit of the NMDA receptor (anti-NMDA R1; Chemicon, Temecula, CA) was used at a 1:100 or 1:250 dilution. As characterized by the manufacturer, anti-NMDA R1 recognizes 4 splice variants of the R1 subunit and does not cross react with any other glutamate receptor subunits.

Control procedures included the replacement of the primary antibody with normal rabbit serum, the elimination of one of the sequential steps in the ICC procedure, and the pre-absorption of anti-NMDA R1 with a synthetic fragment of the receptor subunit (provided by Dr. R. Wenthold, National Institutes of Health). All control procedures resulted in the elimination of the immunoreaction.

All cells displaying ir-NMDA R1 staining were counted at 1000X magnification. Complete counting was insured and double counting avoided by using alternate 5 micrometer serial sections of all NOR-containing brain regions, and counting only those perikarya which contained a large and easily visible nucleus (standard nucleus size = 5 micrometers). The average number of stained NOR cells

and the standard deviation were calculated for males and females of each developmental stage. The differences among groups were compared with one way analysis of variance (ANOVA), and a significant difference was considered to be one with a confidence interval of 99% or a p value of 0.01 or less.

b. Receptor Binding

90 EM and LM platyfish of the genetically defined JP163 stocks born between May 1993 and August 1995, were raised under standard conditions as described above. A minimum of 3 male or female broodmates were sacrificed at a time by decapitation. They were identified and described as immature, pubescent, mature, or senescent by gonad and/or gonopodium examination at autopsy (see description above). Brains were immediately removed from the cranium and frozen at minus 80° C. Assays were performed within 2 weeks according to methods in standard use by Banerjee and colleagues (Banerjee *et al.*, 1995; Lidsky *et al.*, 1993). Tissues were homogenized in 20 volumes of ice cold TRIS-EDTA buffer (pH 7.4) containing 20 mM Hepes using a Polytron PT-20 Homogenizer (Brinkmann Instruments, Inc., Westbury, NY) at half maximal setting for 30 seconds. Homogenates were centrifuged twice at 48,000 g at 4° C for 20 minutes, each time saving the pellet, and then re-suspending it in the same buffer at a concentration of 1-2 mg of tissue per ml (wet weight, equivalent to 0.1-0.2 mg protein per ml).

Total MK-801 binding was determined by incubation of 800 ul of the

membrane fraction with (^{3}H)MK-801 (New England Nuclear, Boston, MA) for 45 minutes at 25° C. (^{3}H)MK-801 concentrations ranged from 14,000 to 150,000 counts per minute (cpm) with a constant specific activity of 22.7 Ci/mM. A parallel set of samples, prepared in the same way but containing a large excess of non-radioactive MK-801, provided non-specific binding. Specific binding (Bsp) is defined as the difference between total binding (Bt) and non-specific binding. All incubations were terminated by rapid filtration through Whatman GF/B glass fiber filters (Brandel, Gaithersburg, MD) at 4° C. Filters were rinsed twice in the same buffer without Hepes (pH 7.4), covered with 3 ml of Aquasol (DuPont, Wilmington, DE), and counted by spectroscopy at 65% efficiency.

The maximum number of binding sites (B_{max}) and the dissociation constants (K_d) were computed with the AccuFit Saturation Program (Beckman Instruments, Inc., Fullerton, CA) using progressive complexity analysis. This is the process of fitting ligand binding data by non-linear least squares regression analysis to progressively more complex models. The program solves the equations describing the binding of labeled ligand to receptor proteins, fits the data to the best binding curve to determine B_{max} and K_d, and indicates whether one or two ligand binding sites are most likely. Variations in binding data among groups were analyzed using ANOVA and differences were considered significant if $p < 0.05$.

c. Antagonist Injections

A total of 88 platyfish from four broods of the early maturing JP 163 stock, born on 6/6/96, 7/12/96, 7/21/96, and 4/10/97 were separated into males and females and then into control and experimental groups at 4 weeks of age (Table 5). Animals were housed and cared for as described above, but in separate tanks. Beginning at 6 weeks of age, all animals were anesthetized three mornings per week by immersion in a 0.04% solution of tricane methane sulfonate (MS-222); they then received intraperitoneal injections of either MK-801 or physiological saline. Animals were weighed weekly and MK-801 concentrations were adjusted to maintain either 5, 10, 20, 40, or 60 *u*g/gm body weight. Injections continued until the first control male was sexually mature, as evidenced by complete metamorphosis of the anal fin into the gonopodium. Between two and three hours after their final injection, all animals were sacrificed by decapitation. Gonads were analyzed at autopsy to confirm developmental state. Differences in development were compared with one way ANOVA and differences of $p < 0.05$ were considered significant.

Three to four brains from each control and experimental group above were pooled and frozen at minus 80° C immediately after dissection from the cranium for use in radioimmunoassay (RIA), described below. Heads of the remaining animals in each group were fixed in Bouin's solution and processed for histology and immunocytochemistry. All gonads were autopsied to confirm stage of development, immediately fixed in Bouin's solution, and also processed for histology and

immunocytochemistry as described above. ICC analysis was performed using antisera to the NMDA R1 subunit and standard methods (see Histology and Immunocytochemistry section for details). Histological analysis was performed on Masson's trichrome-stained sections of gonad and pituitary samples from two representative animals in each group.

d. Radioimmunoassay

Brains of three or four MK-801 injected and control male and female animals were pooled, immediately frozen on dry ice, and stored for not more than 12 months at -80° C. Tissues were suspended in ice cold 2 M acetic acid, homogenized at one half maximal setting for 15 seconds using a Polytron PT-45 Homogenizer (Brinkmann Instruments Inc., Westbury, NY), centrifuged at 10,000 g for 45 min at 4° C, and the supernatant was lyophilized overnight (SpeedVac Concentrator SVC 100H; Savant Instruments Inc., Farmingdale, NY). The pellet was reconstituted in water, centrifuged at 14,000 g for 30 min at 4° C, and supernatant was stored at -80° C for no more than 6 months. Samples were rehydrated in duplicate in phosphate buffered saline (pH 7.0), incubated at room temperature for 30 minutes with a 1:40,000 dilution of anti-mammalian GnRH (mGnRH) (Peninsula Laboratories, Belmont, CA), then incubated at 4° C overnight with a 1:10,000 counts per minute solution of iodinated (^{125}I) mGnRH as previously documented (Sower *et al.,* 1993). Binding was terminated by the addition of dextran coated

charcoal (Sigma Chemical Co., St. Louis, MO), samples were centrifuged at 4000 g for 15 minutes at 4° C, and sample radioactivity was determined with the LKB Wallac 1282 Compugamma Gamma Counter (LKB Nuclear Inc., Gaithersburg, MD). All samples were counted twice.

GnRH concentrations were extrapolated from a standard curve counted with the samples. Data were compared using the Student *t*-test and a difference of $p < 0.1$ was considered significant.

RESULTS

I. Distribution of Ir-NMDA R1

a. Localization in the Platyfish Brain and Pituitary Gland

In general, animals at all four developmental stages studied (Table 1) demonstrated strong immunoreactivity to anti-NMDA R1 only in neuronal cell bodies and fibers of the nucleus olfactoretinalis (NOR) (Table 2 and Figure 4). NMDA R1 was sparsely localized in some neuronal fibers and/or cell bodies of the olfactory lobe (OL), nucleus preopticus (NPO), nucleus preopticus periventricularis (NPP), and nucleus lateralis tuberis (NLT) at the four developmental stages. Cell bodies in the rostral pars distalis of the pituitary gland (RPD) were the only other neuroendocrine structure to consistently express the NMDA R1 protein. The

staining patterns in the different groups are described below.

Immature Animals

All immature fish demonstrated ir-R1 in cell bodies in the NOR (Figures 5C and 5D), in cell bodies and/or fibers in the NPO, and in cell bodies of the RPD of the pituitary gland. In the two brain nuclei, cellular staining was granular in appearance and localized to the cytoplasm. A large unstained nucleus was often visible. Fibers in the NPO had a "beaded" appearance and were oriented in a ventrocaudal direction towards the NLT. In the RPD, cell bodies stained uniformly, with the entire area displaying immunoreactivity of such intensity that individual cells and nuclei were often not visible.

Pubescent Animals

All pubescent animals demonstrated ir-R1 in cell bodies of the NOR (Figures 5E and 5F) and RPD. Cells in the NOR were large and ovoid, as in the immature group, with a stained cytoplasm and a large, round, unstained nucleus. In the NPO and NLT five of seven animals had positive beaded staining in fibers and/or staining in cell bodies. NPO fibers were oriented ventrocaudally toward the NLT, and NLT fibers were oriented ventrally toward the pituitary gland. In the olfactory lobe, all animals had a small number of R1 positive fibers oriented rostrocaudally toward the telencephalon and ventrocaudally toward the NOR. In the RPD, there was an intense ir-reaction in all animals, as in the immature group.

Mature Animals

All mature animals had anti-R1 staining in cell bodies of the NOR (Figures 5G and 5H). Similar to pubescent and immature animals, the mature NOR contained large, ovoid cells with granular ir-material in the cytoplasm. Six of twelve animals had a small number of fibers with beads of ir-material in the OL and eleven of twelve had extensive beaded fibers in the NLT. OL fibers were again oriented rostrocaudally and ventrocaudally toward the NOR, and NLT fibers ventrally toward the pituitary gland. Ir-NMDA R1 was localized in cells and/or fibers of the NPP in 11 of the 12 animals studied. Fibers were oriented ventrocaudally toward the anterior NLT. In the NPO, 6 of 12 animals had R1-positive cell bodies. Cells in the NPP and NPO appeared smaller than those in the NOR and irregularly shaped with a large irregular nucleus. They had a staining pattern similar to that in the NOR; granular cytoplasmic staining and a large, unstained nucleus. Cells in the RPD were positive for ir-R1 in 9 of 12 animals.

Senescent Animals

Anti-R1 staining was found in cell bodies and/or fibers of the NOR in all senescent animals (Figures 5I and 5J). The staining pattern was the same as in mature and pubescent animals (above), but there were also many unstained NOR cell bodies. Fibers in the NOR were beaded with ir-material and oriented caudally toward the NPP. In four of six senescent animals, there were sparsely beaded ir-

fibers in the OL oriented ventrocaudally toward the NOR. Ir-R1 was localized in fibers of the NPP, NPO, and NLT in 2 of 6, 2 of 6, and 4 of 6 animals respectively. As in the other age groups, NPP fibers were oriented dorsocaudally toward the NPO, NPO fibers were oriented ventrocaudally toward the anterior NLT, and NLT fibers were oriented ventrally toward the pituitary gland.

b. Changes with Development

The number of ir-R1 cells in the NOR of immature animals averaged 19 +/- a standard deviation (SD) of 4 for females and 30 +/- 5 for males. In pubescent animals, stained cells averaged 42 +/- 9 for females and 30 +/- 4 for males. In the mature groups averages were 46 +/- 9 for females and 35 +/- 10 for males, and 22 +/- 4 for females and 34 +/- 7 for males in senescent animals (Table 3 and Figures 5 and 6). One way ANOVA revealed a significant difference at a 99% confidence interval ($p < 0.01$) among females of different developmental stages. Differences among male groups were not significant when analyzed by ANOVA, even at a 90% confidence interval of $p < 0.1$.

II. Binding Properties

Standard saturation binding curves were constructed by graphing the amount, in counts per minute, of (^{3}H)MK-801 specifically bound to NMDA receptors (Bsp), versus the amount of free or unbound (^{3}H)MK-801 (Figure 7). Specifically bound MK-801 is calculated by subtracting non-specific binding from

total binding. Total unbound MK-801 is calculated by subtracting non-specific binding from the total counts added to each sample. Each point on each curve is the average value from 2 separate counts. Slopes were calculated for each binding curve using the standard formula and the two points closest to the trendline. Values for females were 0.004, 0.0005, 0.01, and 0.004 for immature, pubescent, mature and senescent groups respectively. Values for males were 0.004 for immature, pubescent, and mature animals, and 0.006 for senescent animals. The maximum number of binding sites (B_{max}) and the dissociation constants (K_d) for the non-competitive NMDA antagonist (^{3}H)MK-801 were estimated using non-linear least squares regression analysis of the binding data.

a. Dissociation Constants

In females, average K_d values +/- standard deviations for immature, pubescent, mature, and senescent animals were 24.4 +/- 8.1, 0.7 +/- 0.4, 25.7 +/- 12.5, and 10.7 +/- 0.5 respectively (Table 4, bottom and Figure 8). When analyzed by ANOVA the female values are significantly different at a confidence interval of 99%, or $p < 0.01$. K_d values for immature, pubescent, mature, and senescent males averaged 7.1 +/- 2.6, 19.5 +/- 8.8, 12.4 +/- 5.9, and 21.8 +/- 0.1 nM respectively. These values are not significantly different even at a decreased confidence interval of 90% or $p < 0.1$.

b. Maximum Binding

Binding maximums and standard deviations for immature, pubescent, mature, and senescent females were 54.9 +/- 18.5, 3.1 +/- 1.9, 177.7 +/- 41.0, and 31.9 +/- 11.4 fm/mg tissue respectively (Table 4, top and Figures 7 and 9). The difference among these groups is significant at a 99% confidence interval ($p < 0.01$) when analyzed by ANOVA. In males, binding maximums were 53.2 +/- 23.4 fm/mg tissue for immature animals, 123.5 +/- 33.8 for pubescents, 77.3 +/- 21.0 for matures, and 105.6 +/- 3.5 for senescent animals. Analysis by ANOVA revealed no significant difference among these 4 groups, even at a confidence interval of 90%, or $p < 0.1$.

