

AN ABSTRACT OF THE THESIS OF

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Title: Characterization and Localization of Arginine  
Vasotocin Receptors in the Brain and Kidney of an  
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Because arginine vasotocin (AVT) activates male sexual behaviors in the rough-skinned newt (Taricha granulosa), quantitative autoradiography with radiolabelled arginine vasopressin ( $^3\text{H-AVP}$ ) was used to localize and characterize putative AVT receptors in the brain of this amphibian. Binding of  $^3\text{H-AVP}$  to sites within the medial pallium was saturable, specific, reversible, of high affinity ( $K_D=1$  nM) and low capacity (57 fmol/mg protein). These binding sites appear to represent authentic central nervous system receptors for AVT. Furthermore, ligand specificity for the binding sites in this amphibian differs from that reported for AVP binding sites in rat brains.

Dense concentrations of specific binding sites were located in the olfactory nerve as it entered the olfactory bulb, within the medial (hippocampal) pallium,

dorsal pallium, and amygdala pars lateralis of the telencephalon, and in the tegmental region of the medulla. Concentrations of binding sites differed significantly among various brain regions. A comparison of male and female newts collected during the breeding season revealed no sexual dimorphism. These areas may represent site(s) of action where AVT elicits sexual behaviors in male T. granulosa.

Gonadectomy of male and female newts (30 days) resulted in significantly reduced concentrations of AVT receptors in the amygdala pars lateralis, compared to concentrations in sham-operated controls. This effect was specific to the amygdala; other brain areas were not affected. These findings support the hypothesis that, in vertebrates in general, one action of gonadal steroid hormones on neural substrates is to maintain receptors to behaviorally active neuropeptides.

Specific binding sites for  $^3\text{H}$ -AVP were localized over the kidney glomeruli. Scatchard analysis showed a single class of receptors with a dissociation constant of 0.77 nM and a binding site concentration of 35 fmol/mg protein. Localization of AVT receptors over kidney glomeruli and ligand specificity of these sites supports the hypothesis that AVT causes antidiuresis in urodele amphibians via a vasoconstricting (pressor) action rather than a tubular antidiuretic response.

CHARACTERIZATION AND LOCALIZATION OF ARGININE  
VASOTOCIN RECEPTORS IN THE BRAIN AND KIDNEY  
OF AN AMPHIBIAN

by

Sunny K. Boyd

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CHARACTERIZATION AND LOCALIZATION OF ARGININE VASOTOCIN  
RECEPTORS IN THE BRAIN AND KIDNEY OF AN AMPHIBIAN

Chapter I

GENERAL INTRODUCTION

Sexual behavior is a complex phenomenon with many points of possible control and modulation by internal and external factors. Steroids are well known for their effects on reproductive behaviors. However, the fact that neuropeptide hormones also play a role in regulating reproductive behaviors is becoming increasingly clear. The causal relationships between neuropeptides and other factors known to influence sexual behavior - such as sensory input and steroid hormone levels - are unknown. These factors may influence neuropeptide action in a variety of ways including changing synthesis and/or release of neuropeptides or modifying the target tissue of the neuropeptides (by affecting receptor levels, capacity for response, etc). The sites of action of neuropeptides must be identified before the complex interactions of neuropeptides and other factors controlling sexual behavior can be investigated in detail.

Arginine vasopressin (AVP) is a nonapeptide synthesized primarily in the supraoptic and paraventricular nuclei of the hypothalamus in the brain of mammals. Axons from cell bodies in these areas then project to the posterior pituitary (where the peptide is stored for eventual release into the circulatory system) and to a variety of extra-hypothalamic areas. AVP is replaced in non-mammalian vertebrates by arginine vasotocin (AVT), which has isoleucine in the 3 position instead of phenylalanine. AVT has also been identified in mammals and may have some biological actions distinct from those of AVP (see review, Zerbe and Robertson, 1981).

There is considerable evidence, as discussed in detail below, that AVT is involved in regulating reproductive behaviors of Taricha granulosa males (Moore and Miller, 1983; Moore and Zoeller, 1979; Zoeller and Moore, 1982). Two other amphibian species also show effects of AVT on reproductive behavior. They are Rana pipiens females (Diakow, 1977; 1978; Diakow and Nemiroff, 1981; Diakow and Raimondi, 1981) and both sexes in Hyla cineria (Penna; personal communication). Additionally, AVT can influence the display of some male newt sexual behaviors in female Taricha (personal observation).

AVP and AVT have also been shown to influence

reproductive behaviors in particular species of mammals (Bohus, 1977; Kihlstrom and Agmo, 1974; Sodersten et al, 1983), fish (Pickford and Strecker, 1977; Wilhelmi et al, 1955), and birds (Kihlstrom and Danninge, 1972). As well, there is extensive evidence in mammals that learning and memory are affected by AVP-like peptides (deWied, 1983; Koob et al, 1981; McGaugh et al, 1982).

The site of action of AVP and AVT in causing these diverse behavioral effects is unclear. The effects of vasopressin on memory have been generally localized in the limbic system of the brain (specifically, the amygdala, hippocampus, septum, and some thalamic areas; see van Wimersma Greidanus et al, 1983a). The site of action on reproductive behaviors is unknown. Although it seems clear that the effect on male T. granulosa is via the central nervous system (CNS), the proposed mechanism of action of AVT in female R. pipiens is through peripheral effects on water uptake and a resultant increase in internal abdominal pressure (Diakow, 1978). The overall importance of the CNS to the activation of reproductive behaviors by AVT (as well as specific areas of the brain perhaps involved in this activation) remains unknown.

AVP-like peptides also influence other CNS functions besides memory and reproductive behaviors.

Following central administration of AVP, heart rate (Meisenberg and Simmons, 1983) and body temperature are altered (Cooper et al, 1979; Kasting et al, 1979). Actions on heart rate appear to be localized to the medulla oblongata and spinal cord, and those on body temperature appear to occur in the septal region. Vasopressin also affects capillary permeability, probably by acting on sites in the hindbrain, to alter blood pressure or osmolality (Swanson and Sawchenko, 1980).

These actions of AVP-like peptides occur by acting on specific cell-surface receptors. Therefore, the first step in determining the sites of action of AVP and AVT depends on localization of neuroanatomical areas possessing these receptors. The purpose of this investigation is to determine which regions within the central nervous system of Taricha granulosa have specific AVT receptors and, therefore are potential sites for the modulation of sexual behaviors of the newt. The influence of gonad removal on AVT receptor populations can then be tested to determine if the interaction between steroids and AVT in controlling reproductive behaviors occurs via steroid influence on AVT receptor distribution, density, or binding characteristics.

ROLE OF ARGININE VASOTOCIN IN AMPHIBIAN REPRODUCTIVE BEHAVIORS

When injected with AVT or AVP, sexually mature T. granulosa males exhibit amplectic clasping behaviors (Moore and Miller, 1983; Moore and Zoeller, 1979; Zoeller and Moore, 1982). Additional evidence that AVT regulates sexual behavior in male newts comes from studies in which endogenous AVT activity is suppressed (Moore and Miller, 1983). An intracranial injection of either an AVP antagonist or anti-AVT immune serum results in a significant decrease in the incidence of amplectic clasping in intact male newts.

The target cells for the behavioral response to AVT in male T. granulosa are most likely in the brain. Male newts are over 10,000 times more sensitive to intracranially administered AVT than systemically administered AVT (Moore and Miller, 1983). Additionally, concentrations of AVT in specific brain areas of male newts are correlated with the occurrence of reproductive behaviors (Zoeller and Moore, 1987). AVT concentrations are significantly higher in sexually active than in sexually inactive males in the dorsal preoptic area, optic tectum, cerebrospinal fluid (CSF), and ventral infundibular nucleus. These studies support the hypothesis that the brain is the site of

action for AVT in facilitating reproductive behaviors in male newts. They also suggest that this effect may be localized in specific parts of the brain.

A complex, and as yet unresolved, interaction exists between AVT and levels of testicular androgens in the control of sexual behavior in male newts. Five and eighteen days after surgery, injection of AVT stimulates clasping behavior in castrated controls (unimplanted or cholesterol-implanted) but not in androgen-implanted, castrated newts. Conversely, AVT injected 33 days after castration increased the incidence of courtship behavior in androgen-implanted males, but not in unimplanted castrates (Zoeller and Moore, 1982). Therefore, although androgens maintain the behavioral responsiveness of newts to AVT, factors such as circulating hormone levels and duration of exposure are clearly important.

Responsiveness of long-term castrates to AVT can also be maintained by very small androgen implants into the hypothalamus (Moore et al, 1981). This suggests that the requirement for androgen is in the brain rather than at the level of peripheral organs. Interestingly, the influx of testosterone into the newt brain can be significantly increased by intraperitoneal injections of AVT, indicating that AVT can enhance the movement of at least one hormone (testosterone) across

the blood-brain barrier (Moore et al, 1981).

The behavioral response of male newts to an injection of AVT is associated with an elevation in plasma androgen concentration (Moore, unpublished). When sexually inactive males are injected with AVT or saline the concentration of androgen in the AVT-injected males that exhibited sexual behaviors was significantly higher compared to that in AVT-injected males that did not exhibit behavior or in saline-injected newts (none of which showed behavior). Although this clearcut correlation exists, cause and effect relationships as well as the mechanism by which these three factors influence each other are not understood.

This AVT-androgen interaction apparently does not occur at the level of the pituitary gland, because hypophysectomy does not abolish the behavioral response to AVT (Zoeller and Moore, 1982). AVT can, however, influence the secretion of prolactin, adrenocorticotrophic hormone, luteinizing hormone, and melanocyte-stimulating hormone from the pituitary gland of mammals (review by Zerbe and Robertson, 1981). Although the behavioral response of newts to AVT injections does not require the release of these hormones, important feedback relationships could exist between these hormones (especially the steroids they

control) and AVT levels and receptor levels. These hormones have also been separately implicated in the modulation of amphibian reproductive behaviors (see review by Moore, 1983).

AVT can also modulate reproductive behaviors in other amphibians. In female R. pipiens, an injection of AVT inhibits release calls; i.e., AVT increases sexual receptivity (Diakow and Nemiroff, 1981). Prostaglandin injection also has this effect in female frogs and, because indomethacin antagonizes this action of AVT, the action of AVT on receptivity appears to involve prostaglandins (Diakow, 1978). Ovariectomy does not alter the inhibitory effect of AVT on release calls, which suggests that ovarian steroids are not required for AVT to activate sexual behaviors in female frogs (Diakow and Raimondi, 1981). This contrasts with the requirement for testicular androgens in male newts. The effect of AVT on R. pipiens behavior is postulated to occur via a peripheral, not central, mechanism whereby AVT stimulates uptake of water and increases intra-abdominal pressure (Diakow, 1978).

AVT is capable of inducing mate-calling, a male reproductive behavior in intact male Hyla cinerea and in androgen-implanted females (Penna, personal communication). In intact and androgen-implanted female T. granulosa, AVT injection can stimulate the

occurrence of male-like amplexic clasping (personal observation). These differences in the ability of AVT to influence male-typical and female-typical reproductive behaviors, even within amphibians, raise important questions about the interactions of different steroids with AVT, and about sex and species differences in AVT modulation of reproduction.

#### LOCALIZATION OF SITES OF ARGININE VASOPRESSIN ACTION

In mammals, vasopressin has a variety of biological actions which are mediated by central nervous system mechanisms. Many of the centrally mediated effects have been shown to be elicited at sites within the brain stem and the limbic system, which is where vasopressin occurs in axons and nerve terminals (Meisenberg and Simmons, 1983).

Among the best studied of these effects is the modulation of memory processes. Several lines of evidence point to a physiological role for the neurohypophyseal hormones in behavior, memory processes and/or cognitive functions: 1) the extrahypothalamic projections of neurohypophyseal axons to various limbic regions; 2) the small amounts of peptide necessary for behavioral effects following central administration; 3) changes in neuropeptide levels in cerebrospinal fluid

of rats in relation to their behavioral performance; 4) the disturbed behavior of rats without adequate endogenous vasopressin (Brattleboro rats and rats receiving a central application of antisera to vasopressin) (Burbach et al, 1983; De Wied, 1983; Kovacs and Telegdy, 1982; van Wimersma Greidanus et al, 1983b; van Wimersma Greidanus, 1984).

In order to localize the site of action of AVP in relation to its effects on learning and memory in mammals, various approaches have been used (see review, van Wimersma Greidanus et al, 1983a). As a result of studies using rats with lesions, as well as studies in which neuropeptides were locally applied into various areas of the brain, it appears that the limbic system (amygdala, hippocampus, septum, and some thalamic areas) plays an essential role in the effect of vasopressin and its derivatives on learning and memory. Neurochemical studies generally indicate that changes occur in catecholamine utilization in the same limbic regions following administration of these neuropeptides. The effects of vasopressin in the terminal regions of the coeruleo-telencephalic noradrenalin system (dorsal septum, dorsal raphe, and dentate gyrus of the dorsal hippocampus) correlate with its effects on consolidation of memory. It is likely that the effects of vasopressin on other transmitter

systems (e.g. dopamine in the amygdala and serotonin in the dentate gyrus of the hippocampal complex) correspond with the effect of this neuropeptide on memory retrieval processes.

These studies suggest such areas are the sites of the behavioral action of AVP, and these regions must therefore contain receptors for AVP or AVP-like peptides. The fact that these sites are important in learning and memory in mammals does not suggest, however, that these same areas are the neural substrate for AVT actions on reproductive behavior. This point remains to be tested empirically.

Other indirect evidence also suggests that discrete areas of the brain contain receptors for AVP and, further, that these areas control different physiological and/or behavioral functions. For example, Tanaka et al. (1977a and b) localized the effects of AVP on catecholamine metabolism in distinct brainstem nuclei and found that the effect of AVP on norepinephrine metabolism is limited to a small proportion of midbrain and lower brain stem nuclei. Specifically, AVP effects occur in the dorsal septal nucleus, medial forebrain bundle, parafasicular nucleus, dorsal raphe nucleus, red nucleus, locus coeruleus and solitary tract nucleus. Furthermore, AVP alters dopamine metabolism in the striatum and dorsal

raphe nucleus (Doris, 1984).

Various central effects of AVP in mammals involve different mechanisms which can be distinguished from one another on the basis of required dose, time-course of action, and structure-activity relationships (Meisenberg and Simmons, 1983). These diverse actions may be localized in separate brain areas. Dehydration in rats induces alterations in AVP content of the neural lobe, anterior pituitary, and median eminence but not in the supraoptic nucleus, paraventricular nucleus, suprachiasmatic nucleus, or organum vasculosum laminae terminalis (Samson, 1985). Also, use of a mRNA probe for vasopressin in rat brain has shown effects of osmotic stress on supraoptic nucleus and paraventricular nucleus mRNA content but no effect on the suprachiasmatic nucleus (Burbach et al, 1984). Separate regulation may reflect the existence of two independent vasopressin systems associated with its peripheral and central functions, respectively. Research in male Taricha also shows that water deprivation affects AVT levels in different areas of the brain than those regulating sexual activity (Zoeller and Moore, 1987).

IMMUNOREACTIVE ARGININE VASOPRESSIN AND VASOTOCIN  
WITHIN THE BRAIN

In mammals, immunoreactive AVP is distributed among a wide range of brain areas. There are distinct pathways of AVP-containing neurons and regional specificity in AVP concentrations. Use of immunohistochemical methods on mammalian brains reveals three major sites of AVP-containing perikarya (nerve cell bodies) -- the supraoptic nucleus (SON), paraventricular nucleus (PVN), and suprachiasmatic nucleus (SCN) (see review, Dierickx, 1980; Swaab et al, 1975a,b; Vandesande and Dierickx, 1975; Vandesande et al, 1975; 1977). As well, a few accessory magnocellular neurons, separate from the SON, PVN, and SCN proper but scattered between these nuclei, also contain vasopressin and project to the posterior pituitary (Kelly and Swanson, 1980; Sofroniew and Weindl, 1978; Zimmerman, 1976; 1981). Recent studies also describe AVP immunoreactive cell bodies outside the hypothalamus in the bed nucleus of the stria terminalis (DeVries and Buijs, 1983; Van Leeuwen and Caffee, 1983).

The main, if not sole, projection of AVP-immunoreactive fibers from the SON is to the posterior pituitary (Zimmerman, 1981; Zimmerman et al, 1984). The PVN sends fewer vasopressinergic fibers to the posterior pituitary (Swanson and Sawchenko, 1980;

Wiegand and Price, 1980; Zimmerman, 1981); it also has significant projections to the zona externa (portal capillary bed) of the median eminence (Armstrong and Hatton, 1980; Kelly and Swanson, 1980; Swanson and Sawchenko, 1980; Wiegand and Price, 1980) and extrahypothalamic sites in the forebrain, brain stem, and spinal cord (see review, Zimmerman et al, 1984).

AVP-containing neurons of the SCN do not project to the pituitary but instead project to extrahypothalamic sites in the forebrain and hindbrain (Buijs, 1978; Sofroniew and Weindl, 1978, 1980; Zimmerman, 1981). Although some investigators report that most extrahypothalamic sites in the forebrain are innervated from the SCN (Sofroniew, 1980), others believe the contributions of the SCN are more limited to specific areas such as the lateral septum, lateral habenula, and the organum vasculosum of the lamina terminalis (Buijs, 1978; Buijs and Swaab, 1979; Buijs et al, 1978).

In fish and amphibians, the neurosecretory nuclei of the hypothalamus include the two hypothalamic magnocellular preoptic nuclei, located bilaterally in the wall of the prechiasmatic part of the diencephalon (Dierickx, 1980). In reptiles, birds, and mammals, each magnocellular preoptic nucleus has evolved into two nuclei, namely the SON and PVN. Only mammals, have additional neurosecretory cells in the SCN (Sterba et

al, 1981).

Vasotocin has been visualized immunocytochemically in the neurosecretory nuclei of representative species of non-mammalian vertebrates (Blahser, 1981a,b; Bons, 1983; Gossens et al, 1977a,b,c; 1978; 1979). Extrahypothalamic neurosecretory pathways similar to those in mammalian species are present in non-mammalian vertebrates as well (Sterba et al, 1979; 1980; van den Dungen et al, 1982).

Immunocytochemical staining for AVT has been done in several species of amphibian, all anurans. Vasotocinergic cell bodies occur in the preoptic area and have fibers projecting to the pars nervosa, pars intermedia, and external region of the median eminence (Dierickx, 1980; Dierickx and Vandesande, 1976; 1977; Fisher et al, 1981; Vandesande and Dierickx, 1976; Van Vossel et al, 1976; 1977). A few cell bodies immunoreactive for AVT occur outside the preoptic area -- in the pars ventralis of the tuber cinereum (Dierickx and Vandesande, 1977).

Recent immunocytochemical work in the toad Bufo japonicus, shows perikarya of AVT neurons primarily in the ventral magnocellular part of the preoptic nucleus, where they form laminar cell clusters (Jokura and Urano, 1985; Takami et al, 1984). A small number of AVT-containing neurons also occur in the dorsal

magnocellular and suprachiasmatic parts of the preoptic nucleus. Many of the fibers arising from the ventral POA project to the median eminence and pars nervosa via the infundibulum. In addition to this neurosecretory pathway, fibers project anteriorly from the magnocellular POA to the anterior preoptic nucleus and many continue to the medial septal region, mesencephalic reticular formation, optic tectum, and medulla oblongata (Urano et al, 1985). This group did not detect seasonal variations in immunoreactive AVT (Jokura and Urano, 1985) which is an unexpected finding since the volume of the magnocellular preoptic nucleus varied seasonally in this toad (Takami and Urano, 1984) and the magnocellular neurosecretory neurons show seasonal morphological changes in R. temporaria (Dierickx and Vandesande, 1965).

In mammals, the regional distribution of AVP detected by radioimmunoassay is generally compatible with immunohistochemical findings. Significant amounts of AVP have been measured in the median eminence, retrochiasmatic area, SON, PVN, SCN, and arcuate nucleus (George and Jacobowitz, 1975). Use of a more sensitive assay has revealed, in addition, AVP in the thalamus, cerebellum, medulla, amygdala, substantia nigra, hippocampus, pons, spinal cord, occipital lobe, caudate putamen, and frontal lobe (Glick and

Brownstein, 1980; Hawthorn et al, 1980). Summy-Long et al (1978) found AVP in the sub-fornical organ and hippocampus-fornix, and Dogterom et al (1978a) detected AVP in the limbic areas, parafascicular nucleus, cortex, choroid plexus and medulla. In addition, AVP has been found in the CSF of several species (Dogterom et al, 1978b; Jenkins et al, 1980; Luerssen et al, 1977).