III. NMDA Antagonism

a. Effect on Puberty

Treatment with the NMDA receptor antagonist MK-801 caused a dose-dependent decrease in the number of female platyfish that reached puberty. The same treatment in males had no significant effect (Tables 5 and 6, and Figure 10). When broodmates received 5 *u*g/gm body weight of MK-801, 2 of 5 females and 3 of 5 males had entered puberty by 24 weeks; all control females and 4 of 5 control males were pubescent at this time. When broodmates received 10 *u*g/gm, the number of females entering puberty decreased to 1 of 5. In males, 3 of 4 were pubescent. All but 1 of 14 controls were pubescent in this group. At 20, 40, and 60 *u*g/gm MK-801, no females were pubescent, while all controls were. In males,

from one third to one half of the controls and from one half to all treated animals were pubescent at these higher doses. One way ANOVA showed a significant difference between control and experimental females and among females treated at the different doses at $p < 0.01$. Among male groups there was no significant difference.

b. Effect on Weight Gain

Per cent weight gain in platyfish was also affected by MK-801 treatment (Table 7 and Figure 11). In control females, the average increase in body weight was 269 +/- 25% at 24 weeks of age. The average body weight increase in treated females was 191 +/- 76%. When analyzed with the Student *t*-test, these differences in weight gain are significant at $p < 0.05$. In control males, body weight increased by 248 +/- 41% and males receiving MK-801 increased by 212 +/- 42%. These differences are not significant, even at $p < 0.1$.

c. Gonad and Pituitary Gland Histology

Samples from two representative control females showed mature ovaries, containing many large, round oocytes filled with yolk (Figure 12). Smaller follicular cells surrounded the mature ova. In two typical MK-801-treated females, the ovaries were immature, and only one developing oocyte with a small amount of deposited yolk was visible. Follicular and interstitial cells surrounded abundant immature ova. In males, there were no apparent differences in standard histological

analysis of gonads from treated and control animals. All samples contained abundant seminiferous tubules with evidence of active sperm production occurring within them.

The pituitary glands from the control females contained a relatively large gonadotropic zone, visible on the ventral surface of the gland in mid-sagittal section (Figure 13). In the treated females, there was no evidence of proliferation of the gonadotropic zone. Only a thin layer of cells was present on the pituitary ventral surface. In both control and treated males, the pituitary contained a developing gonadotropic zone with some cell proliferation. There were no obvious differences in pituitary gland morphology between the control and treated males.

d. GnRH Radioimmunoassay

The amounts of GnRH in pg/mg brain tissue were calculated from readings in counts per minute from one sample of pooled tissue for control males and females and one for MK-801-treated males and females. The average values from 2 counts were 0.16 and 0.27 in control and MK-801-treated females and 0.48 and 0.63 in control and MK-801-treated males respectively (Figure 14). In control and injected males GnRH values and standard deviations were 0.48 +/- 0.25 and 0.63 +/- 0.35 respectively. Application of the Student *t*-test revealed a significant difference at $p < 0.1$ for females, but no significant difference between GnRH concentrations in treated and control males. (Table 8 for summary.)

DISCUSSION

It has been firmly established that the P gene regulates the age at maturity in platyfish (see Introduction). We have now taken a new approach to the persistent question of what regulates the cascade of GnRH appearances in the neuroendocrine nuclei and pituitary gland of the platyfish. To the over twenty years of investigation using a variety of probes and tools, we now add this study of age-related variations in the distribution and activity of a receptor molecule. We have studied the NMDA-specific glutamate receptor and how it relates to the neuroendocrine events that modulate maturation, function, and decline of the platyfish reproductive system.

Our investigation documents gender-specific changes in several characteristics of the NMDA receptor in the platyfish at several important developmental periods in the lifespan. We have localized the R1 subunit of the receptor to cells in the brain that contain GnRH (Figures 4 and 5), an important neurohormone regulator of the reproductive system (Flynn *et al.,* 1997). We have shown a sexually dimorphic variation during development in NMDA receptor distribution in the NOR, the first nucleus to contain GnRH (Figure 6). We report an increase in the number of NMDA-containing NOR cells in females as they become mature, and then a decrease at senescence. Variation in NMDA affinity for the antagonist MK-801 also occurs as females develop, with pubescent animals

exhibiting a significant increase in this receptor property (Figure 8) (Flynn *et al.*, 1996; Flynn *et al.*, 1998). We have also shown a sexual dimorphism in the concentration of activated NMDA receptors in platyfish brain, with females having a higher concentration of open or stimulated NMDA receptors when they are mature than at any other time in their life cycle (Figure 9). We have further shown that chronic *in vivo* antagonism of NMDA receptor-mediated ion movement, by blocking the cation channel with a noncompetitive inhibitor, inhibits puberty in females only (Figure 10). We observed an increased concentration of GnRH in protein extracts of brain tissue from antagonist-treated compared to untreated females (Figure 14). Interestingly, all of these NMDA properties remained unchanged throughout the male lifespan. Taken together, these data strongly suggest that the NMDA receptor plays a crucial role in determining the timing of reproductive system development, maturation, and senescence in female platyfish, but not in males.

We have localized the R1 subunit of the NMDA receptor to the NOR of the platyfish and shown that the number of NMDA-containing NOR cells is increased in females that are pubescent and mature (Table 3 and Figure 6). In the light of evidence that glutamate stimulates GnRH release from the mammalian hypothalamus (see Introduction), it is tempting to speculate that perhaps the increased number of NMDA receptors that we report may be the mechanism which allows increased glutamate binding, and subsequent increased GnRH release from the platyfish NOR. We suspect that this may be among the primary mechanism(s) for the initiation of puberty in the female.

There are several lines of evidence indicating a glutamate influence through NMDA receptors on GnRH and therefore on reproductive system activity (see Introduction). The mechanism of glutamate communication with GnRH cells is, however, unclear. There is conflicting evidence with regard to a direct glutamate message received through NMDA receptors on GnRH-containing/secreting cells. We have reported sexually dimorphic localization of the R1 subunit in GnRH cells of the platyfish NOR (Flynn *et al.,* 1997; Flynn *et al.,* 1998), and we have colocalized R1 and GnRH in the same NOR cells (unpublished data). In the electric fish *A. leptorhynchus*, R1 mRNA is abundant in hypothalamic nuclei, but no relationship to GnRH localization has been demonstrated (Bottai *et al.,* 1997). In rats, a recent study suggests that less than 5% of GnRH-containing hypothalamic cells also express R1 mRNA (Smith and Abbud, 1995). However, Gore and colleagues recently showed colocalization of GnRH and R1 mRNA in the hypothalamus of female rats (Gore *et al.,* 1996). This apparently contradictory data from rats is very interesting because it raises the issue of the developmental stage of the animal, which in our study we show is an important physiological variable. In the study by Smith and colleagues, where there is less than 5% colocalization, male and non-reproductive females were used. In the latter study, R1 mRNA on GnRH cells was most abundant in post pubertal females.

Although the structure of the platyfish NOR is set early in life when the number of cells reaches a maximum, a plethora of functional changes based on immunocytochemical and physiological studies has been shown to occur with

development [(Schreibman and Margolis-Nunno, 1987; Schreibman *et al.*, 1991) for review]. In teleosts, the NOR receives input from several environmental sensors, including the retina, the pineal gland, and the olfactory system (Münz *et al.*, 1981). The NOR also communicates with several other neuroendocrine nuclei (ibid.). These anatomical and physiological features make the NOR likely to be the network of neurons that regulates GnRH release and therefore determines the timing of reproductive system development. It is our contention that some stimulatory, or removal of inhibitory, activity on the NOR cells initiates the cascade of neural and endocrine events that culminate in puberty.

We now add increased expression of the NMDA receptor to this list of cytophysiological changes that occur in the NOR. Our ICC results demonstrate that in female platyfish the number of cells expressing ir-NMDA R1 in the NOR is low at immaturity, increases at puberty, stays high at maturity, then decreases at senescence. Both the localization of R1 on GnRH-containing cells and this pattern of NMDA R1 expression suggest a relationship between the timing of receptor availability and the neuroendocrine activation that initiates and maintains sexual maturation.

Our observation of decreased R1 in senescent females leads us to contemplate a relationship between decreased NMDA stimulation and aging of the female reproductive system. In addition to regulating developmental events, the age-related demise of neuroendocrine systems is suspected of initiating the faltering of other physiological systems, including the BPG axis (Timiras, 1989). In humans,

concentrations of several hypothalamic, pituitary, and endocrine gland hormones decrease before characteristic senescence begins (Bjorntorp, 1995). In platyfish, there are several age-related changes in neurohormone and neurotransmitter localization in the BPG axis [(Schreibman and Margolis-Nunno, 1989) for review]. In the female pond snail, *L. stangalis*, the cessation of egg laying ability coincides with decreased branching in neuroendocrine cells (Janse *et al.*, 1996).

Changes in several neurotransmitter and receptor properties have also been shown to coincide with neuroendocrine aging. In the rat hypothalamus, there is a decrease in glutamate release following dopamine stimulation that occurs with increasing age (Exposito *et al.*, 1995; Porras and Mora, 1995). The age-related termination of estrous cycling in the rat has been linked to a decrease in hypothalamic norepinephrine levels (Mohankumar *et al.*, 1995). In the cortex and hippocampus of aged mice, NMDA receptor function has been shown to decrease with age, as binding properties of the receptor change (Magnusson, 1995).

Gender-specific changes in neurotransmitter activity in the BPG axis are not restricted to mammals but can be seen in other vertebrates, including platyfish, where reports by Margolis-Nunno and colleagues of increased ir-serotonin staining in the brain and pituitary gland of aging males bolster the suggestion that neurotransmitter changes may be associated with reproductive senescence (Margolis-Nunno *et al.*, 1986). The decrease in ir-NMDA R1 that we now report at senescence in the female platyfish is consistent with the literature and may be directly related to our previous reports that there is a more prominent decrease in

female reproductive capability with increasing age than there is in males (Schreibman *et al.,* 1983; Schreibman *et al.,* 1991).

It is interesting to note that there was only one area, other than the NOR, of consistent ir-R1 localization in the platyfish. This was in the olfactory bulb. It has been demonstrated by Schreibman and colleagues that the olfactory system of platyfish undergoes dramatic changes at the time of sexual maturation which are coincident with the appearance of GnRH in the olfactory bulb (Schreibman *et al.,* 1986). In pre-pubescent platyfish, the olfactory epithelium consists of a mass of poorly vascularized, uniform, undifferentiated cells. As the animal approaches and proceeds through puberty, dramatic changes occur in the olfactory epithelium transforming it into a highly vascularized structure populated by specialized cell types (Schreibman *et al.,* 1984). The role of glutamate in this process, if any, is unknown.

The association between the olfactory system and GnRH neural networks is well documented. GnRH neurons have their embryonic origins in the olfactory placode. During development of both rats and humans, these neurons migrate from the placode to their destination in various brain nuclei (Schwanzel-Fukuda *et al.,* 1996; Wray *et al.,* 1994). In addition, light and electron microscope studies have established that neural processes from olfactory cells synapse with the cells of the NOR (Schreibman and Margolis-Nunno, 1987). Our demonstration of ir-NMDA R1 in olfactory bulb fibers is of interest in light of these structural and functional

links between the olfactory system and the neuroendocrine structures connected to reproductive system development.

Maturation of the BPG axis is genetically regulated in the platyfish (Kallman *et al.,* 1973; Schreibman and Kallman, 1978; Schreibman *et al.,* 1989). The age at which puberty occurs is determined by alleles at the P locus, located on the sex chromosomes [(Kallman *et al.,* 1973; Schreibman and Kallman, 1977; Schreibman and Kallman, 1978) and see Introduction]. The exact mechanism(s) of P gene activity however, remains unknown. Examination of the nature of the P gene has been a slow and methodical process, though strides in resolving this mystery are currently being made. In addition to efforts to identify the means by which this genetic locus exerts its control on the maturational process, the isolation and cloning of the gene is also underway (Schreibman *et al.,* 1994).

Previous data have suggested that the P gene activates a "switch" that resides in the NOR (Halpern-Sebold and Schreibman, 1983), the first brain center to contain ir-GnRH. The NOR is believed to be the site of the initiation of a cascade of neural signals that descend upon the pituitary gland and result in the secretion of pituitary gonadotropin and the subsequent maturation of the gonads (Figure 2). Numerous studies from Schreibman and colleagues support a communication network among the GnRH nuclei of the platyfish brain [(Schreibman and Kallman, 1977; Schreibman *et al.,* 1990; Schreibman *et al.,* 1991) for review]. Since the NOR functions in a genetically-determined and age-dependent manner, beginning its production of GnRH only at a specific time in the life cycle, it is likely that at least

some of the neurotransmitter signals coming into and going out of the NOR are also age-dependent.

In both early and late maturing platyfish genotypes, the appearance of GnRH in the NPP and NLT may be the result of GnRH moving caudally along axonal fibers from the NOR and then either collecting at the NPP and NLT, or acting as a neurotransmitter, communicating a message to these nuclei to begin synthesis of their own GnRH. Alternative explanations for the appearance of GnRH in the more caudal neuroendocrine nuclei include new and independent GnRH synthesis at these sites, or communication using other neurotransmitters from the NOR to the NPP and NLT, or transmitter input coming to these nuclei directly from other brain centers. It may also be that new nerve fibers need to be generated to these nuclei, a concept not yet explored experimentally. Any or all of these are possibilities.

Immunocytochemical evidence from our laboratory showing several neuropeptides localized in fibers between the NOR, NPP, and NLT supports the idea of neurotransmitter communication among these GnRH-containing nuclei (Magliulo-Cepriano *et al.,* 1993; Magliulo-Cepriano and Schreibman, 1993). GnRH movement from the NOR however, is also likely to contribute to GnRH appearance in the more caudal nuclei. Our laboratory has documented GnRH in fibers that connect the NOR to the NPP, NLT, and pituitary gland (Halpern-Sebold and Schreibman, 1983; Halpern-Sebold *et al.,* 1986). We have also shown the age-related appearance of GnRH in these fibers (ibid.). The sexually dimorphic and age-related change in NMDA R1 localization on GnRH cells of the platyfish NOR that

we now show suggests a possible mechanism for impacting on the timing of GnRH release from these cells.