Arginine vasotocin immunoreactivity has been demonstrated in the brain of male T. granulosa (Zoeller and Moore, 1986) using microdissection and radioimmunoassay techniques. High levels were observed in the hypothalamo-hypophyseal system, including the preoptic area, ventral and dorsal hypothalamic areas, and the pars distalis and pars nervosa. Detectable quantities were also observed in extrahypothalamic areas such as the pallium, amygdala, and striatum in the forebrain, dorsal and ventral thalamus, superior and inferior colliculi, and nucleus tubercularis posterioris of the midbrain, and the secondary visceral nucleus, interpeduncular nucleus, and ventrolateral hindbrain in the medulla oblongata. No AVT was detected in the olfactory bulb, rostral telencephalon, or specific areas of the caudal telencephalon and diencephalon. These results demonstrate AVT distribution across a wide range of brain areas and

regional specificity in AVT concentration in male newts. The presence of AVT in several extrahypothalamic brain regions may reflect the functioning of vasotocinergic neurons in the regulation of diverse functions such as hydromineral balance and the expression of sexual behaviors.

The potential importance of an AVT-steroid interaction is supported by the observation that sex steroids are concentrated in areas of the amphibian brain that also contain AVT (e.g., thalamic and tectal areas, Kelley, 1980; Kelley et al, 1978). In addition, the concentration of AVT in some brain areas of male newts (dorsal POA, optic tectum, cerebrospinal fluid, and ventral infundibulum) are correlated with the occurrence of sexual behavior (Zoeller and Moore, 1987). Dehydration, on the other hand, influences AVT levels in the ventral POA and interpeduncular nucleus. Seasonal differences exist in AVT in the optic tectum (Zoeller and Moore, 1986). The physiological or behavioral significance of these correlations has not been determined.

Significant amounts of AVT have been reported in goldfish telencephalon, hypothalamus, and pituitary (Hontela and Lederis, 1985). Presence of arginine vasotocin has been reported in the pineal body of some teleosts by radioimmunoassay and bioassay, but was not

detected by immunofluorescence techniques (Holder et al, 1979; Vivien-Roels et al, 1979). AVT has also been localized with radioimmunoassay techniques in reptilian brains (Vivien-Roels et al, 1979).

In rat, in addition to immunocytochemical and radioimmunoassay data, PVN projections to the amygdala, lateral septum, periaqueductal gray of midbrain, and medulla have been found by electrophysiological methods (Pittman et al, 1981). Neurons sensitive to topical application of neurohypophyseal peptides are present in the hippocampus, the supraoptic nucleus, locus coeruleus, brainstem and spinal cord (Muhlethaler and Dreifuss, 1984; Muhlethaler et al, 1982). These sensitive neurons may be noradrenergic, given that noradrenergic fibers innervate the area of the PVN where vasopressinergic neurons predominate (Kawata and Sano, 1984).

#### ARGININE VASOPRESSIN RECEPTORS IN THE BRAIN

Specific binding sites for tritiated AVP have been identified in the brain of adult male rats, using in vitro autoradiography. Within the hypothalamus, dense AVP binding sites are seen in the SON, PVN, and SCN. High specific binding is also apparent in the median eminence tubero-infundibular region and in the

posterior pituitary. Other sites with specific receptors for AVP include the hippocampus, lateral septum, superficial cortex, cerebellum, nucleus tractus solitarius, anterior pituitary, spinal cord, olfactory nucleus, central and medial amygdala, medial geniculate nucleus, nucleus accumbens, diagonal band of Broca, and nucleus interstitialis striae terminalis (Baskin et al, 1983; Biegon et al, 1984; Brinton et al, 1984; deKloet et al, 1984; Dorsa et al, 1983; Lawrence et al, 1984; Pearlmutter et al, 1983; Van Leeuwen and Wolters, 1983; Yamamura et al, 1983). Autoradiographic localization of binding sites for vasotocin has not been reported for any species.

The brain vasopressin receptors have been recently investigated in membrane preparations from rat hippocampus, amygdala, and dorsal hindbrain (Audigier and Barberis, 1985; Cornett and Dorsa, 1985; Dorsa et al, 1984). Scatchard analysis of AVP binding to receptors in these areas showed a single class of binding sites, dissociation constants of around 1 nM, and maximal binding capacities between 33 and 40 fmol/mg protein.

Response of brain AVP receptors to selective antagonists has shown them to be different from kidney receptors (V2 subtype, see next section). Brain tissue receptors show relatively higher affinities for pressor

(V1 subtype) antagonists compared to antidiuretic analogues (Audigier and Barberis, 1985; Cornett and Dorsa, 1985; Dorsa et al, 1984). Brain receptors, however, may still be shown to differ from V1 receptors as well and represent a third receptor subtype.

Studies with antagonists in hippocampal membranes identify two types of binding sites for AVP (Audigier and Barberis, 1985). The  $K_d$  is the same for both, however one site also binds oxytocin with high affinity and the other site does not. These two sites also show very different affinities for several analogs of AVP and oxytocin. Quantitative analysis of autoradiographs of AVP binding to rat brain suggests other brain areas may have high and low affinity AVP receptors (Brinton et al, 1984). Other groups have also reported Scatchard analyses consistent with this possibility (Pearlmutter et al, 1983; Lawrence et al, 1984).

In brain tissue preparations, adenylate cyclase activity is generally not affected by vasopressin (Audigier and Barberis, 1985; Dorsa et al, 1983). Electrophysiological studies, however, show that the AVP-induced depolarization of supraoptic neurons is directly linked to an adenylate cyclase system (Abe et al, 1983).

Several lines of evidence suggest that AVP binding sites in rat brain represent binding to receptors and not the vasopressin-specific neurophysin. First,

autoradiographic distribution of binding sites in homozygous Brattleboro rats, which are genetically deficient in both AVP and neurophysin, is similar to that seen in normal rats (Brinton et al, 1984). Secondly, the optimal pH for vasopressin binding to its neurophysin is 5.5 (Gainer, 1982) and all studies cited here were carried out at pH 7.4 to 8.

## MECHANISM OF ACTION OF ARGININE VASOPRESSIN IN NON-NEURAL TISSUE

The bulk of AVP produced in the hypothalamus is transported down axons for storage in nerve terminals found in the posterior pituitary (pars nervosa). This AVP is released into the general circulation where it has three main physiological actions: It induces the contraction or relaxation of certain types of vascular smooth muscle, promotes the movement of water across responsive renal epithelial tissues, and stimulates glycogenolysis in the liver. Some hypothalamic axons, in addition, project to the external zone of the median eminence where AVP may enter the portal system and stimulate ACTH secretion from the anterior pituitary (Hadley, 1984).

### General Receptor Characteristics

As with other peptide hormones, AVP interacts with target tissue cellular membrane receptors. The vasopressin receptor appears somewhat analogous to the adrenergic and histamine receptors in having one subclass of receptors which controls the cells by a rise in cytosolic  $Ca^{+2}$  concentration and another population of receptors which controls the cells by adenylate cyclase (Michell et al, 1979).

Vasopressin receptors are best studied in liver and kidney membranes. Despite marked functional

differences between liver and kidney receptors, there are no appreciable differences in the molecular size of the receptors (Guillon et al, 1980). Solubilized receptors from rat liver and kidney membranes both show standard sedimentation coefficients of 3.7 S. Molecular weights of the receptors are 83,000 and 80,000 for liver and kidney, respectively. Dissociation constants for vasopressin binding to kidney receptors and to liver receptors are similar, approximately 4 nM (Butlen et al, 1978; Cantau et al, 1980). Guanyl nucleotides increase the dissociation rate of vasopressin (and all analogs) specifically bound to liver and kidney membranes (Guillon et al, 1980).

Several factors that may control vasopressin receptors have been described for kidney tissue. Adrenal steroids exert a dual action on the vasopressin-sensitive adenylate cyclase of the rat kidney. These actions are (a) modulation of the number of receptor sites (adrenalectomy reduces receptor number and aldosterone or dexamethasone treatment restores receptor number) and (b) control of synthesis of an unknown component which enhances the receptor-adenylate cyclase coupling efficiency (Rajerison et al, 1974). In normal animals the receptor-adenylate cyclase coupling did not appear to be maximally stimulated.

Of unclear physiological significance are two further observations. First, administration of thyroxine increases rat renal medullary vasopressin-sensitive adenylate cyclase activity. Secondly, insulin and serum increase the number of receptors for vasopressin in a cell line derived from pig kidney without changing the apparent affinity of the receptors for vasopressin ( $K_d = 10$  nM) or the coupling between receptors and adenylate cyclase (Roy et al, 1980). However, manipulations of adrenal steroids and thyroid hormones, which reportedly affect renal receptors in intact animals, had no effect on kidney cells in culture.

Several structural features of the AVP molecule that are important for receptor binding and activity have been determined in the course of producing specific analogs. Native mammalian arginine vasopressin has the following structure: Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-(NH<sub>2</sub>)<sub>2</sub>, except for pigs where lysine is substituted for arginine in the 8 position (Acher, 1980). There is a single disulfide bridge between amino acids 1 and 6. The structural requirements for activity can be dissociated quite clearly from the requirements for binding (Roy et al, 1975). Based on analog studies, vasopressin is believed to have a rather featureless hydrophobic side that is responsible for binding to tissue receptors and

a cluster of hydrophilic groups on the other side that are responsible for receptor activation. The hydrophobic side interacts with binding sites that are similar in all types of receptors. The hydrophilic groups may determine tissue specificity (Sawyer et al, 1981; Sawyer and Manning, 1984). The importance of the basic group on the 8-amino acid of vasopressin in determining potency as either a vasopressor or an antidiuretic agent suggests that at least this part of the peptide does contribute to specificity.

#### Evidence for Receptor Subclasses

Despite obvious similarities between liver and kidney vasopressin receptors, marked functional differences between these receptors have been demonstrated. Vasopressin binding to kidney membranes triggers adenylate cyclase activation but no such activation was found after vasopressin binding to liver membranes (Cantau et al, 1980; Rajerison et al, 1974).

The relative potencies of a series of structural vasopressin analogs for binding to liver and kidney receptors are clearly distinguishable, which indicates that hormone binding sites on each type of receptor are different (Butlen et al, 1978; Cantau et al, 1980). At least two types of vasopressin receptor have been well characterized: these have been termed the V1 and V2 receptors. Analogs have been developed which can specifically inhibit the antidiuretic response to

vasopressin (V2 receptors) without any apparent action on pressor receptors (V1) and vice versa (Sawyer and Manning, 1984). There is a striking correlation between the potency of vasopressin analogs in stimulating glycogenolysis in isolated hepatocytes and their vasopressor potency.

Antagonistic analogs that block the vasopressor response also block stimulation of glycogenolysis, and their association constants as estimated from vasopressor assays and from glycogenolysis assays agree closely (Keppens and DeWulf, 1979). Thus, the V2 activation results in stimulation of membrane-bound adenylate cyclase (Handler and Orloff, 1981).

Recently, functionally active V1 receptors have been identified in zona glomerulosa cells from the rat adrenal gland (Balla et al, 1985). Identification of these receptors as the pressor (V1) subtype was based on binding displacement experiments with specific analogs. In these cells, vasopressin stimulated turnover of phosphatidylinositol as it does in the liver, and increased aldosterone production.

Vasopressin receptors have also been observed in the rat testis (Meidan and Hseuh, 1985). These receptors appear to be the V1 subtype and are found in association with Leydig cells. Vasopressin has been shown to have a direct action on testicular steroidogenesis, decreasing androgen production and

increasing pregnenolone and progesterone biosynthesis (Adashi et al, 1984).

There is also limited evidence for other subclasses of vasopressin receptor. Pituitary corticotrope cells respond to AVP and corticotropin-releasing hormone synergistically to cause ACTH secretion. The response of corticotrophes to specific antagonists for V1 and V2 receptors is variable (Antoni et al, 1984). Specific antidiuretic (V2) agonists (and oxytocin) had low affinities for both liver and pituitary binding sites, and a pressor (V1) antagonist was active in liver but not pituitary receptors. This suggests that ligand specificity of rat pituitary AVP receptors is distinct from those of previously characterized V1, V2, and oxytocin binding sites in the rat (Antoni, 1984).

Behavioral responses to AVP analogs also provide insights into brain receptor subtypes. The limited data available on structure-activity relations indicates that these putative central receptors may be neither the V1 nor V2 type (Delanoy et al, 1978; Walter et al, 1978; see previous section also).

#### Mechanism of Vasopressin Action in the Kidney

Primary Action on Renal Epithelia. The permeability effects of AVP are initiated by interaction with specific receptors in the basolateral plasma membrane of responsive epithelial cells (Handler and Orloff, 1981). The interaction results in

stimulation of adenylate cyclase in that plasma membrane and elevation of the concentration of cAMP within the cell (Chase and Aurbach, 1968). Cyclic AMP (or its analogs) as well as phosphodiesterase inhibitors enhance water permeability, thus supporting a role for cAMP as the intracellular messenger of AVP in the kidney. Vasopressin increases the maximal velocity of adenylate cyclase without changing the affinity of the enzyme for its substrate (Jard, 1983; Jard et al, 1975). The high affinity for AVP that is shown by receptors is the result of a low dissociation rate constant of hormone bound to receptor rather than a high association rate constant (Bergman and Hechter, 1978). A considerable fraction of maximal adenylate cyclase activation occurs when a relatively small fraction of binding sites are occupied by vasopressin. The only mechanism by which cAMP has been shown to mediate the effect of hormones involves protein phosphorylation. Hander and Orloff (1981) note that the idea that cAMP elicits changes in membrane permeability by activation of phosphorylation of membrane proteins (or other proteins) is an attractive hypothesis but one with little direct support. The exact site of action of permeability effects is the membrane of the mucosal (luminal) side of renal epithelial cells with AVP receptors being located only on the serosal side of the tubules (Hadley, 1984).

At this time, most evidence favors the concept that increased water permeability, in response to AVP, results from opening small aqueous channels in the apical membrane (Gluck and Al-Awqati, 1980). Considerable correlative evidence relates the appearance of intramembranous particle aggregates to the vasopressin-elicited water-permeability change. These aggregates have been seen in mammalian cortical collecting ducts after AVP treatment as well as in toad skin and frog urinary bladder (Chevalier et, 1974). The particles are arranged in rows with a corresponding series of parallel groves in the face of the membrane. The appearance of aggregates in the apical membrane is independent of water flow per se; they appear in AVP-treated toad urinary bladders with or without an osmotic gradient. AVP increases the number of aggregates and their total area without altering their size distribution. There is a high correlation between the magnitude of the AVP-elicited water permeability and the number and area of aggregates. The aggregates are clearly unrelated to the sodium-transport or urea-permeability response (Handler and Orloff, 1981). Particle aggregates similar to those in the apical membrane after AVP have been found in the membrane of intracellular vacuoles of unstimulated cells, leading to the suggestion that the particle aggregates exist in a preformed state and are inserted into the apical

membrane in response to AVP (Humbert et al, 1977; Wade, 1978). Studies using cytochalasin-B and colchicine experiments suggest that microtubules are involved in the AVP-elicited fusion of particle-aggregate-containing cytoplasmic membranes into the plasma membrane. Microfilaments are not involved in the process of membrane fusion but in the movement of particle aggregates from the intracellular membranes to the apical plasma membrane after fusion (Muller et al, 1980).

Vasopressin stimulates sodium transport across renal membranes and increases permeability to urea and related small amides (Andreoli and Schafer, 1976). The independence of the water-permeability, sodium-transport, and urea-permeability responses has been well demonstrated (Handler and Orloff, 1981). It has been suggested that there are three AVP-sensitive adenylate cyclase systems and three pools of cAMP in epithelial cells. Exogenous PGE<sub>1</sub> and PGE<sub>2</sub> cause a marked suppression of vasopressin-stimulated water flow, while treatment with cyclooxygenase inhibitors augment the action of vasopressin on water transport (Currie and Needleman, 1984). It is generally accepted that PGE inhibits the stimulation of adenylate cyclase activity by AVP. This interpretation is based on the observation that PGE does not inhibit the water-permeability response to cAMP and that

vasopressin-elicited accumulation of cAMP in epithelial cells is reduced in the presence of low concentrations of PGE (Handler and Orloff, 1981). A nonpressor antidiuretic analog of vasopressin fails to acutely stimulate PGE<sub>2</sub> synthesis in kidney, and the stimulation of PGE<sub>2</sub> synthesis by vasopressin is inhibited by a specific pressor antagonist (Beck et al, 1980; Campbell et al, 1982; Currie and Needleman, 1984). These findings suggest that vasopressin stimulation of renal PGs is related primarily to its pressor and not its antidiuretic activity.

Vasopressin and Adrenal Steroid Interactions in the Kidney. Adrenal steroid hormones enhance the water-permeability response to vasopressin by three mechanisms. One is inhibition of cyclic nucleotide phosphodiesterase activity (Schwartz and Kokko, 1980; Stoff et al, 1973). The second effect is inhibition of PGE biosynthesis from endogenous stores of arachidonic acid (Zusman et al, 1978). The third mechanism is the effect on receptors mentioned previously.

#### Mechanism of Vasopressin Action in the Liver

At present, vasopressin is the most potent known hormonal activator of glycogen phosphorylase (Kirk et al, 1981). This glycogenolytic effect does not involve cAMP-initiated series of enzymatic activations. There is no rise in cAMP content of vasopressin-perfused liver; neither phosphorylase kinase nor protein kinase

are activated. The mode of action of vasopressin in liver cells most probably involves an increase in cytosolic  $Ca^{+2}$  (Keppens and deWulf, 1979). The pressor effects of vasopressin also depend on calcium (Altura and Altura, 1977).

Vasopressin also evokes a large increase in the incorporation of labeled phosphate into phosphatidylinositol in rat hepatocytes and promotes phosphatidylinositol breakdown (Kirk et al, 1981). This stimulation of phosphatidylinositol metabolism is rapid (within 1-2 minutes). Half-maximal effects on phosphatidylinositol occur at a concentration of vasopressin at which approximately half of the hepatic receptors are occupied but which is much greater than is needed to produce half-maximal activation of glycogen phosphorylase. Although the incorporation of labeled phosphate into lipids decreases when cells are incubated in a calcium free medium, vasopressin still provokes substantial stimulation of phosphatidylinositol labeling under these conditions. In addition, influx of  $Ca^{+2}$  into hepatocytes with the ionophore A23187 does not affect phosphatidylinositol metabolism (Michell et al, 1979). This action, then, does not appear to be a consequence of calcium mobilization in the cytosol. At present, the most widely considered possible function for vasopressin-stimulated phosphatidylinositol turnover is

as a coupling reaction in the mobilization of  $Ca^{+2}$  that is brought about by stimulation of many membrane receptors (Kirk et al, 1981; Michell et al, 1979).

#### SUMMARY

Arginine vasopressin and AVT are known to influence the behavior of vertebrates. In the case of reproductive behavior, there is evidence that AVT acts on neurons in the brain and that steroid hormones influence this action of AVT. The site of action of AVT, as well as the nature of the interactions of AVT and gonadal steroids, is unknown. A thorough understanding of the mechanism of AVT action on the central nervous system and behavior therefore requires answers to questions about AVT receptors. In particular, does the brain contain specific receptors for AVT? What is the neuroanatomical distribution of these receptors? What are the binding characteristics of these receptors? Are AVT receptors influenced by circulating gonadal steroid hormones? How do central nervous system receptors compare with receptors in the kidney? The purpose of this thesis is to examine these issues in detail for the rough-skinned newt, Taricha granulosa.

Characterization of brain neuropeptide receptors has been hampered by small amounts of tissue and low

receptor number. The use of in vitro quantitative autoradiography here allows for more sensitive measurement of binding than previously possible, using techniques of radioligand binding to membrane fractions.