Since the NMDA molecule is a heterodimer, composed of R1 and R2 subunits, the presence of the NMDA R1 protein alone does not indicate the presence of a fully functional receptor. While it has been demonstrated that R1 alone is capable of conducting a Ca^{2+} current, a receptor composed of both R1 and R2 subunits shows a markedly increased response to NMDA and/or glutamate stimulation and is believed to be the physiologically active form of the receptor (Moriyoshi *et al.,* 1991). We used binding of the non-competitive NMDA antagonist (^{3}H)MK-801 to investigate functional NMDA receptors in male and female platyfish at the four previously defined developmental stages. MK-801 is a synthetic polycyclic carbon compound whose binding site on the NMDA molecule is not the ligand site, but is instead a site within the open ion channel, comprising parts of both the R1 and R2 subunits. In order for MK-801 to bind, therefore, R1 and R2 must be assembled as a functional receptor, and the receptor complex must be in the open conformation (Figure 1) (Anson *et al.,* 1998; Larsen and Monaghan, 1997).

We calculated the affinity of (^{3}H)MK-801 for the platyfish NMDA receptor and the concentration of activated NMDA receptors by measuring (^{3}H)MK-801 binding in membrane preparations from platyfish brain homogenates (Figure 8). One could argue that whole brain homogenates are not a reliable indicator of NMDA activity on GnRH cells because of the widespread NMDA receptor distribution. It is important to note however, that a ubiquitous distribution for the

NMDA receptor (which includes localization in the hypothalamus) appears limited to mammals. In rats, R1 localization studies show high receptor concentrations in hippocampus, cerebellum, hypothalamus, and brainstem (Petralia *et al.,* 1994). In humans there is widespread distribution of both NMDA R1 and R2 with especially high concentrations of R2 reported in the hippocampus (Huang *et al.,* 1995).

Several studies in fish however, report a much more limited distribution for NMDA receptors. We have shown in platyfish that NMDA R1 is localized in some scattered telencephalon cell bodies, but the only clusters of intense cell body staining occur in neuroendocrine areas analogous to the mammalian hypothalamus (Flynn *et al.,* 1997). In rainbow trout, a stimulatory action by NMDA on GnRH cells has been demonstrated (Flett *et al.,* 1994) but no localization studies have been performed. In *A. leptorhynchus*, a weakly electric fish, NMDA R1 localization studies show highly enriched areas in the hypothalamus and very limited distribution in the cerebellum and brainstem (Bottai *et al.,* 1997; Monaghan and Maler, 1991). Especially in the light of the very limited distribution that we show in platyfish, we feel confident that whole brain NMDA studies reliably represent NMDA receptors in platyfish neuroendocrine nuclei, particularly the NOR.

In male and female platyfish we report a consistent NMDA affinity for (^{3}H)MK-801 throughout the lifespan, except for pubescent females who display a markedly increased affinity for this antagonist. The consistent affinity coincides with other reports of consistent receptor binding properties in several brain areas and in several species. In frogs, glutamate binding is the same in juvenile and adult animals

(Cline *et al.*, 1994). In male rats it was recently demonstrated that in the cortex, glutamate affinity at the NMDA receptor is unchanged throughout the lifespan (Jasek and Griffith, 1998). In humans NMDA receptor binding in the hippocampus is unchanged from 24 weeks gestational age through 94 years (Johnson *et al.*, 1996).

It is important to note that the affinity of a receptor for any given ligand is considered an inherent receptor property, a changed affinity is thought to be possible only under different conditions. Many receptors are known to exist in several isoforms and these are known to have different properties (see Introduction). The various isoforms of a receptor are also likely to have different affinities, possibly the result of different protein conformation. Varied affinities have been shown for NMDA receptors in different areas of the mature male rat brain, and this is interpreted as the expression of different R1 and R2 isoforms (Bresink *et al.*, 1995). The changed affinity in pubescent females that we report then, may be the result of the expression of another of the several R1 and R2 isoforms known to exist (Larsen and Monaghan, 1997; Moriyoshi *et al.*, 1991). A search of the literature, which we believe to be thorough, suggests that the only explanation for changed affinity of a given receptor is a changed isoform expression.

There are several examples where changes in the isoform composition of receptors occur as animals develop. This is a possible mechanism for mediating hormonal and neurotransmitter regulation of developmental changes. A varied isoform expression of several different receptors has been repeatedly shown with

development in the muscle systems of several species, including trout skeletal muscle (Gauvry and Fauconneau, 1996) and cultured skeletal, cardiac, and smooth muscle from the rat (Ziober *et al.,* 1997). Expression of varied NMDA receptor isoforms occurs with development in the mouse brain, including cerebellum (Muzet and Dupont, 1996) and cortex and hippocampus (Magnusson, 1995). In insect muscle, isoform expression was further shown to be regulated by the steroid hormone environment. During the metamorphosis from pupa to adult, steroid concentrations change and stimulate a change in the isoform composition of the ecdysone receptor (Hegstrom *et al.,* 1998).

This raises the possibility of the same type of hormone-receptor interaction between the sex steroids and the NMDA receptor in the platyfish. There is ample evidence in the rat of gonadal steroids, and estrogen in particular, feeding back to regulate GnRH (Brown *et al.,* 1994; Pinilla *et al.,* 1995). In mammals, estrogen exerts both a positive and negative feedback on GnRH secretion but the mechanisms controlling this complex communication remain a mystery.

Our data suggest that in the platyfish NOR there is an isoform of the NMDA receptor expressed *only* in pubescent females. The appearance of this receptor coincides with increasing estrogen production by the developing ovaries and increased estrogen feedback to GnRH cells. The increased expression of NMDA receptors and the change in NMDA receptor isoform that we show in developing female platyfish occurs at the same time that the GnRH-containing nuclei are undergoing dramatic changes (i.e., appearance of GnRH in the NPP, NLT, and

pituitary). These concurrent changes may be related.

The possibility of NMDA properties in pubescent females resulting from increasing estrogen levels is an intriguing one that has support in reports in the literature that estrogens exert multiple effects on neuroendocrine and other brain areas [(McCarthy *et al.*, 1997), (Zanisi and Messi, 1991) for review]. A future study to see if estrogen is required for NMDA receptor development could be done by ovariectomizing immature platyfish females and/or by giving estrogen to immature males. An amelioration of NMDA change in the former case, and an attenuation in the latter, would be strong evidence of an estrogen requirement for developmental changes in NMDA.

We have also reported a significantly higher binding maximum for (^{3}H)MK-801 in membrane preparations from the brains of reproductively mature female platyfish compared with binding maxima in immature, pubescent, and senescent animals (Figures 7 and 9). The binding maximum is the amount of specific binding to NMDA receptors that occurs when (^{3}H)MK-801 is present in saturating concentrations. This number is generally indicative of the concentration of receptor molecules in a given tissue sample, but in the case of MK-801, B_{max} is more specifically a measure of the concentration of open NMDA ion channels. This suggests that the increase in B_{max} in mature female platyfish represents an increase in the concentration of physiologically activated receptors.

In the mammalian literature, changes in binding properties of NMDA receptors are reported in several species. In rat visual cortex there is an increase in

maximum binding to NMDA, kainate, and AMPA receptors as young animals mature (Gordon *et al.*, 1997). In the human cortex and hippocampus there is decreased binding of glutamate to both NMDA and non-NMDA receptors with advancing age (Johnson *et al.*, 1996). A relationship between decreased NMDA activity and memory loss and decreased learning ability in older adults has been suggested, making age-related changes of NMDA receptors a very relevant topic for humans. In fish however, this is the first study of variation in NMDA receptor binding properties with development.

The increased (^{3}H)MK-801 binding maximum that we report in mature female platyfish indicates an increase in the concentration of open, or physiologically activated, NMDA receptors. We propose that as a result of the increased activity there is an increased stimulation of GnRH-containing cells in the NOR when females are mature. It is true that GnRH appears in cell bodies of the platyfish NOR at a much earlier age (5 - 8 weeks), and it's therefore unlikely that NMDA stimulation is directly influencing GnRH expression in the NOR. The appearance of GnRH in the more caudal NPP and NLT however, does not occur until puberty and continues through maturity. As discussed earlier (p. 38), this caudal "movement" of GnRH may be the result of *de novo* GnRH synthesis, or it may be an actual movement of GnRH molecules. The increased glutamate stimulation of NOR cells that we report at female maturity may, therefore, be influencing GnRH release or perhaps the growth of GnRH-containing neural fibers from the NOR.

It would be interesting to know if the concentration of GnRH changes

during platyfish development. While our laboratory has studied developmental changes in GnRH distribution and in the distribution of other neuropeptides and pituitary hormones (Magliulo-Cepriano *et al.*, 1993; Magliulo-Cepriano and Schreibman, 1993; Margolis-Kazan *et al.*, 1981; Yousha *et al.*, 1995) [(Schreibman *et al.*, 1994) for review], we have not looked at the amount of GnRH in the brain during development. In the goldfish hypothalamus however, a decreased GnRH concentration in female brain during times of reproductive activity has been demonstrated (Aamodt *et al.*, 1995). This decrease in neural GnRH concentration at a time of a known increase in GnRH activity can be explained by the movement of GnRH from neuroendocrine areas to the gonadotropic cells of the anterior pituitary. Recall that in teleosts there is a direct innervation of the adenohypophysis by NOR cells. Stimulation of the NOR by NMDA then, could result in the movement of previously synthesized GnRH directly into the pituitary gland. Support for this explanation is offered by our pilot study showing increased GnRH levels in the female platyfish brain following NMDA inhibition (Figure 14).

We further investigated the physiological role(s) of NMDA receptor activation on BPG axis development by injecting immature platyfish broodmates with the NMDA receptor antagonist MK-801. We observed that chronic NMDA antagonism causes a striking, dose-dependent inhibition of puberty *in females only* (Figure 10). Interestingly, a recent study in Siberian hamsters also shows that MK-801 injections inhibit puberty, but only males were tested (Ebling and Cronin, 1998). In this species puberty is regulated by photoperiod, thus a different

mechanism for NMDA influence is possible. A direct relationship has been established between photoperiod and serum melatonin in another hamster species, *P. sungorus* (Niklowitz *et al.*, 1994). MK-801 has also been linked to light cycle effects in rats, where it has been shown to alter melatonin receptor expression (Gauer *et al.*, 1994). The different response to MK-801 in hamsters and fish may be due to a melatonin effect on reproduction in the hamster. This is further supported by reports that physiological doses of melatonin have no effect on the BPG axis in fish (Marchant *et al.*, 1989). MK-801 treatment has also been shown to affect other parameters in several species, including learning in fishes (Davis and Klinger, 1995) and multiple sensorimotor behaviors in rats (Wozniak *et al.*, 1990).

Histological examination of gonads from MK-801-treated and control platyfish groups revealed only small, white oocytes in the undeveloped ovaries of treated females, and large, yolky ones in the developed ovaries of the untreated groups (Figure 12). In both control and experimental males, gonads were mature, with active spermatogenesis and numerous seminiferous tubules. We looked for, but did not find, any immunocytochemical evidence of NMDA receptors in the gonads themselves. This is consistent with the literature where there is also no evidence for NMDA receptor localization in gonadal tissue of either mammals or fishes. This indicates that there is no direct, NMDA-mediated interaction between glutamate and gonad development.

In standard histological preparations of the pituitary gland from treated and control groups, we observed a small gonadotropic zone typical of immature animals

in MK-801 treated females, and a greatly enlarged, multilayered gonadotropic zone typical of mature animals in untreated females (Figure 13) [(Schreibman, 1964; Schreibman *et al.*, 1973; Schreibman, 1986) for pituitary gland review]. An expanded gonadotropic zone is a characteristic of puberty and indicates increased GtH production, and increased stimulation of the gonads (Margolis-Kazan *et al.*, 1981; Margolis-Kazan and Schreibman, 1984). In platyfish and other teleosts, the many cell types of the anterior pituitary gland are grouped into discrete and separate areas of the gland allowing easy identification of, for example, somatotropic, gonadotropic, and lactotropic zones (see Introduction). As a model system to study pituitary function, this anatomical design offers some important advantages over the mammalian pituitary where the many cell types are intermingled.

The value of the structure of the teleost pituitary gland for study is illustrated in the following example. In rats, immunocytochemical studies have localized scattered NMDA R1 receptor subunits in the adenohypophysis of mature males (Petralia *et al.*, 1994). No colocalization studies have indicated that the NMDA R1 is on GtH cells, however, and it remains unclear which cell type they are on. In the platyfish, we have reported NMDA receptor localization in the pituitary gland only on the prolactin cells of the rostral pars distalis (Flynn *et al.*, 1997). This is consistent with the reports in several vertebrate species of an NMDA influence on prolactin secretion (Abbud and Smith, 1993; Kochman *et al.*, 1993; Lincoln and Clarke, 1994; Pinilla *et al.*, 1995). Despite the localization of R1 to the anterior pituitary there is no evidence in platyfish or other species of NMDA receptors on

gonadotropic cells. We suggest therefore that the immature gonadotropic zone in MK-801 treated female platyfish is not the result of direct MK-801 activity at the pituitary, but rather an effect of MK-801 activity in the neural component of the BPG axis.

We looked at MK-801-treated and control animals with standard immunocytochemical preparations of brain sections and found similar NMDA receptor distribution in both. We also did a pilot radioimmunoassay (RIA) study to determine GnRH concentration in protein extracts from the pooled brains of treated and untreated animals. In the treated group we found a significantly higher concentration of GnRH than in the controls (Figure 14). Although limited by the scope of the experiment, this could suggest that when NMDA receptors are blocked in the female, GnRH is not released from neuroendocrine cells, thus leading to an increased concentration of neural GnRH. This data further suggests that the decrease in neural GnRH under normal conditions is due to GnRH movement from neural structures to the pituitary gland.

It seems likely that MK-801 antagonism interferes with NMDA regulation at the neural level of the BPG axis; in platyfish, the NOR. Our report of a lack of pituitary and gonad development in MK-801-treated animals may be explained because MK-801 treatment inhibited the release of GnRH from neuroendocrine cells that is required for the initiation of the cascade of development. Studies in several species throughout the animal kingdom indicate an NMDA modulation of the GnRH cells of the BPG axis (see Introduction), and our data supports this in platyfish as

well. Our *in vivo* study of the effects of MK-801 treatment on development is consistent with earlier reports in female rats (Urbanski and Ojeda, 1990), and adds to our understanding of NMDA-GnRH communication.

We have also reported that chronic MK-801 treatment results in decreased weight gain in female platyfish (Figure 11). It has been demonstrated in several mammals that the glutamate agonist NMDA stimulates growth hormone (GH) release, suggesting that NMDA antagonism would decrease release of this hormone. In bulls, a single NMDA injection results in increased serum GH (Shahab *et al.*, 1993). This appears due, at least in part, to a direct action on GH cells. In somatotrophs isolated from the pituitary gland of the male rat, it has been demonstrated that NMDA application results in a dose-dependent and immediate secretion of GH (Lindstrom and Ohlsson, 1992).

In fish however, the NMDA-GH interaction is less clear. NMDA treatment stimulated GH release in sexually immature male trout primed with testosterone (Flett *et al.*, 1994). In contrast, NMDA injections were shown to inhibit GH secretion in mature female goldfish (Trudeau *et al.*, 1996), but also to stimulate GH secretion in the same animal (Marchant *et al.*, 1989). The contradictory effects of GH may be related to a sexual dimorphism resulting from different steroid environments. A later study in immature trout looked at both males and females and at steroid hormone environments. It showed that while both NMDA and GnRH independently stimulate GH secretion, only in estrogen-treated animals does

concurrent treatment with an NMDA antagonist abolish the GH response (Holloway and Leatherland, 1997).

The confusion of data when steroid hormones come into play may indicate that there is more than one influence on GH secretion. Our data suggests that NMDA influences growth in females only. It is not clear if there is a direct action by NMDA on pituitary somatotrophs or if the influence is indirect. NMDA may be increasing GnRH, which is in turn increasing GH levels. A future study to clarify the mechanism of decreased growth in platyfish females would be valuable as the current literature is contradictory and can support both possibilities. In any case, and despite all of the apparent contradictions, it is clear that some communication between growth hormone and glutamate is occurring both in fishes and in mammals.

We are confronted with an apparent paradox when trying to reconcile the sexually dimorphic properties of NMDA in the BPG axis with our previous reports that sexual maturation in platyfish is similar in both sexes. It is important to recognize that our earlier studies have documented similar genetic controls over the *timing* of reproductive system development in males and females, and although the end results may be the same, specific neuroendocrine mechanisms may be different. Our laboratory has previously shown that there are significant gender-specific differences in neuroendocrine neurotransmitters that occur with platyfish development. These include an increased number of gonadotropes in the adenohypophysis (Margolis-Kazan and Schreibman, 1984), increased ir-GnRH staining intensity and distribution in the brain (Schreibman *et al.,* 1985), and varied

somatostatin and serotonin distribution (Margolis-Nunno *et al.,* 1986; Margolis-Nunno *et al.,* 1987).

There is considerable recent research documenting sexually dimorphic and age-related changes in several other widely divergent systems as well. These include human skeletal growth where there is sexually dimorphic variation in the rate and duration of growth in different parts of the cranium, (Humphrey, 1998), and toadfish muscle development where the histological structure of myofibrils is different in the embryo, juvenile, and adult stages (Loesser *et al.,* 1997). In the starling brain, there is an increase in the size of song control nuclei with development in males only (Bernard *et al.,* 1996). Our report of a sexually dimorphic regulation by NMDA of platyfish reproductive system development and its timing, is further evidence that sexual dimorphism in development may be the result of the sexually dimorphic properties of both neurotransmitters and receptors.

This variation may originate at the level of the P gene in the platyfish. The location of this gene on the sex chromosomes is highly suggestive of a sexually dimorphic or sex-linked activity. We now suggest that one possible way that the P gene may operate is through NMDA receptors, in this way controlling the timing, quantity, and perhaps type of glutamate stimulation to GnRH cells. There are a number of ways to influence the availability of the NMDA R1 and R2 proteins, including rates of gene transcription, translation, and/or post-translational modifications. The platyfish P gene may indeed operate at any one of these points. Further studies could clarify the site of P gene influence and the exact location of a

sexually dimorphic mechanism.

Very exciting recent work in our laboratory and others shows that in the neuroendocrine system, the apparent use of similar chemical messengers (GnRH, GtH) may not be so similar. More and more variations of these neurohormones have been characterized, shown to have different distributions, and age-related changes have been shown to occur (Amano *et al.,* 1994; Magliulo-Cepriano *et al.,* 1994; Sower *et al.,* 1993). It is becoming clear that these subtle chemical differences are probably significant, and may in fact be a mechanism for sexually dimorphic control. Since most work has been done in males, studies to clarify and expand these findings would no doubt illuminate the role(s) of the various forms of neurohormones and neurotransmitters in gender-specific regulatory processes.

Our recent work supports and enhances our contention that there is a clearly defined sequence of developmental events, under genetic control, that orchestrate the spatial and temporal events of reproductive system development and decline. It should not be surprising that the reproductive system, the most obvious gender-specific system, should also be regulated in a gender-specific way. However there is still considerable ground to cover before we can fully comprehend the influences of the genome, the environment, the brain, and the endocrine system in the developmental changes of the reproductive system.

Genetic, environmental, and physiological influences on age-related processes have been studied for many years in platyfish (Kallman and Borkoski, 1978; Margolis-Kazan and Schreibman, 1984; Margolis-Nunno *et al.,* 1986;

Margolis-Nunno *et al.,* 1987) providing an extensive body of fundamental knowledge and one of the most complete longitudinal studies available for any vertebrate. While it is clear that the P gene determines age at maturity, we have not been able to define what factors determine the timing of the critical appearance of GnRH in the more caudal neuroendocrine nuclei, the NPP and NLT, and ultimately in the pituitary gland. Nor do we know if neurotransmitter changes in the NOR precede and therefore perhaps influence GnRH changes or if neurotransmitter change is a consequence of GnRH. We have not been able to determine cause and effect. Furthermore, we still don't know where in the neuroendocrine cascade of GnRH required for reproductive system maturation the P gene might act to stimulate, or remove inhibition on, GnRH expression.

We now propose that GnRH activity in females is influenced by the relative types and/or amounts of the NMDA proteins in NOR cells, and that the timing of the expression of these receptor proteins could be determined by P gene composition. The genetic mechanisms are themselves likely influenced by numerous other factors, probably including interactions with other hormones. This would be consistent with our observations that NMDA receptors modulate GnRH in females only.

It is clear from the many studies in our laboratory and others, that regulatory substances may play different roles at different times in the lifespan. We demonstrate here, once again, how difficult it is to postulate a role for a particular substance when looking at but one small segment of the life cycle continuum of only

one sex of an organism. Since the development, maturity, and eventual demise of the BPG axis is but one example of similar age-related processes that occur throughout the body, these studies may form a more general model for a genetically controlled and sexually dimorphic neuroendocrine regulation of physiological aging.

The findings of this study further demonstrate that sexual maturation, activity, and decline result from a complicated series of interactions that involve a genetic component, at least three major organs, a number of neuropeptide and amino acid modulators, multiple forms of GnRH and GtH, and responsiveness to internal and external environmental changes. We now postulate a sexually dimorphic and developmentally-dependent role for the NMDA type of glutamate receptor in BPG axis regulation, and we discuss the possible influence of this receptor in the brain nuclei known to be involved in the control of reproductive system function in the platyfish.

APPENDIX

Table 1: Definitions of developmental stages. The wide range of ages within each stage reflects our use of early and late maturing genotypes. Stages were identified by gross and microscopic examination of the gonad at autopsy and/or by examination of the gonopodium (see Materials and Methods).

DEVELOPMENTAL STAGE	AGE	ANATOMICAL STATE
immature (imm)	1 – 6 months	undifferentiated gonad no anal fin metamorphosis
pubescent (pubc)	4 – 5 months	some gonad development partial anal fin metamorphosis
mature (mat)	4 – 10 months	large mature gonads fully developed gonopodium
senescent (sen)	24 – 36 months	some gonad atresia at least 2 years old

Table 2: Distribution of ir-NMDAR1 in the brain and pituitary gland. Staining in platyfish at 4 developmental stages. 0 = no staining, Fs = staining in fibers, CBs = staining in cell bodies. OL = olfactory lobe, NOR = nucleus olfactoretinalis, NPO = nucleus preopticus, NPP = nucleus preopticus periventricularis, NLT = nucleus lateralis tuberis, RPD = rostral pars distalis of the pituitary gland.

	IMMATURE	PUBESCENT	MATURE	SENESCENT
OL	Fs\CBs	Fs	Fs	Fs
NOR	CBs	CBs	CBs	Fs\CBs
NPP	0	0	Fs\CBs	0
NPO	Fs\CBs	Fs\CBs	Fs\CBs	0
NLT	Fs	Fs	Fs	Fs
RPD	CBs	CBs	CBs	CBs

Table 3: Number of ir-NMDAR1 cells in the NOR. Average values +/- SD for counts of all stained cells in alternate sections of the nucleus olfactoretinalis in males and females at 4 developmental stages. For immature and pubescent animals n = 14 (7 male, 7 female) at each stage, for mature n = 11 (6 male, 5 female), and for senescent n = 9 (4 male, 5 female).

FEMALES		MALES	
imm	19 +/- 4	imm	30 +/-5
pube	42 +/- 9	pube	30 +/- 4
mat	46 +/- 9	mat	35 +/- 10
sen	22 +/- 4	sen	34 +/- 7

Table 4: Binding maxima (B_{max}) and dissociation constants (K_d) for (^{3}H)MK-801. Average values +/- SD in males and females at 4 developmental stages. For immature and pubescent animals n = 6 (3 male, 3 female) at each stage, for mature n = 11 (5 male, 6 female), and for senescent n = 4 (2 male, 2 female).

Bmax (fm/mg) at 4 stages of development				
FEMALES			MALES	
imm	54.9 +/- 18.5		imm	53.2 +/- 3.5
pube	3.1 +/- 1.9		pube	123.5 +/- 33.8
mat	177.7 +/- 41.0		mat	77.3 +/- 21.0
sen	31.9 +/- 11.4		sen	105.6 +/- 3.5

Kd (nM) at 4 stages of development				
FEMALES			MALES	
imm	24.4 +/- 8.4		imm	7.1 +/- 2.6
pube	0.7 +/- 0.04		pube	19.5 +/- 8.8
mat	25.7 +/- 12.5		mat	12.4 +/- 5.9
sen	10.7 +/- 0.05		sen	21.8 +/- 0.1

Table 5: Summary of MK-801 injections. Four broods of platyfish were divided into five groups. In group 1 n = 24 (10 males and 14 females), group 2 n = 23 (9 males and 14 females), group 3 n = 14 (7 males and 7 females), group 4 n = 19 (13 males and 6 females), and group 5 n = 12 (7 males and 5 females).

GROUP	DATE OF BIRTH	MK-801 DOSE	INJECTION PERIOD	AGE AT DEATH
1	4/10/97	5 *u*g/gm	7.5 weeks	3 months
2	4/10/97	10 *u*g/gm	7.5 weeks	3 months
3	6/6/96	20 *u*g/gm	8.5 weeks	3.5 months
4	7/21/96	40 *u*g/gm	15 weeks	5 months
5	7/12/96	60 *u*g/gm	9.5 weeks	3 .5 months

Table 6: Effects of chronic MK-801 treatment on puberty. Animals received intraperitoneal injections of MK-801 at 5, 10, 20, 40, or 60 *u*g/gm body weight or of physiological saline. Injections began at 6 weeks of age and continued 3x/week until one control male reached sexual maturity.

MK-801 DOSE	FEMALES IN PUBERTY		MALES IN PUBERTY	
	control	experimental	control	experimental
5 *u*g/gm	9/9	2/5	4/5	3/5
10	9/9	1/5	4/5	3/4
20	2/2	0/5	2/4	3/3
40	3/3	0/3	3/5	7/8
60	2/2	0/3	1/3	2/4

Table 7: Effects of chronic MK-801 injections on weight gain. Animals received intraperitoneal injections of MK-801 at 5, 10, 20, 40, or 60 *u*g/gm body weight or of physiological saline. Injections began at 6 weeks of age and continued 3x/week until one control male reached sexual maturity.

MK-801 DOSE	% WEIGHT GAIN	
	females	males
5 *u*g/gm	196 %	240 %
10	292	245
20	233	233
40	122	200
60	110	144

Table 8: Summary. There are anatomical, biochemical, and physiological changes in NMDA receptor properties throughout the female platyfish lifespan.

	FEMALES	MALES
# of cells containing ir-NMDAR1	high at puberty high at maturity	unchanged
receptor affinity [1/(^{3}H)MK-801 K_d]	high at puberty	unchanged
# of receptors [(^{3}H)MK-801 B_{max}]	low at puberty high at maturity	unchanged
response to MK-801	decreased # in puberty decreased growth increased [GnRH] in brain	unchanged

Figure 1: The NMDA receptor. This schematic diagram shows several important receptor properties including the R1 and R2 subunits, the glutamate and glycine binding sites, the cation channel, and the site of MK-801 binding. (Adapted from Research Biochemicals, Inc., 1995 catalog.)