The first manuscript (Chapter II) characterizes AVP binding sites in a single region of the newt brain -- the medial pallium. These studies both validate the procedures and present evidence that the brain of newts contains authentic receptors for AVT. The second manuscript (Chapter III) is primarily descriptive: brain receptors are localized in specific neuroanatomical regions, receptor concentrations are quantified, and the distribution and density of receptors in male newts is compared with that in female newts. The third manuscript (Chapter IV) describes the effects of gonad removal on AVT receptors in the brain of males and females. Evidence presented in Chapter IV supports the hypothesis that gonadal steroid hormones maintain receptors for AVT in certain brain regions. And, finally, the fourth manuscript (Chapter V) examines AVT receptor characteristics in a non-neural tissue of the newt, the kidney, as an independent validation of the in vitro autoradiography. These studies of kidney receptors demonstrate important differences between AVT receptors in brain and kidney in this species, and also yield new information about

the mechanism of AVT action in causing antidiuresis in urodele amphibians.

AUTORADIOGRAPHIC CHARACTERIZATION OF VASOPRESSIN  
BINDING SITES IN THE BRAIN OF A URODELE AMPHIBIAN

Chapter II

INTRODUCTION

There is considerable evidence that arginine vasotocin (AVT) is involved in regulating reproductive behaviors of male rough-skinned newts, Taricha granulosa (Moore and Miller, 1983; Moore and Zoeller, 1979; Zoeller and Moore, 1982) and female leopard frogs, Rana pipiens (Diakow, 1978). In other vertebrates, AVT and/or arginine vasopressin (AVP) have been shown to influence reproductive behaviors in two species of mammals (Bohus, 1977; Kihlstrom and Agmo, 1974; Sodersten et al., 1983) and birds (Kihlstrom and Danninge, 1972), and in one fish species (Pickford and Strecker, 1977; Wilhelmi et al., 1955).

The sites of action for these behavioral effects of AVT and AVP are unknown, although specific binding sites for tritiated AVP have been identified in the brain of adult male rats using autoradiography and membrane preparations (Audigier and Barberis, 1985; Baskin et al., 1983; Biegon et al., 1984; Brinton et al., 1984; Cornett and Dorsa, 1985; DeKloet et al., 1984; Dorsa et al., 1983; Dorsa et al., 1984;

Pearlmutter et al., 1983; Van Leeuwan and Wolters, 1983). These studies have shown that AVP receptors are localized in many hypothalamic and extra-hypothalamic sites.

Despite the many reports of behavioral actions of AVT, the distribution and characteristics of AVT receptors in the brains of non-mammalian vertebrates have not been reported. The present study used quantitative autoradiography to describe the binding of radiolabelled vasopressin to putative AVT receptors in the forebrain of male rough-skinned newts.

## METHODS AND MATERIALS

Tissue Preparation. Forty adult male newts (T. granulosa) were collected locally in October and maintained overnight in a large laboratory tank (4.85 x 1.0 x 0.36 m) of dechlorinated water at 20°C. The next day they were decapitated; brains were removed, frozen on dry ice, and embedded in Tissue Tek O.C.T. compound. Brains were stored at -80°C.

Tissues were sectioned (16  $\mu$ m; coronal orientation) using a microtome (Harris) at -20°C. Two sections through the telencephalon were placed on a slide to permit "duplicate" determinations on each animal (neuroanatomical terminology see Herrick, 1948; see Fig. 1). Alternate sections were placed on another slide so that total and nonspecific binding could be determined on comparable sections. Tissue sections were thaw-mounted, and slides were dried for 2 hr in a vacuum desiccator at 0 to -5°C. Slides were stored up to 4 days at -20°C. Pairs of slides (total and nonspecific binding slides) were assigned randomly to treatment groups to control for differences among animals. A pilot study, using serial sections of the telencephalon from four newts, showed that dense binding occurs in the medial pallium and that binding levels within the medial pallium do not change significantly from the rostral to caudal regions

(one-way analysis of variance on data from 10 different neuroanatomical levels;  $F=1.92$ ;  $p=0.091$ ). Since there is little myelination of fibers in the newt forebrain (Herrick, 1948) and no major fiber tracts through the medial pallium, quenching by white matter probably is not a significant factor in these studies.

Binding Procedures. The incubation procedures used were similar to those of Brinton et al. (1984) and Dorsa et al. (1983). Briefly, tissue sections were incubated with  $10 \times 10^{-9}$  M  $^3\text{H-AVP}$  (New England Nuclear, specific activity 40 Ci/mmol) in 200  $\mu\text{l}$  of 50 mM Tris-HCl (pH 7.4) containing 5 mM  $\text{MgCl}_2$ , 2 mg/ml bovine serum albumin, 0.5 mg/ml bacitracin, and 10  $\mu\text{g/ml}$  aprotinin (Sigma Chemical Co.). Blanks (nonspecific binding sections) were incubated in the same medium with the addition of 1  $\mu\text{M}$  unlabelled AVT (Sigma). Tissue preincubation did not significantly enhance binding in preliminary experiments and therefore was not used in studies reported here. Following 30 min of incubation at  $23^\circ\text{C}$ , slides were washed in two 30-sec rinses of ice cold buffer (10 mM Tris-HCl, 1 mM  $\text{MgCl}_2$ , and 1 mg/ml bovine serum albumin) followed by brief dipping in double distilled water and drying on a slide warmer at  $40^\circ\text{C}$ . Slides were stored desiccated overnight then apposed to tritium-sensitive Ultrofilm (LKB Instruments) in X-ray cassettes and exposed for 2 months at room

temperature. Films were developed in Kodak D-19 at 21°C for 4 min. Tissue sections were lightly stained with cresyl violet.

Displacement studies used AVP, AVT, oxytocin (OXY), AVP fragment 4-9 (AVP4-9), desGly(NH<sub>2</sub>)AVP (all from Sigma), mesotocin (MT), [1-deaminopenicillamine, 2-O-methyltyrosine]AVP (or [dPen<sup>1</sup>Tyr(Me)<sup>2</sup>]AVP), d(CH<sub>2</sub>)<sub>5</sub>[Tyr(Me)<sup>2</sup>]AVP, and pressinoic acid (PA) (all from Bachem Chemical Co.).

Autoradiogram Analysis. Differences in optical densities generated by binding of tritiated AVP to the medial pallium of newt brains were determined using the IBM PC/AT-based DUMAS system (Circon microvideo camera) and BRAIN software developed at the Drexel Image Processing Center (Gallistel and Tretiak, 1985). This system quantified average optical density for each tissue section after focusing on 0.1 mm<sup>2</sup> in the central one-third, approximately, of the medial pallia on each side of the brain. These values were converted to fmol <sup>3</sup>H-AVP/mg protein using brain-mash standards prepared from newts using the procedure of Rainbow et al. (1984). Sections through five different standards were made at 16 μm and mounted on slides. One set of standards was included in each cassette. Standards had optical densities that ranged from 0.073 to 0.865, values which are within detectable limits of the film and camera. The coefficient of variation for the

standards on different films was less than 2.5%. Other sections of brain-mash standards were homogenized in double distilled water and used for liquid scintillation counting or protein determinations (Bradford, 1976).

Data from kinetic and competition experiments were analyzed using LIGAND (Munson and Rodbard, 1980).

## RESULTS

Localization of Binding Sites. Measurements of optical density revealed discrete areas in the forebrain of T. granulosa with high concentrations of  $^3\text{H}$ -AVP binding sites (Fig. 2). The neuroanatomical location of these sites within the medial (hippocampal) pallium is primarily over the neuropil. This binding was specific; addition of unlabelled AVT to the incubation medium abolished binding of  $^3\text{H}$ -AVP in these areas and resulted in homogeneous background binding across the sections.

Binding Characteristics. Binding of  $^3\text{H}$ -AVP in the medial pallium reached equilibrium within 30 min at 23°C (Fig. 3). This binding was temperature dependent, reaching equilibrium more slowly at 5°C than 23°C (Fig. 3). The observed rate constant at 23°C ( $K_{\text{obs}}$ ) was  $0.07 \pm 0.01 \text{ min}^{-1}$ . Specifically bound  $^3\text{H}$ -AVP was released in a time-dependent manner following a 1000-fold dilution of incubation buffer (Fig. 4). After 60 minutes, less than 12% of the radioactivity bound at zero-time remained attached to slices. The calculated dissociation rate constant ( $K_{\text{off}}$ ) at 23°C was  $0.03 \pm 0.006 \text{ min}^{-1}$ . Both the association and dissociation time-courses were mono-exponential processes (see insets figures 3, 4).

The kinetically derived dissociation constant ( $K_D$ ) was 5 nM which is similar to the  $K_D$  determined from saturation binding isotherms (see below).

Binding of  $^3\text{H}$ -AVP in the newt medial pallium also was saturable and of high affinity. A direct plot of binding isotherm data shows saturation of specific binding at a concentration of approximately 10 nM (Fig. 5). Signal-to-noise ratio was maximal at a concentration of 10 nM radiolabeled AVP (67% specific binding) so this concentration was used in further experiments. Nonspecific binding was linear. A Scatchard replot of specific binding data best fit a single site model and yielded a  $K_D$  of  $1.0 \pm 0.1$  nM and maximal binding capacity ( $B_{\text{max}}$ ) of  $57 \pm 7.4$  fmol/mg protein (Fig. 5). The Hill coefficient was 0.99.

Inhibition of Binding. The rank order of potency of peptides competing for  $^3\text{H}$ -AVP binding sites in the newt medial pallium was AVT >  $\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$  > AVP = oxytocin =  $[\text{dPen}^1\text{Tyr}(\text{Me})^2]\text{AVP}$  > mesotocin >> desGly( $\text{NH}_2$ )AVP, AVP fragment 4-9, and pressinoic acid (Fig. 6a,b). Calculated inhibition constants ( $K_I$ ) for these peptides are in Table 1.