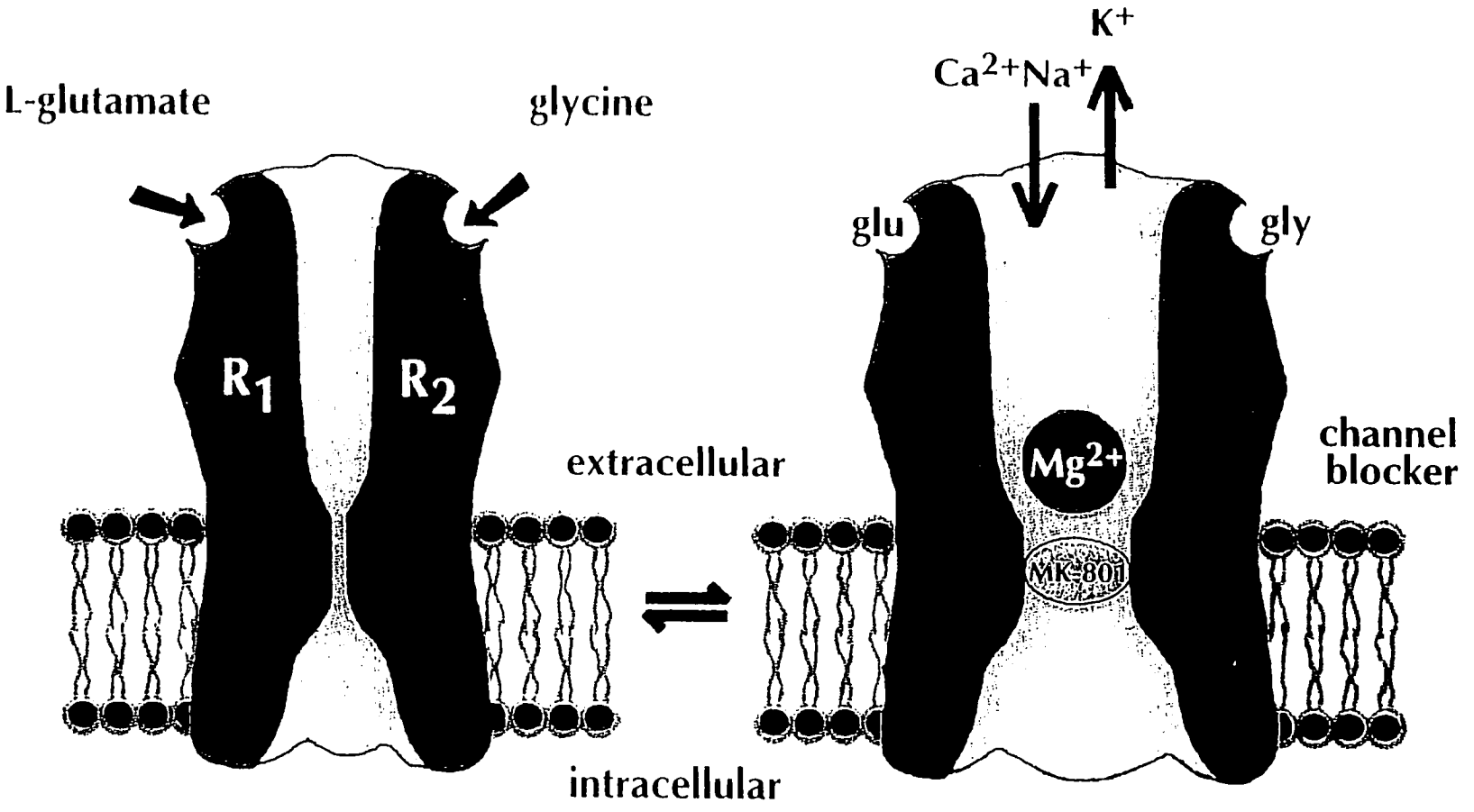


Figure 2: Schematic of the brain-pituitary-gonad (BPG) axis. This diagram shows the interrelationship among three GnRH containing nuclei in platyfish brain, the NOR, NPP, and NLT, as well as their communication with the pituitary gland, and ultimately with the gonads (see Introduction). (Adapted from Schreibman *et al.*, 1994.)

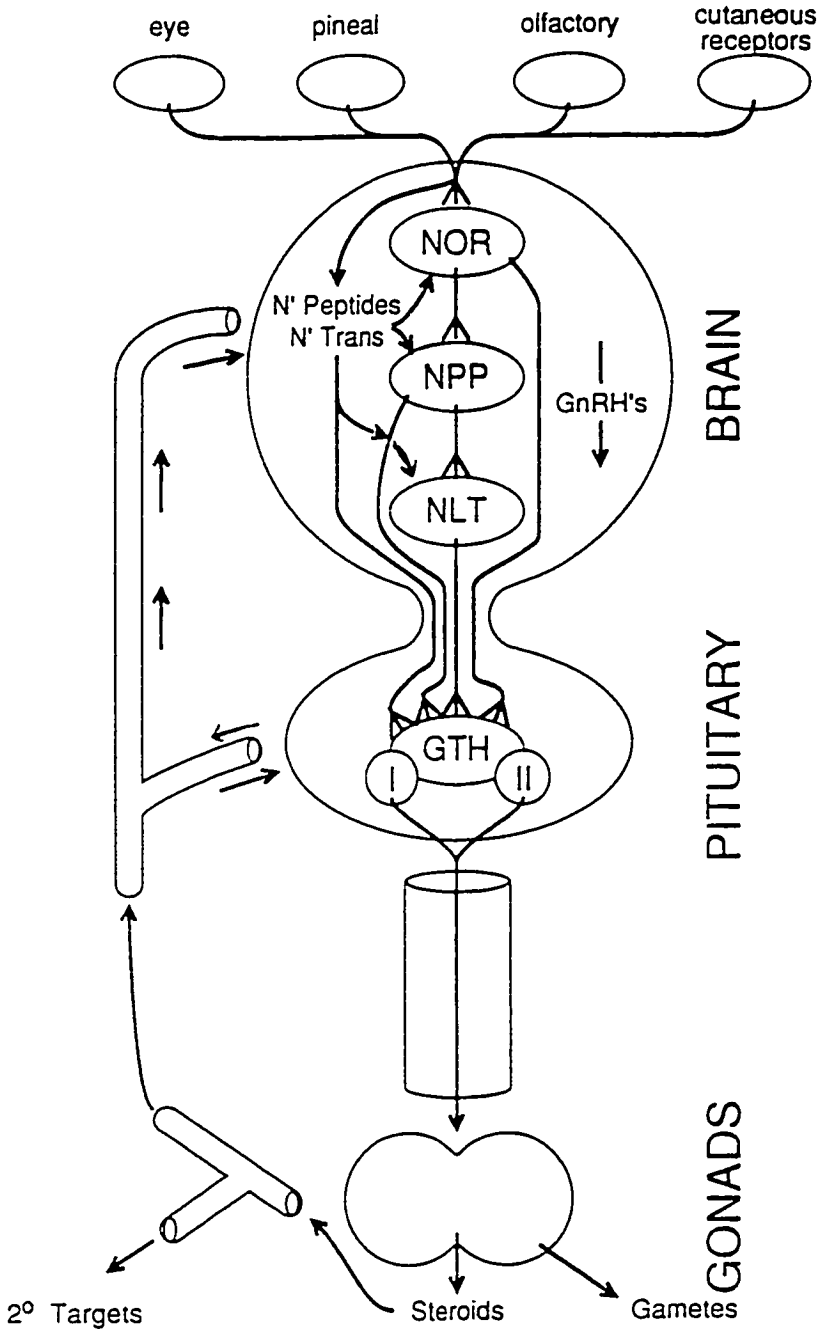


Figure 3: Metamorphosis of the male anal fin. The development of the gonopodium (B) from the anal fin (A) occurs in 6 discrete stages, under the influence of androgens, as male platyfish begin puberty (1 - 6 below). (Adapted from Schreibman, unpublished observation.)

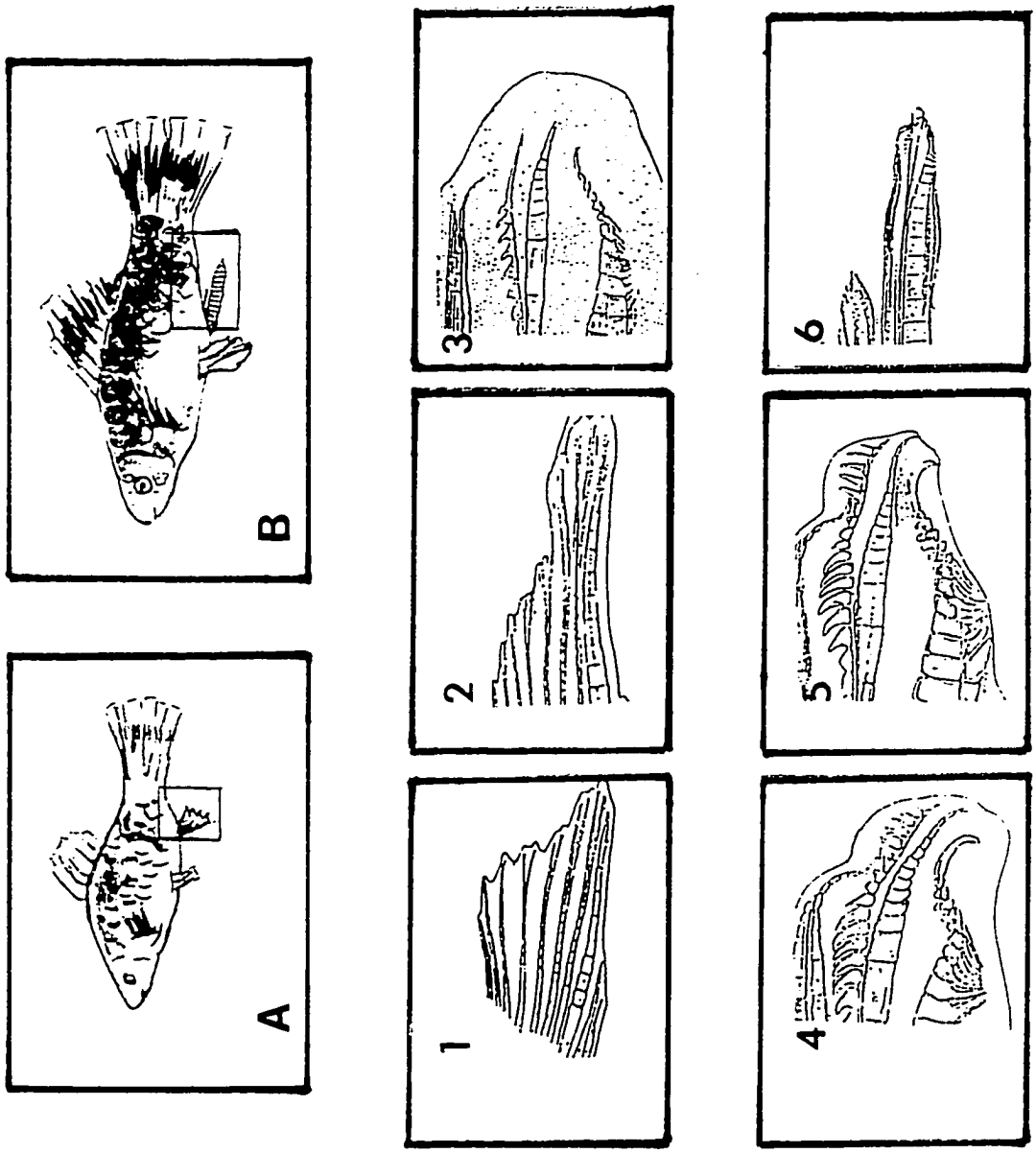
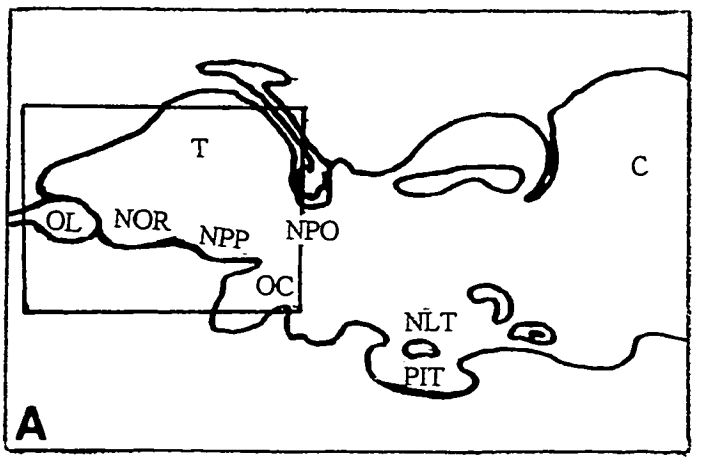


Figure 4: Ir-NMDAR1 distribution in the platyfish brain. (A) is an illustration of a mid-sagittal brain section. Anterior is to the left. OL = olfactory lobe, OC = optic chiasm, NOR = nucleus olfactoretinalis, NPO = nucleus preopticus, NPP = nucleus preopticus periventricularis, NLT = nucleus lateralis tuberis, T= telencephalon, C = cerebellum. (B) Magnification of boxed area in (A) in photomicrograph of the brain of a typical pubescent male, note staining localized in the NOR (arrowhead). Bar = 250 microns.



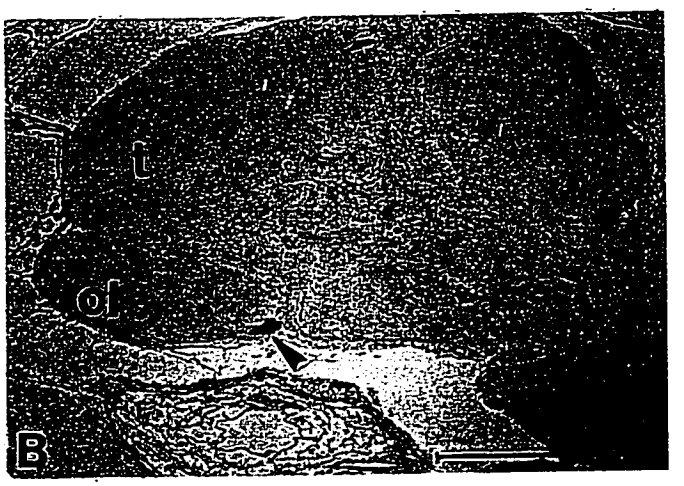


Figure 5: Ir-NMDAR1 at 4 developmental stages. (A) Illustration of mid-sagittal section of the platyfish brain. (B) Magnification of boxed area in (A). (C - J) Magnification of boxed area in (B) at different stages. An immature female and male (C,D), a pubescent pair (E,F), a mature pair (G,H), and a senescent pair (I,J), note differences in the number of cell bodies (arrowheads) stained in the NOR. Anterior is to the left. Bars = 25 microns.

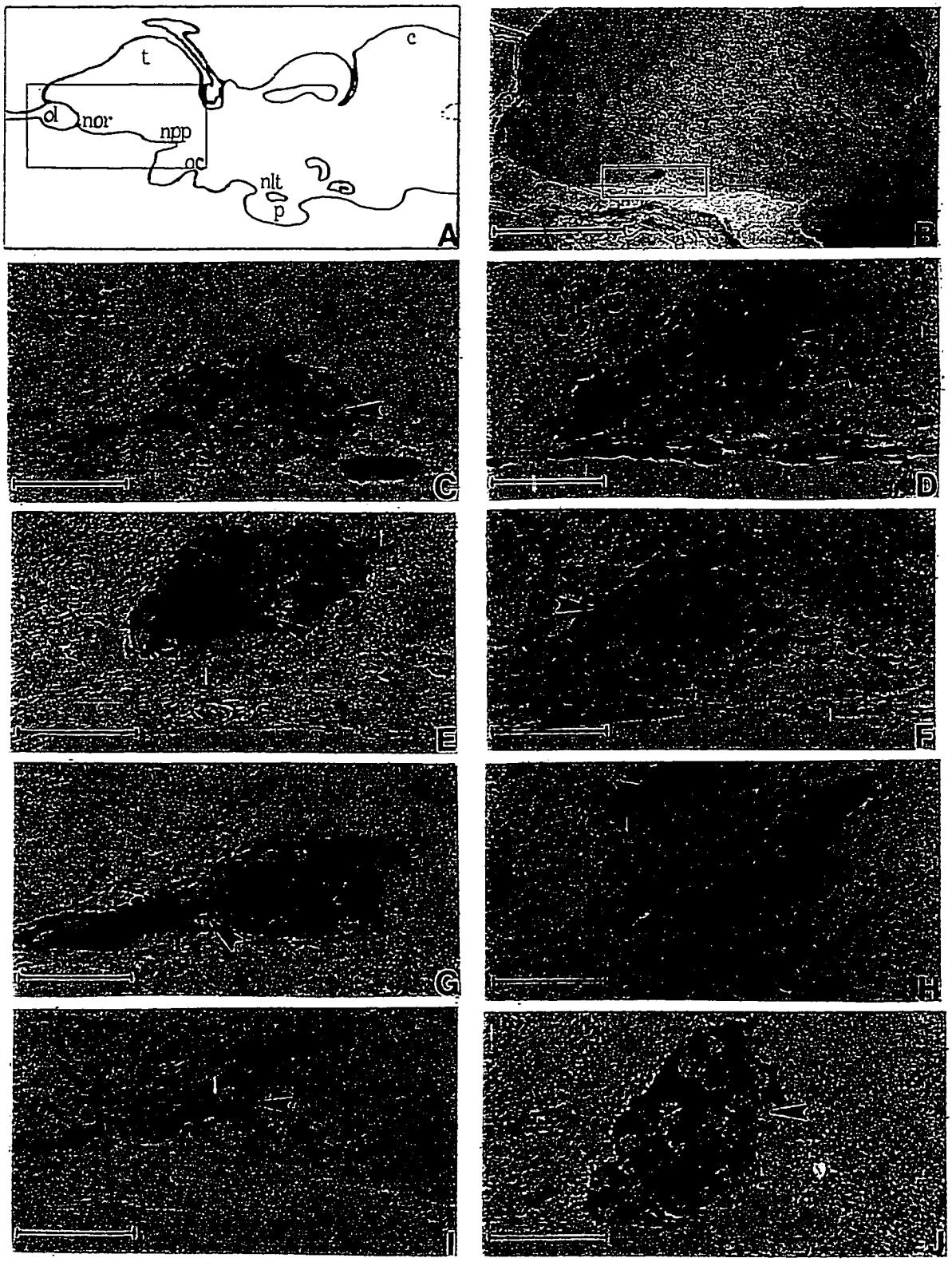


Figure 6: Ir-NMDAR1 in NOR cell bodies. Immature (imm), pubescent (pube), mature (mat), and senescent (sen) females (black bars) and males (white bars). The number of cell bodies stained with ir-NMDAR1 in the NOR is significantly higher ($p < 0.01$) in pubescent and mature females compared to immature and senescent ones. There is no significant difference ($p < 0.1$) between immature and senescent females nor between pubescent and mature females, and no difference among the male groups nor between any female stage and any male stage.

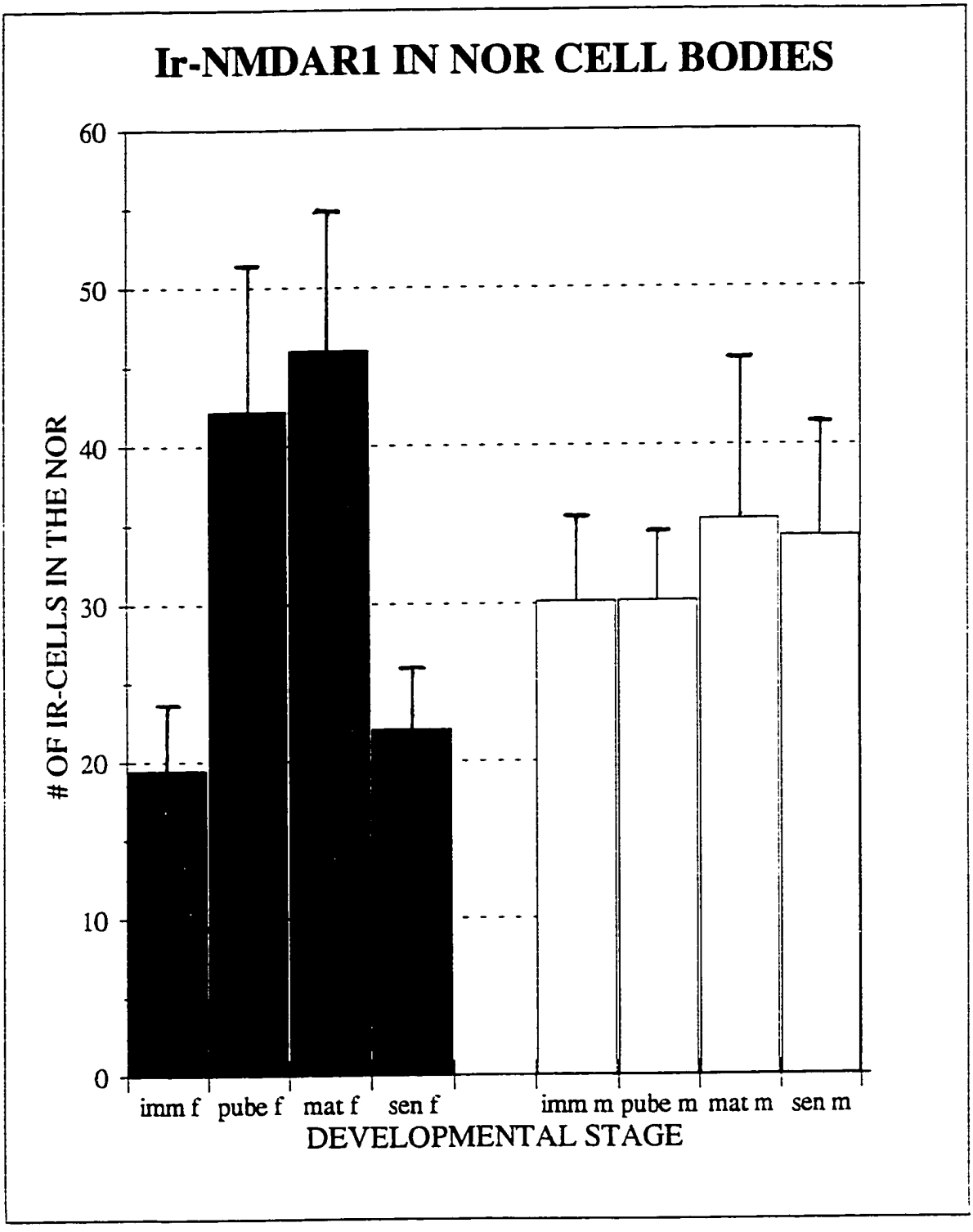


Figure 7: (^{3}H)MK-801 binding to NMDA receptors. Binding curves for typical male and female animals of each developmental stage. Each point is the average of 2 runs; all points are in counts per minute (cpm), and the best possible curve is drawn through all values.

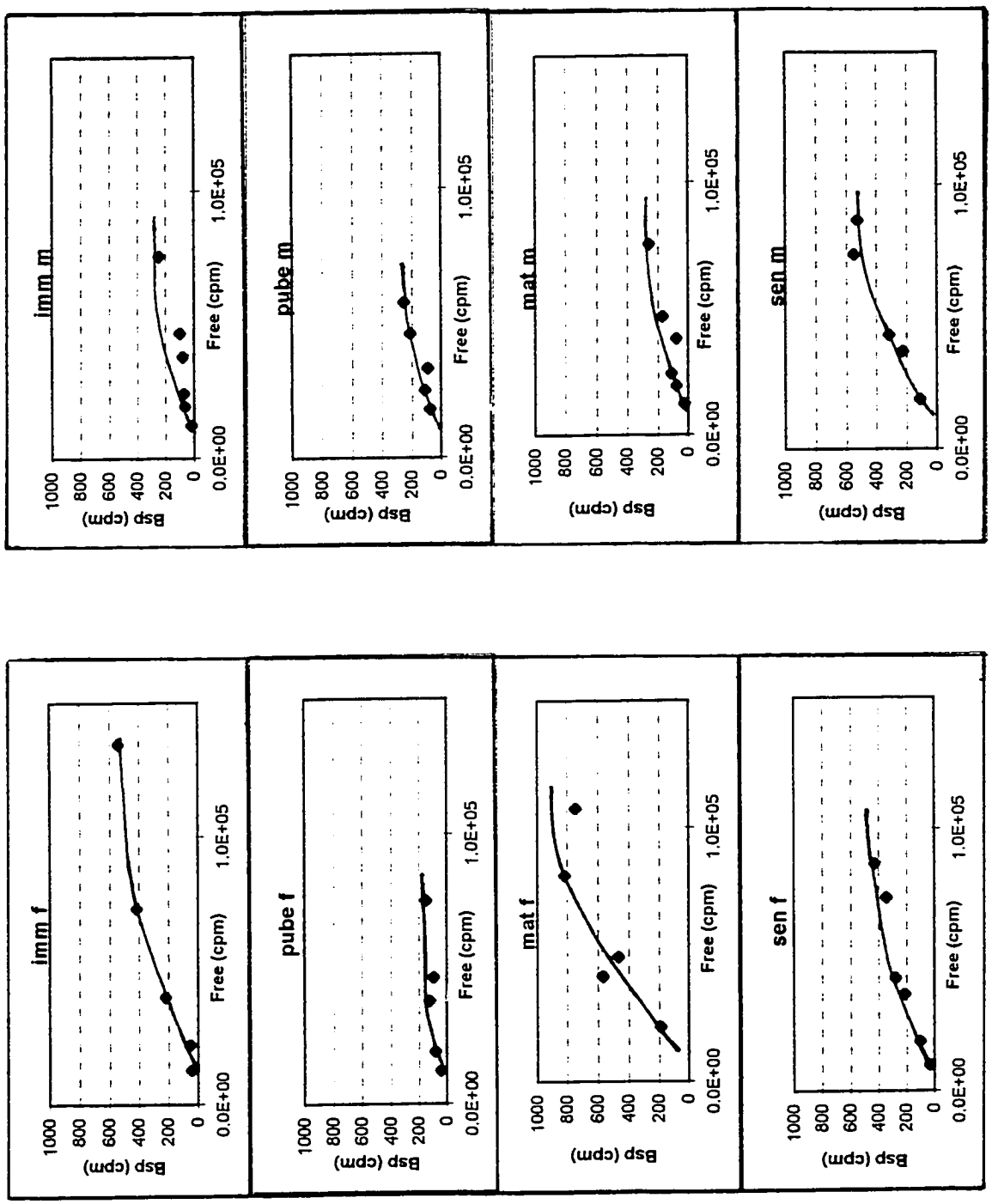


Figure 8: NMDA affinity for (^{3}H)MK-801. Summary graph of binding data from all assays shows significantly increased affinity (decreased K_d) for MK-801 ($p < 0.01$) in pubescent females compared to females of all other stages. There is no significant difference ($p < 0.1$) among any of the male groups, any of the other female groups, nor between males and females.