## DISCUSSION

The central vasotocinergic system of amphibians has been implicated in the control of diverse functions such as sexual behavior (Diakow, 1978; Moore and Miller, 1983) and hydromineral balance (Nouwen and Kuhn, 1981; 1983; Rosenbloom and Fisher, 1974). Specific and discretely-distributed  $^3\text{H}$ -AVP binding sites identified here within the brain of a urodele amphibian may represent the precise sites of action of AVT in controlling these events. These sites have several defining characteristics of authentic receptors: saturability, reversibility, and time- and temperature-dependence of binding. They have a high affinity and low capacity for  $^3\text{H}$ -AVP. These putative receptors are localized to several neuroanatomical areas within the forebrain of male rough-skinned newts -- specifically, the medial (hippocampal) pallium, dorsal pallium, and amygdala pars lateralis. These same areas contain detectable concentrations of immunoreactive AVT as measured by radioimmunoassay in male newts (Zoeller and Moore, 1986).

The ability of AVT to compete for  $^3\text{H}$ -AVP binding sites in newts is greater than that of AVP. As well, the  $K_I$  for AVT in newt brains is an order of magnitude less than the  $K_D$  for AVP in the same tissue indicating that the binding sites observed here

represent authentic, specific AVT receptors.

The  $^3\text{H}$ -AVP binding sites in newt brain tissue differ in several important respects from binding sites previously reported in rat brain tissue. Vasopressin and vasotocin have very similar inhibition constants in rat hippocampal preparations (Audigier and Barberis, 1985). In newt brain tissue, on the other hand, the displacement curve for AVP is shallow, suggesting that AVP binds to more than one site in the amphibian. These two sites may represent specific receptors for AVT and mesotocin. These two neurohypophyseal peptides, which differ by only one amino acid, are present in amphibians (Acher, 1983) and, as our results show, are clearly different in their ability to displace  $^3\text{H}$ -AVP from binding sites in newts. In contrast, the inhibition constants for AVP and oxytocin in newt brain tissue are very similar. These peptides are the mammalian homologues of AVT and mesotocin (Acher, 1983). In mammals, AVP and oxytocin have different affinities for  $^3\text{H}$ -AVP binding sites in the dorsal hindbrain and amygdala (Cornett and Dorsa, 1985; Dorsa et al., 1984). However, there are apparently two types of AVP receptors in rat hippocampus: one receptor type has a high affinity for vasopressin and low affinity for oxytocin; the other type has the same affinity for both AVP and oxytocin (Audigier and Barberis, 1985). This latter type is perhaps the

ancestral receptor type, considering that in this amphibian AVP and oxytocin competed equally for the  $^3\text{H}$ -AVP binding sites in the medial pallium.

Dissociation constants for  $^3\text{H}$ -AVP determined from saturation isotherms and kinetic experiments were found to be significantly different from the  $K_I$  for AVP calculated from displacement studies. This finding may be due to the difference in the competing ligands in the two situations. As mentioned above, the shape of the displacement curve for AVP suggests that  $^3\text{H}$ -AVP binds to more than one site in newt brain slices. One possible explanation for the difference between  $K_D$  and  $K_I$  could be that AVT only recognized one of these sites and when AVT was used to define nonspecific binding (as in kinetic and saturation experiments), only a single class of high affinity ( $K_D=1-5$  nM) sites was revealed. This is supported by mono-exponential kinetics (Fig. 3, 4) and linearity of Scatchard plot (Fig. 5). In displacement experiments, on the other hand, unlabelled AVP was used to displace  $^3\text{H}$ -AVP. In this case, the radiolabelled AVP was displaced from more than one site. Therefore, the  $K_I$  reported is an "average" value and likely includes inhibition constants for several sites with a range of affinities for AVP.

The  $K_D$  and  $B_{\text{max}}$  observed in newt pallial tissues are similar to those previously reported for

rat brain tissue (Audigier and Barberis, 1985; Cornett and Dorsa, 1985; Dorsa et al., 1984). In addition, the synthetic analogs  $d(CH_2)_5[Tyr(Me)_2]AVP$  and  $[dPen^1Tyr(Me)_2]AVP$  have similar affinities for  $^3H$ -AVP binding sites in newt brain tissue and rat brain tissue (our data compared to Cornett and Dorsa, 1985 and Dorsa et al., 1984). These peptides are both specific antagonists for the pressor subtype ( $V_1$ ) of AVP receptor found in mammalian hepatocytes and vascular smooth muscle (Sawyer and Manning, 1984; Sawyer et al., 1981). Their ability to compete for  $^3H$ -AVP binding sites in newt pallium suggests that these sites more closely resemble the  $V_1$  receptor subtype of mammals than the antidiuretic subtype ( $V_2$ ). Rank order potency of these two analogs also differs from that observed in mammalian pituitary gland (Antoni, 1984; Antoni et al., 1984). The  $^3H$ -AVP binding sites in newt and rat brain, therefore, appear to differ from mammalian antidiuretic and pituitary binding sites in ligand specificity.

The ability of several peptides to displace  $^3H$ -AVP from binding sites in the medial pallium corresponds to their ability to alter sexual behavior in male newts. Arginine vasotocin is a potent stimulator of newt amplexic clasping and  $d(CH_2)_5[Tyr(Me)_2]AVP$  inhibits clasping in low doses (Moore and Miller, 1983; Moore and Zoeller, 1979;

Zoeller and Moore, 1982). Both of these compounds compete effectively for  $^3\text{H}$ -AVP binding sites in male newts. AVP competes less effectively than AVT for  $^3\text{H}$ -AVP binding in newts and, likewise, high doses of AVP are required for a behavioral response to be observed (Moore and Zoeller, 1979). Mesotocin is a poor displacer at these sites and, when injected systemically, has failed to induce sexual behaviors in male newts (Moore, unpublished results).

The  $^3\text{H}$ -AVP binding in newt medial pallium is not displaced by two vasopressin analogs that have potent effects on learning and memory in rats. Vasopressin fragment AVP 4-9 is highly selective for alterations of memory processes without having significant antidiuretic, pressor or uterus stimulating (oxytocin-like) activity (Burbach et al., 1983; DeWied et al., 1984). The desglycinamide derivative of AVP [desGly(NH<sub>2</sub>)AVP] also affects memory without pressor effects (DeWied et al., 1984). Possible effects of these peptides on behavior in amphibians has not been reported.

Characteristics of binding sites in the newt medial pallium may not be the same as sites in other parts of the amphibian brain. This is the case in rat brains where populations of AVP receptors in dorsal hindbrain, amygdala, and hippocampus differ slightly from one another (Audigier and Barberis, 1985; Cornett

and Dorsa, 1985; Dorsa et al., 1984). Of particular importance is the observation that specific binding sites for labelled AVP 4-9, distinct from AVP and oxytocin sites, have been identified in the rat (Brinton et al., 1986; DeKloet et al., 1985). Whether amphibians possess such sites as well is unknown.

The binding sites for  $^3\text{H}$ -AVP in the newt medial pallium may represent the neural substrate for the regulation of newt sexual behavior by AVT. The medial pallium of amphibians is probably homologous to the mammalian hippocampus (Kicliter and Ebbesson, 1976). In rats, the hippocampus and related limbic structures are likely sites for some of the behavioral actions of AVP-like peptides (van Wimersma Greidanus et al., 1983). In amphibians, the medial pallium receives input from both visual and olfactory systems (Kicliter and Ebbesson, 1976; Wicht and Himstedt, 1986) and may function to integrate this information for the effective control of reproductive processes.

## FIGURE 1

Schematic diagram of the newt brain. Sections were taken between levels 1 and 3. Diagram 1a is an exterior side view. Diagrams 1b, c, and d represent typical frontal sections at the levels indicated on 1a. Abbreviations: amy, amygdala pars lateralis; b.ol., olfactory bulb; cb, cerebellum; dp, dorsal pallium; hyth, hypothalamus; lp, lateral pallium; mp, medial pallium; na, nucleus accumbens; pit, anterior pituitary; poa, preoptic area; sep, septal nucleus; II-X, cranial nerves.