RECEPTOR AFFINITY FOR (3H)MK-801

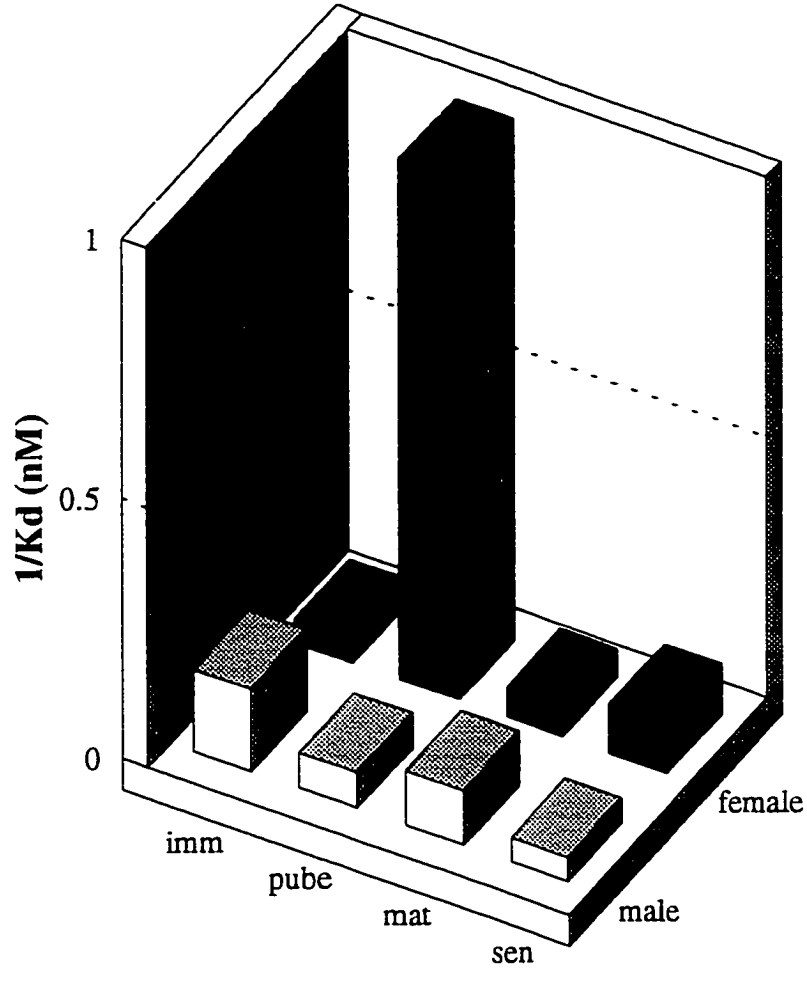


Figure 9: Maximum binding of (^{3}H)MK-801. Immature (imm), pubescent (pube), mature (mat), and senescent (sen) females (black bars) and males (white bars). Summary graph of binding data from all assays shows significantly ($p < 0.01$) decreased B_{max} in pubescent females and increased B_{max} in mature females compared to females at other developmental stages. There is no significant difference ($p < 0.1$) among any of the male groups nor between immature and senescent females.

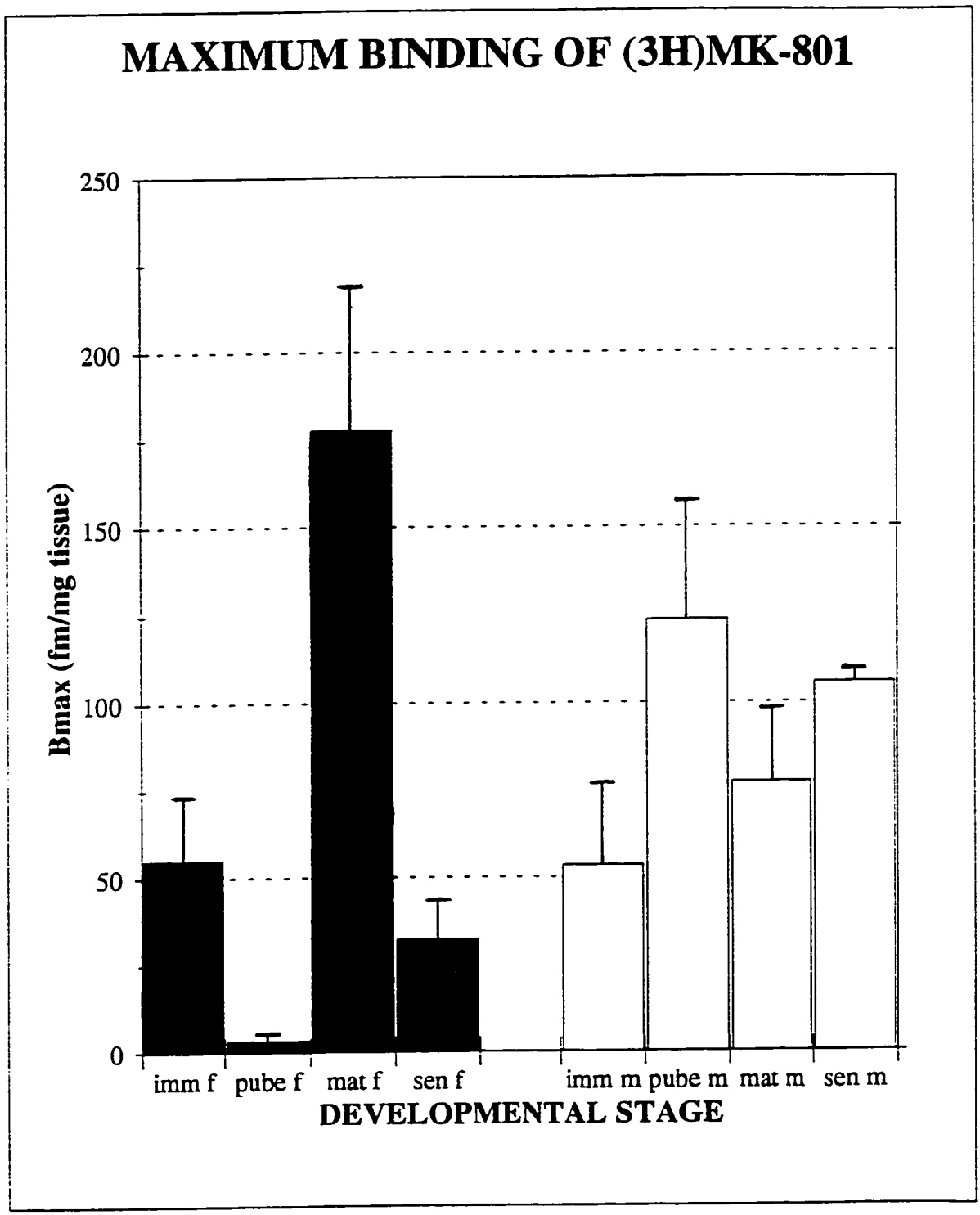
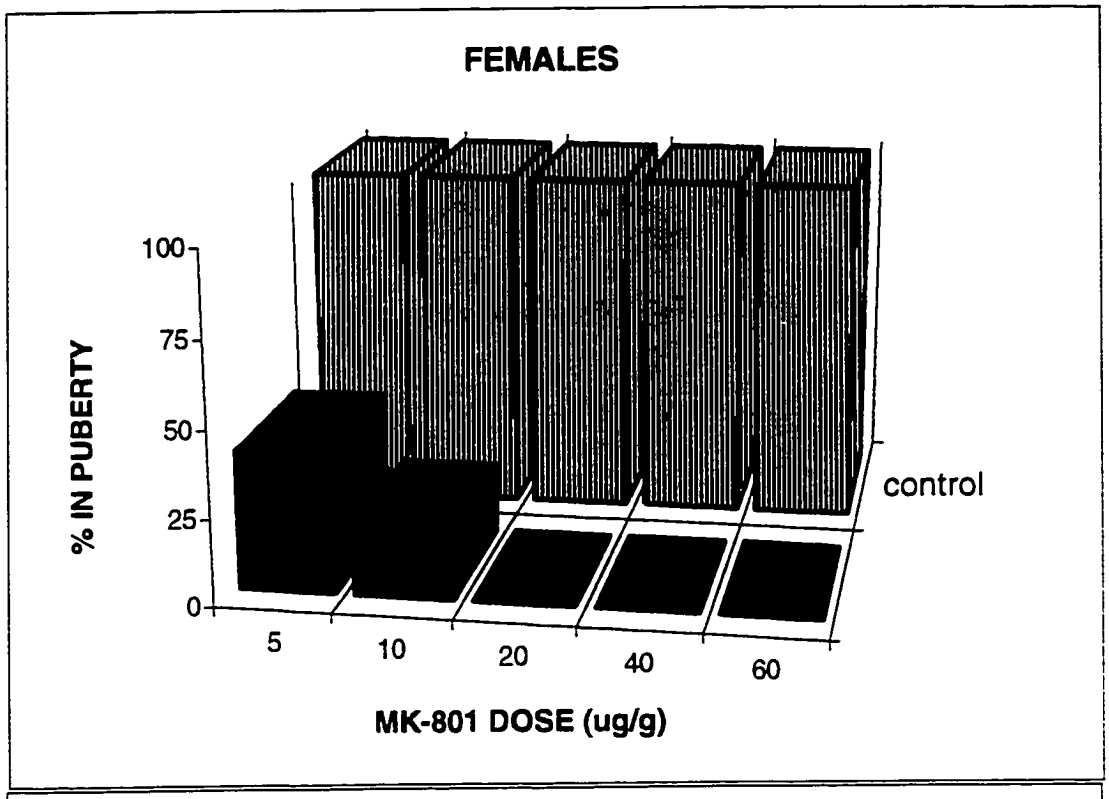


Figure 10: The effect of MK-801 on puberty. Percentage of female (black bars) and male (white bars) animals in puberty after chronic treatment with one of 5 doses of MK-801. Differences among the females are dose dependent and significant at $p < 0.01$, differences among the males are not significant.

THE EFFECT OF MK-801 ON PUBERTY



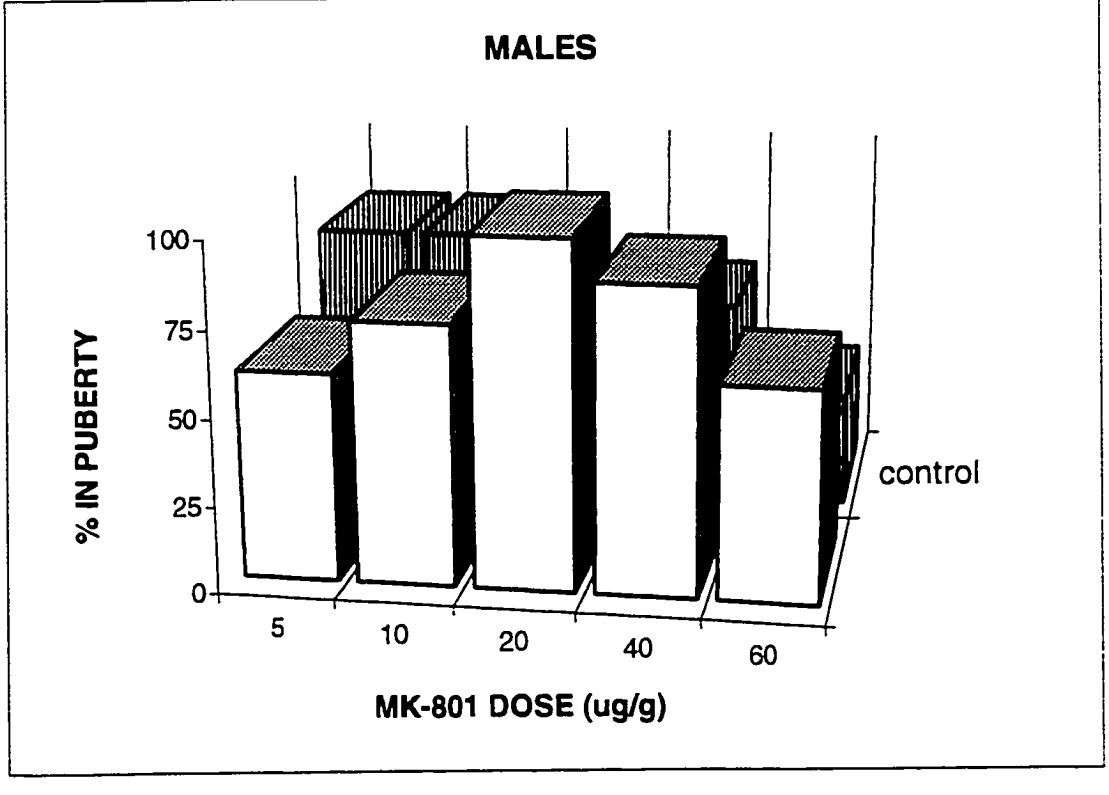


Figure 11: The effect of MK-801 on growth. Percent increase in weight during injection period in control and MK-801 treated males and females. MK-801 doses ranged from 5 to 60 ug/gm body weight. Female data are significantly different at $p < 0.05$ (*), male data are not different at $p < 0.1$.

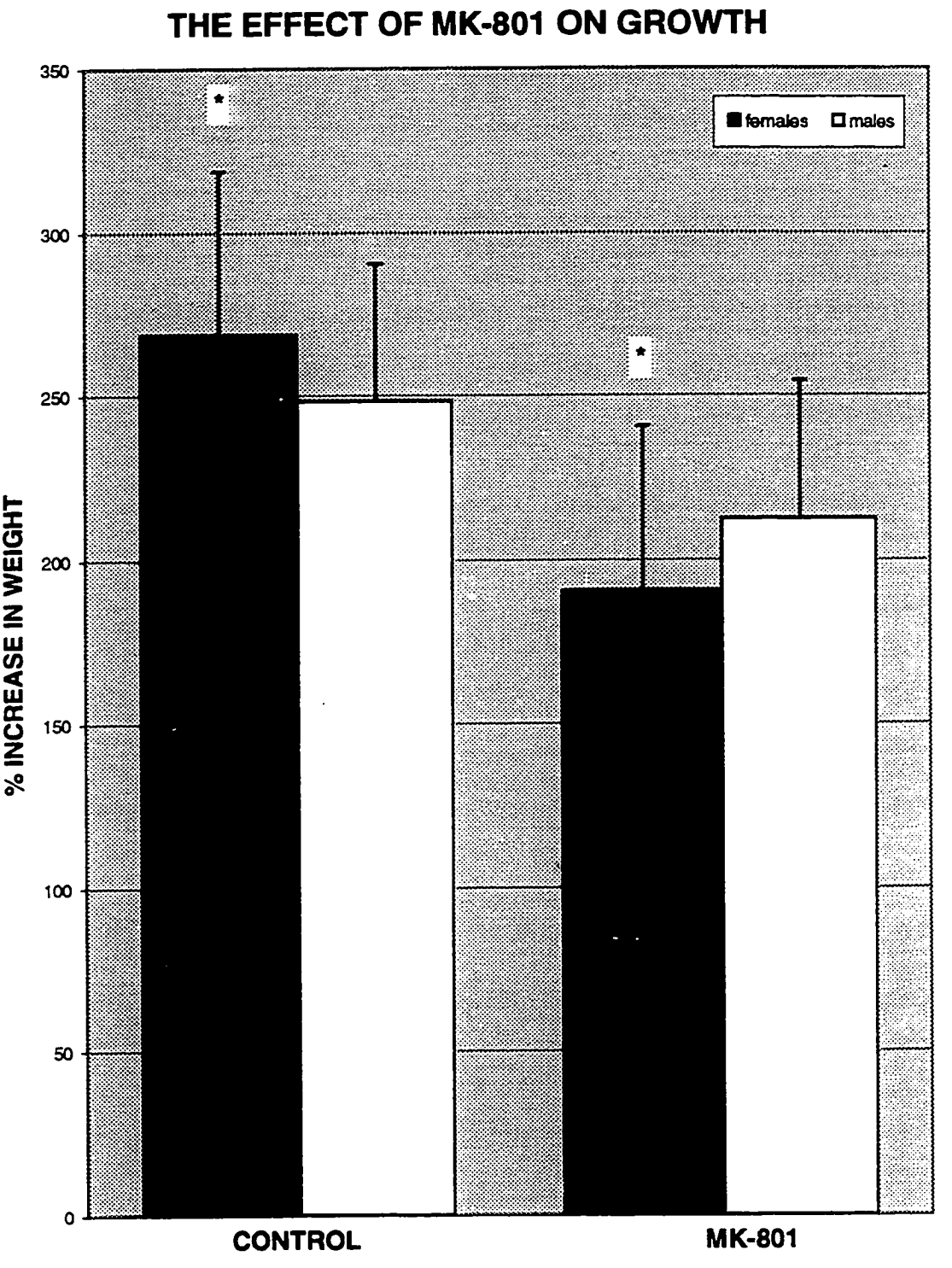


Figure 12: The effect of MK-801 on gonad histology. Gonad sections are stained with Masson's trichrome. (A) A control female, oocytes (o) are large and mature, small follicular cells (f) are around them. (B) An experimental female, small immature oocytes and follicular cells. (C and D) Testes from pubescent control and treated animals respectively show many mature, sperm-containing spermatozeugmata (arrowheads). Bars = 100 microns.

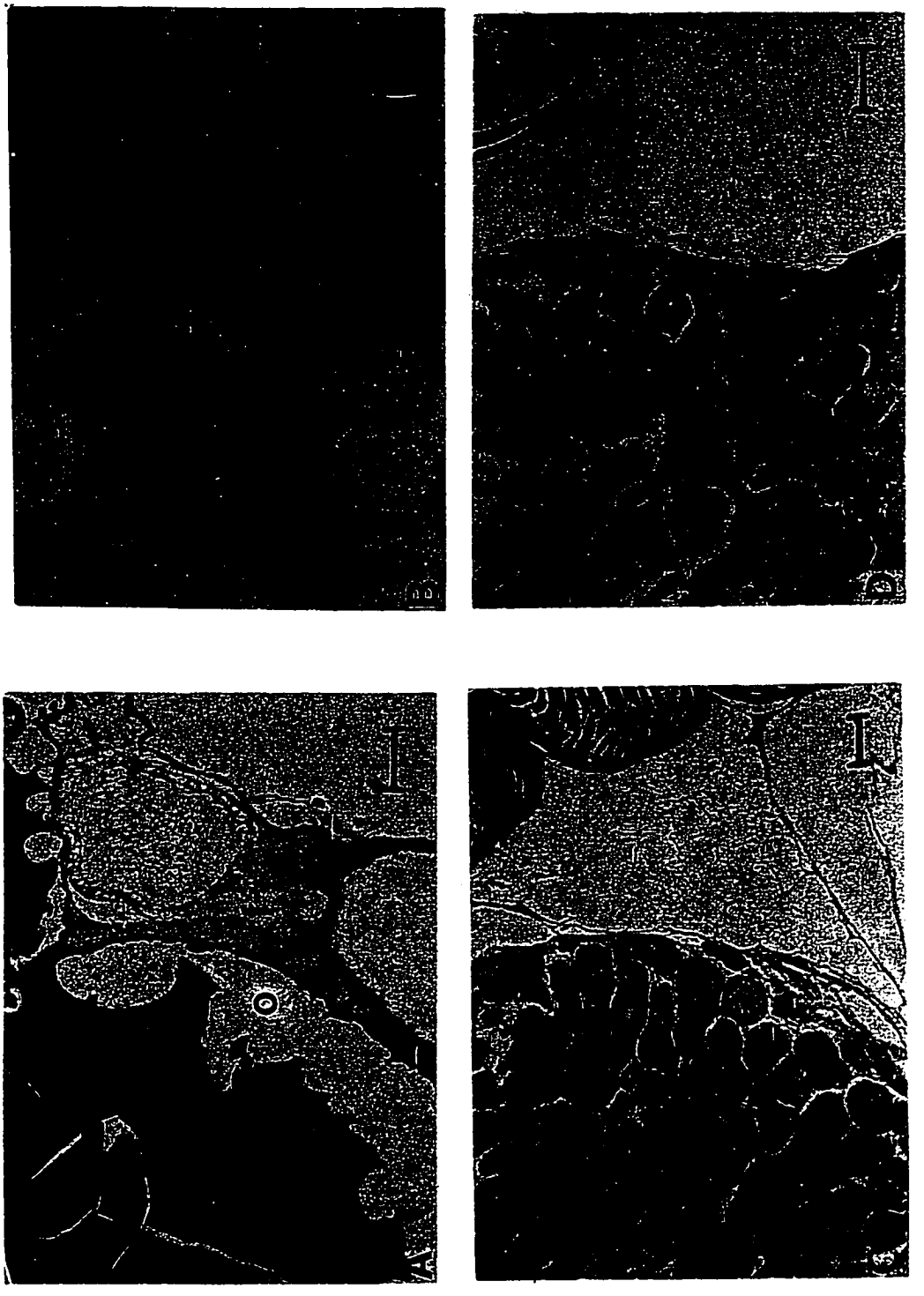


Figure 13: The effect of MK-801 on pituitary histology. Mid-sagittal sections of the pituitary gland stained with DAB. (A) A control female; the ventral portion of the gland is large where a thick layer of gonadotropic cells is visible (arrowheads). (B) An experimental female; the ventral gonadotropic layer is thin and undeveloped. (C and D) A control and experimental male respectively, both with narrow bands of gonadotropic cells visible in the ventral part of the gland. Bars = 100 microns.

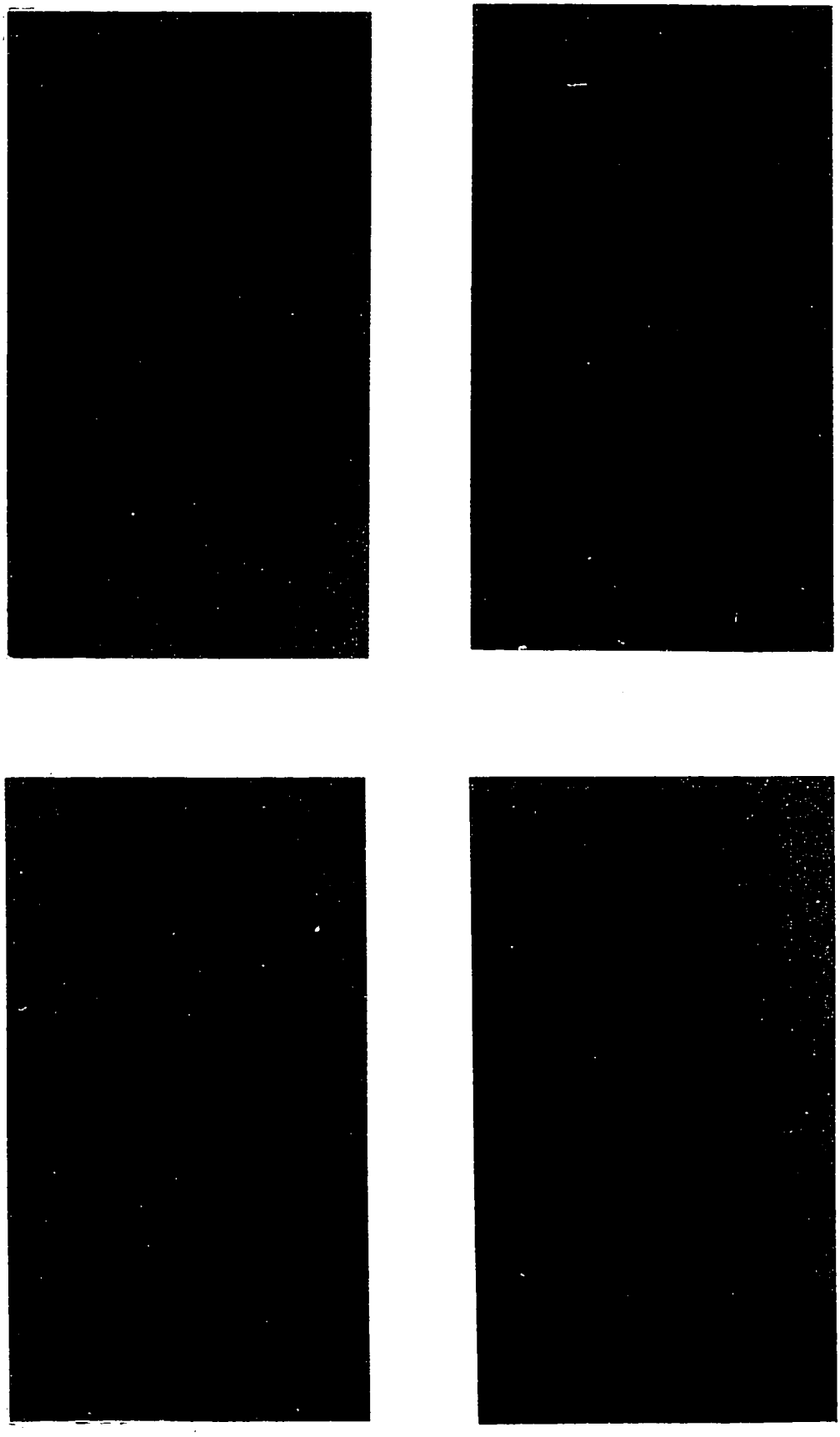


Figure 14: The effect of MK-801 on GnRH. Pilot study used radioimmunoassay to quantify concentration of GnRH in 1 or 2 samples of pooled tissue from each of the four treatment groups. Student *t*-test shows a significant difference between the female values at $p < 0.1$ (*). There is no difference between the males.

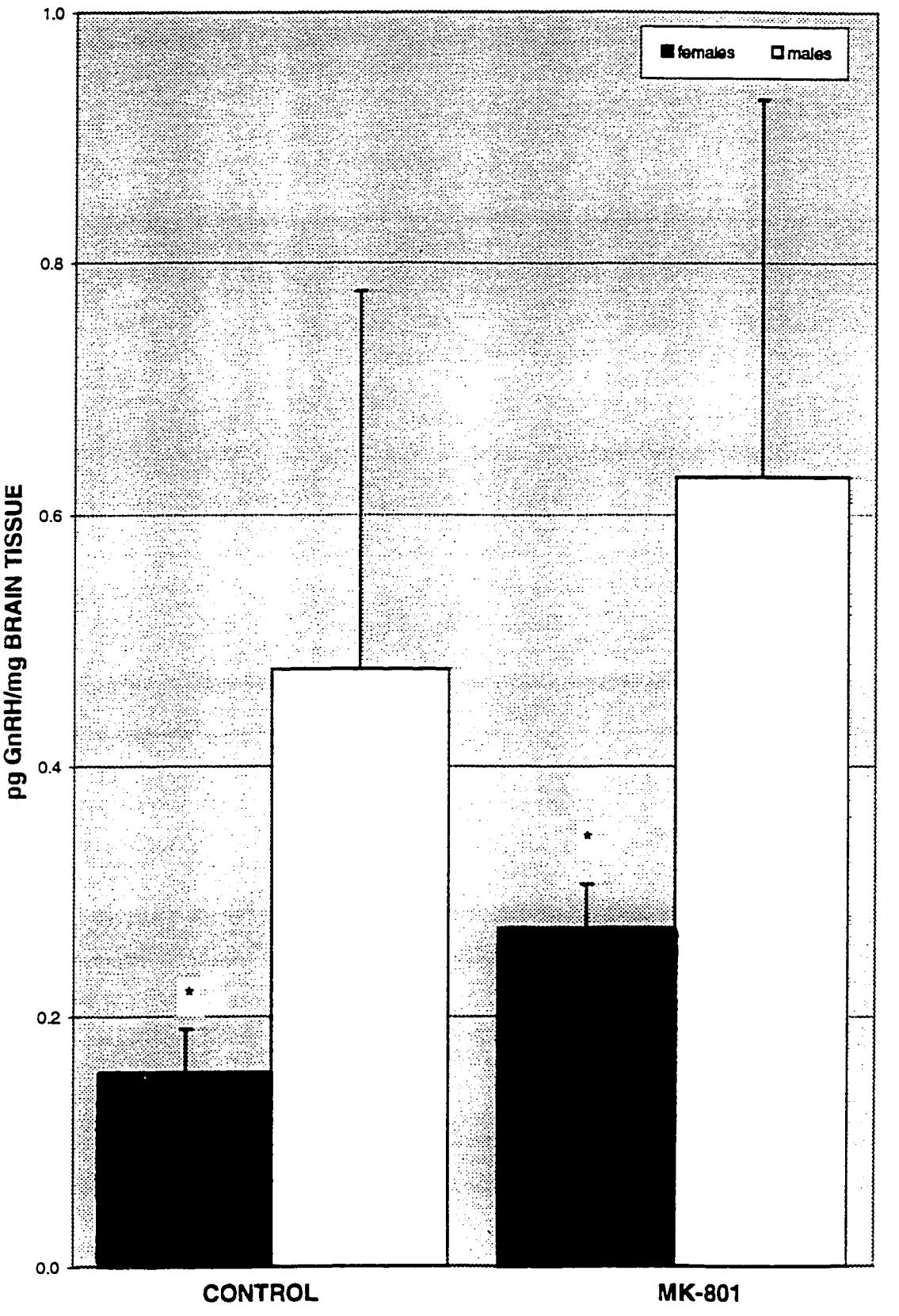


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IMAGE EVALUATION TEST TARGET (QA-3)

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