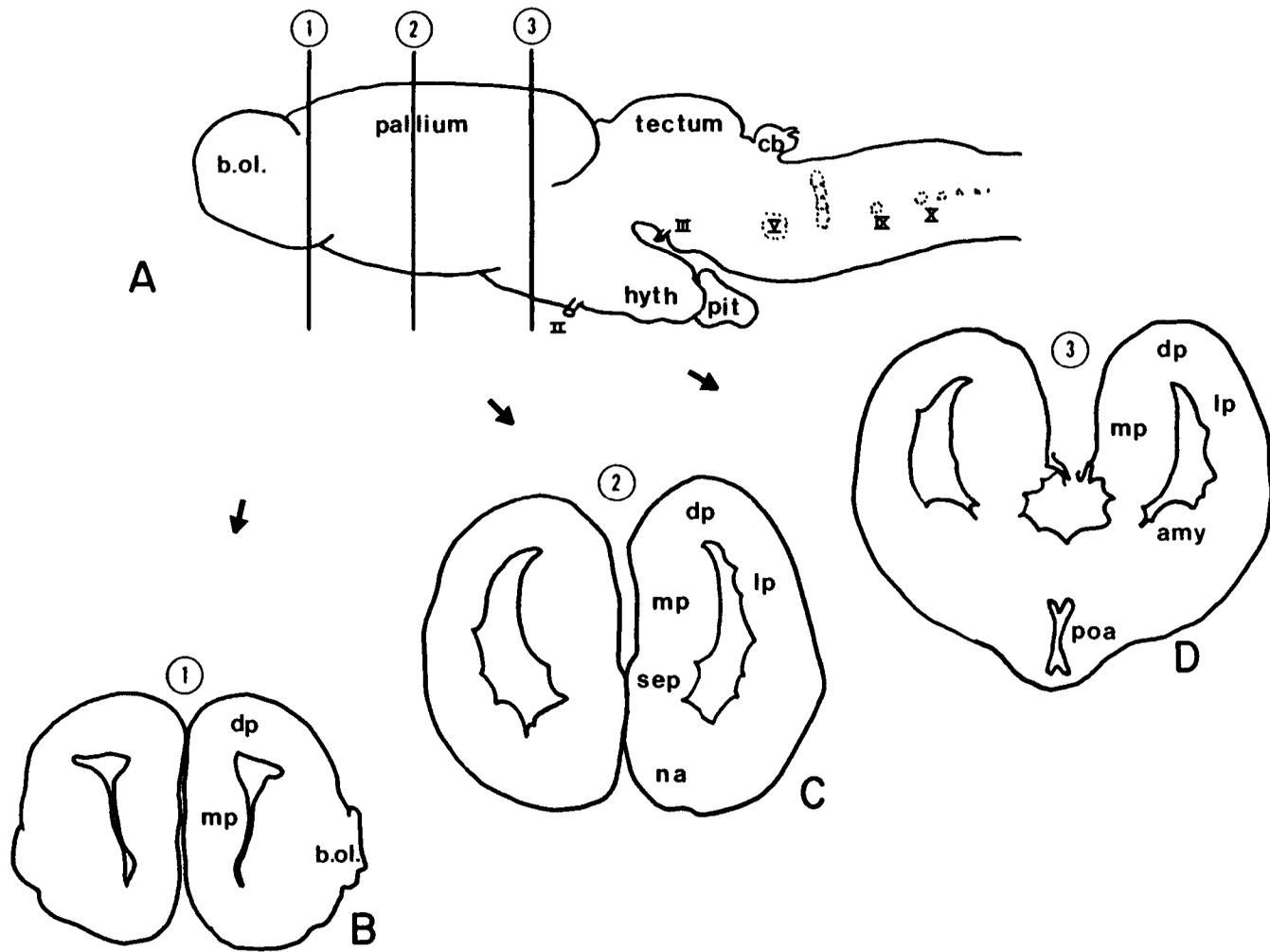
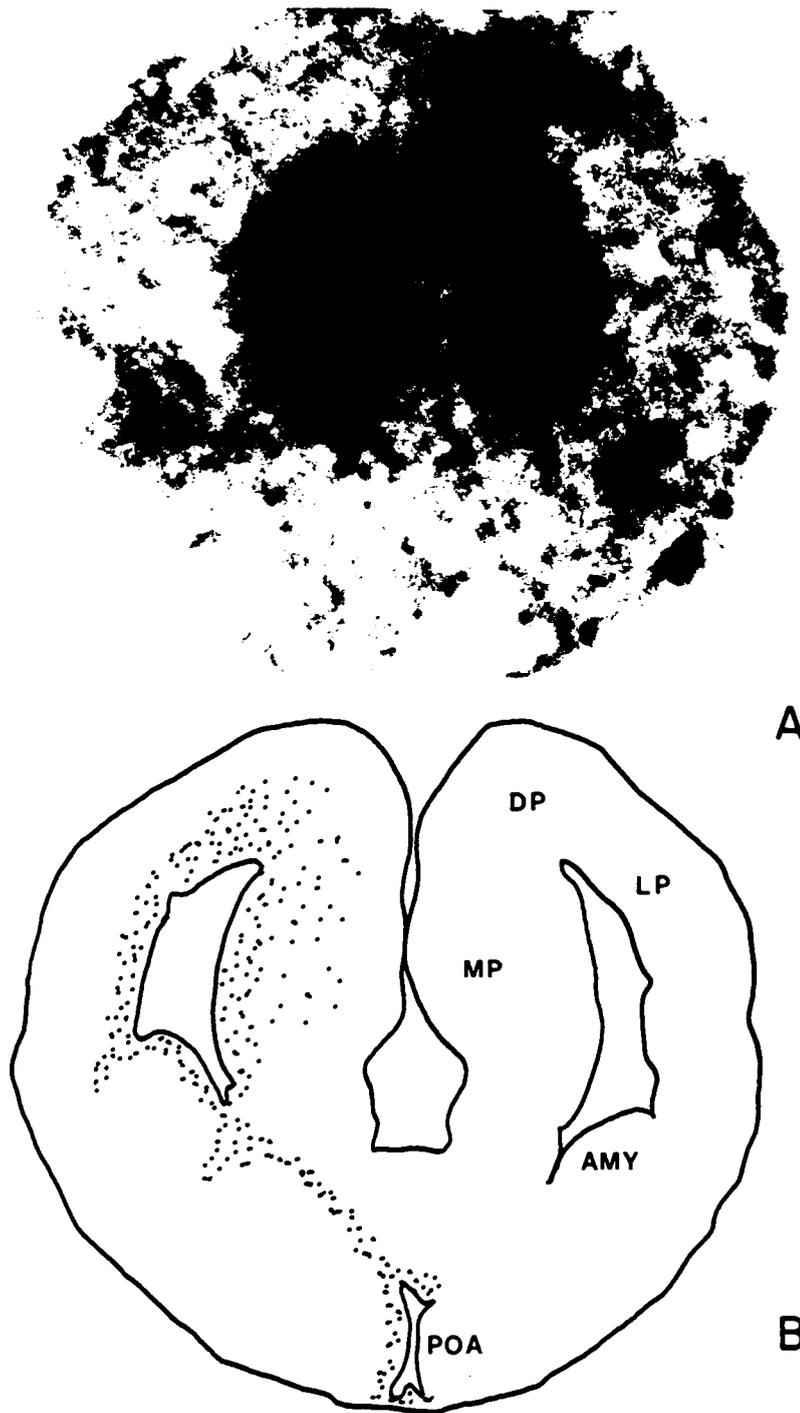


FIGURE 1

## FIGURE 2

Autoradiogram (a) and schematic diagram of neuroanatomical structures (b) from a section at approximately level 3 in figure 1. Autoradiogram depicts  $^3\text{H}$ -AVP binding in the medial pallium (mp), dorsal pallium (dp), and amygdala pars lateralis (amy). The right side of this section is slightly more rostral than the left. Dots on the left side of 2b represent the locations of all types of cell bodies as observed in a cresyl violet stained section. See figure 1 legend for explanation of other abbreviations.

FIGURE 2



## FIGURE 3

Time course of  $^3\text{H}$ -AVP binding to the medial pallium of male newts. Brain slices were incubated with 5 nM  $^3\text{H}$ -AVP at either 5°C or 23°C for the indicated times (other procedural details in Methods section). Each point represents the mean of determinations from six different brains (SEM was less than 10% of the mean). Curve fitting analysis indicated that the relationship between time and specific binding (nCi/mg tissue) at 5°C could best be expressed as a linear function ( $r=0.96$ ; slope=0.04; y-intercept=0.03). This relationship at 23°C best fit a log function ( $y=a + b\text{LN}x$ ;  $r=0.94$ ; slope=0.8; y-intercept=-1).

Inset: Pseudo-first-order association rate plot at 23°C. The observed rate constant ( $K_{\text{obs}}$ ) was determined from the slope of this line, which was linear ( $r=0.98$ ; slope=0.07; y-intercept=-0.4).

Abbreviations: Bt, specifically bound  $^3\text{H}$ -AVP at indicated time; Beq, specifically bound  $^3\text{H}$ -AVP at equilibrium (mean of values at 45, 60, and 90 min).

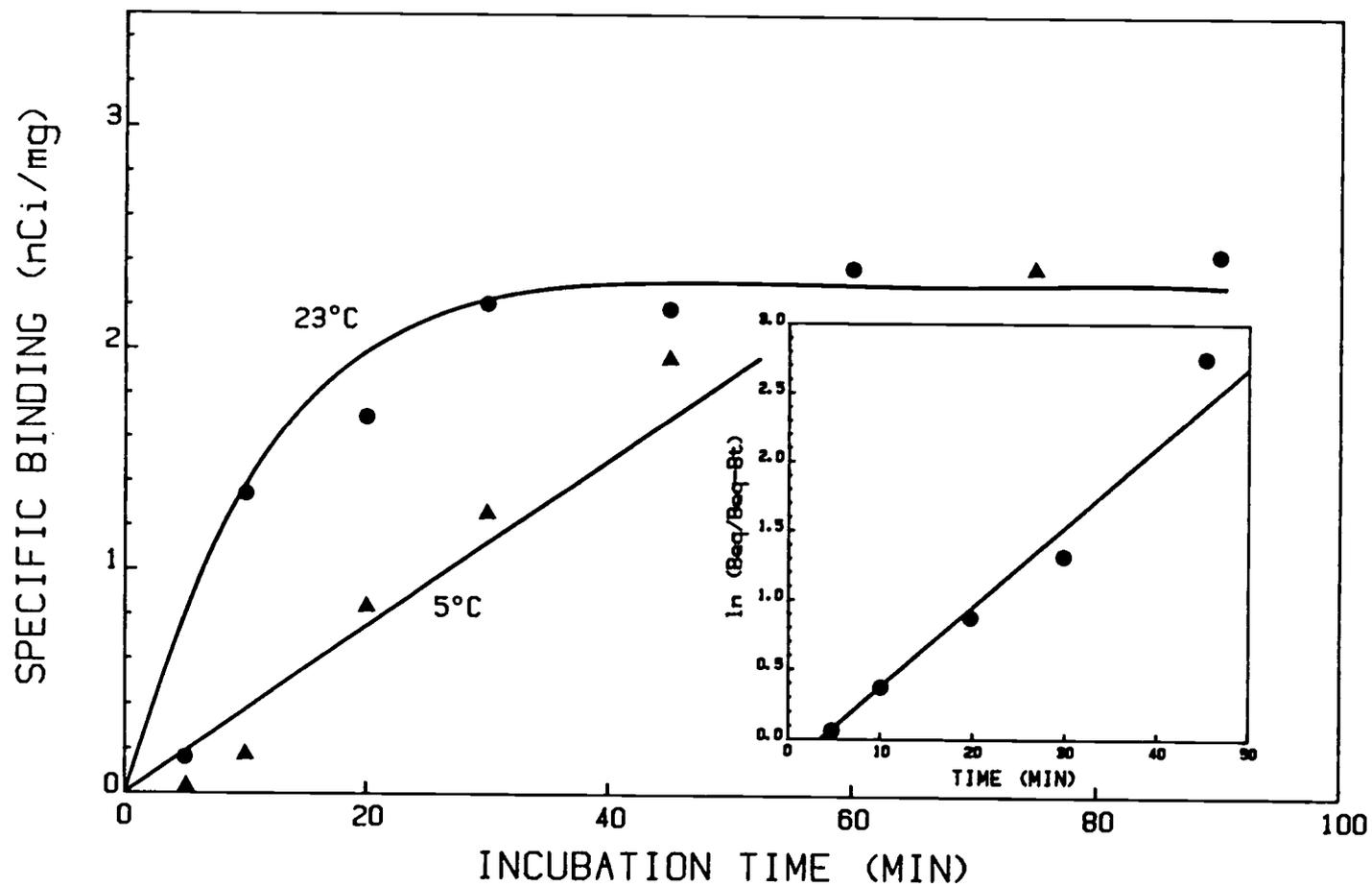


FIGURE 3

## FIGURE 4

Reversibility of  $^3\text{H}$ -AVP binding to newt medial pallium. Brain slices were first incubated with 5 nM  $^3\text{H}$ -AVP for 30 min at  $23^\circ\text{C}$  followed by immersion in buffer alone to dilute the effective concentration of labeled peptide. Slices were removed from this wash after varying times and specific binding determined as described in Methods. Each point represents the mean of determinations from six different brains (SEM was less than 10% of the mean). Curve fitting analysis indicated that the relationship between time and specific binding could best be expressed as a log function ( $r=0.99$ ; slope=-0.8; y-intercept=3.6).

Inset: First-order dissociation rate plot. The rate constant for dissociation ( $K_{\text{off}}$ ) was determined by linear regression ( $r=0.95$ ; slope=-0.03; y-intercept=1.1).

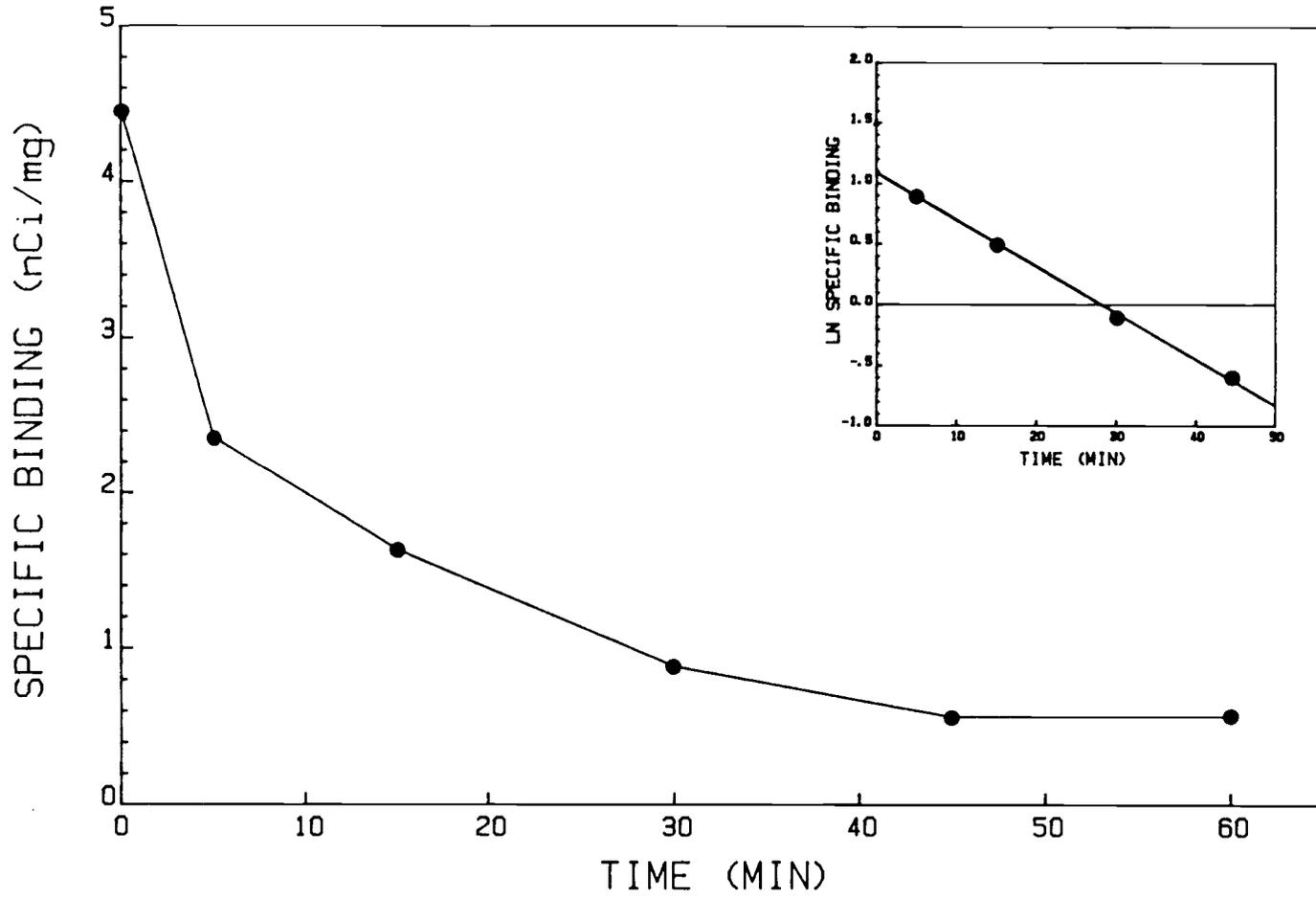


FIGURE 4

## FIGURE 5

Direct plot of results from saturation binding experiment. Each point represents the mean of determinations from six different brains (SEM was less than 10% of the mean). Specific binding (SB) could best be expressed as a log function ( $r=0.94$ ). Nonspecific binding (NSB) was linear ( $r=.99$ ).

Inset: Scatchard replot of these data which was linear ( $r=0.99$ ). Equation for the line yielded a  $K_D$  of  $1.0 \pm 0.1$  nM and a  $B_{max}$  of  $57 \pm 7.4$  fmol/mg protein.

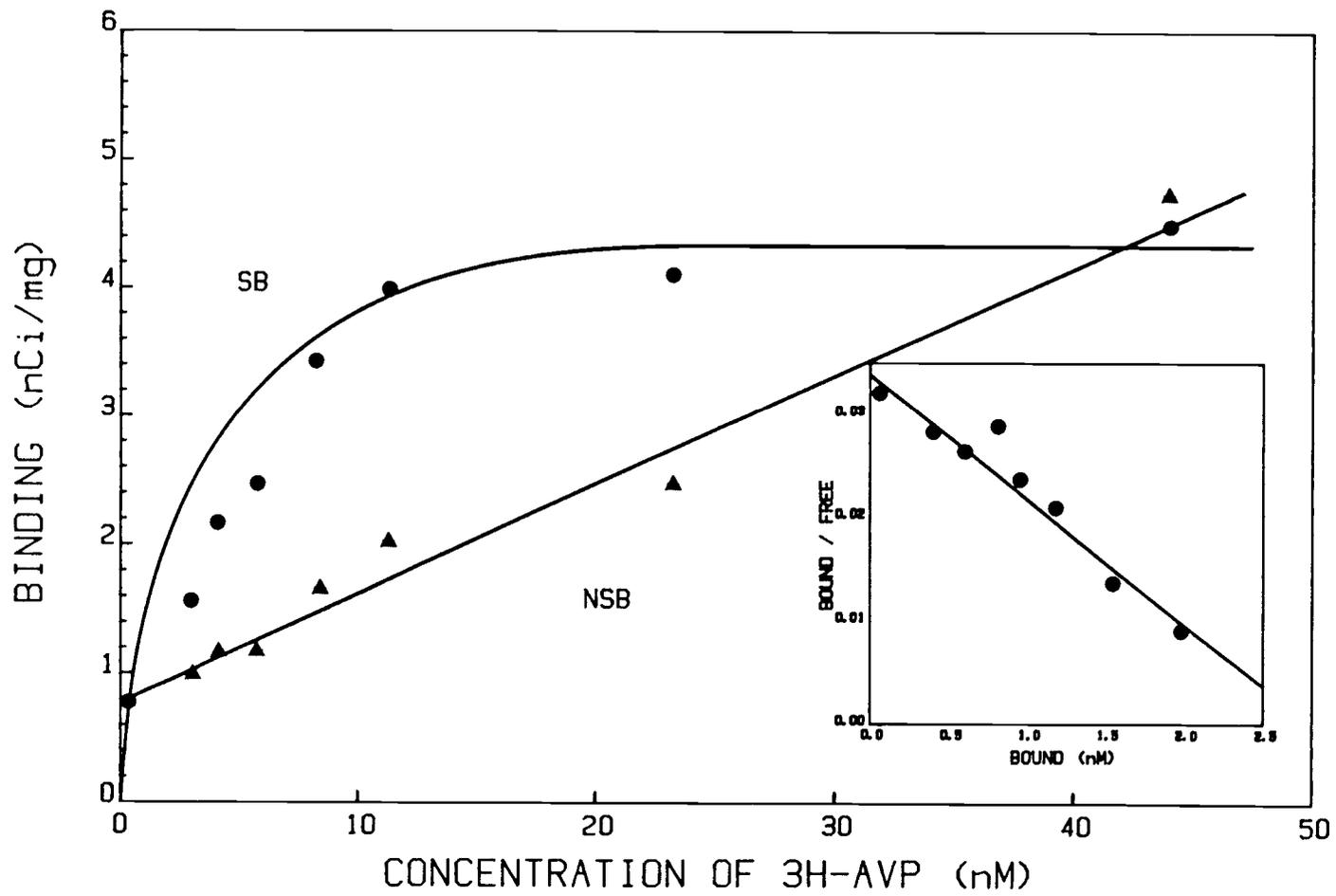


FIGURE 5

## FIGURE 6

Dose-dependent inhibition of  $^3\text{H}$ -AVP binding to newt medial pallium by unlabelled peptides. Brain slices were incubated in the presence of 10 nM  $^3\text{H}$ -AVP and increasing amounts of unlabelled peptides. Values measured in the presence of unlabelled peptides were expressed as a percentage of binding in the absence of competitor (0% was defined in the presence of 1  $\mu\text{M}$  AVT).

Figure 6a: Inhibition by AVT and other naturally occurring related peptides (OXY=oxytocin, MT=mesotocin).

Figure 6b: Inhibition by synthetic forms of AVT and related peptides (PA=pressinoic acid, dG-AVP=desGly(NH<sub>2</sub>)AVP, dPen-AVP=[dPen<sup>1</sup>Tyr(Me)<sup>2</sup>]AVP, MC=d(CH<sub>2</sub>)<sub>5</sub>[Tyr(Me)<sup>2</sup>]AVP).

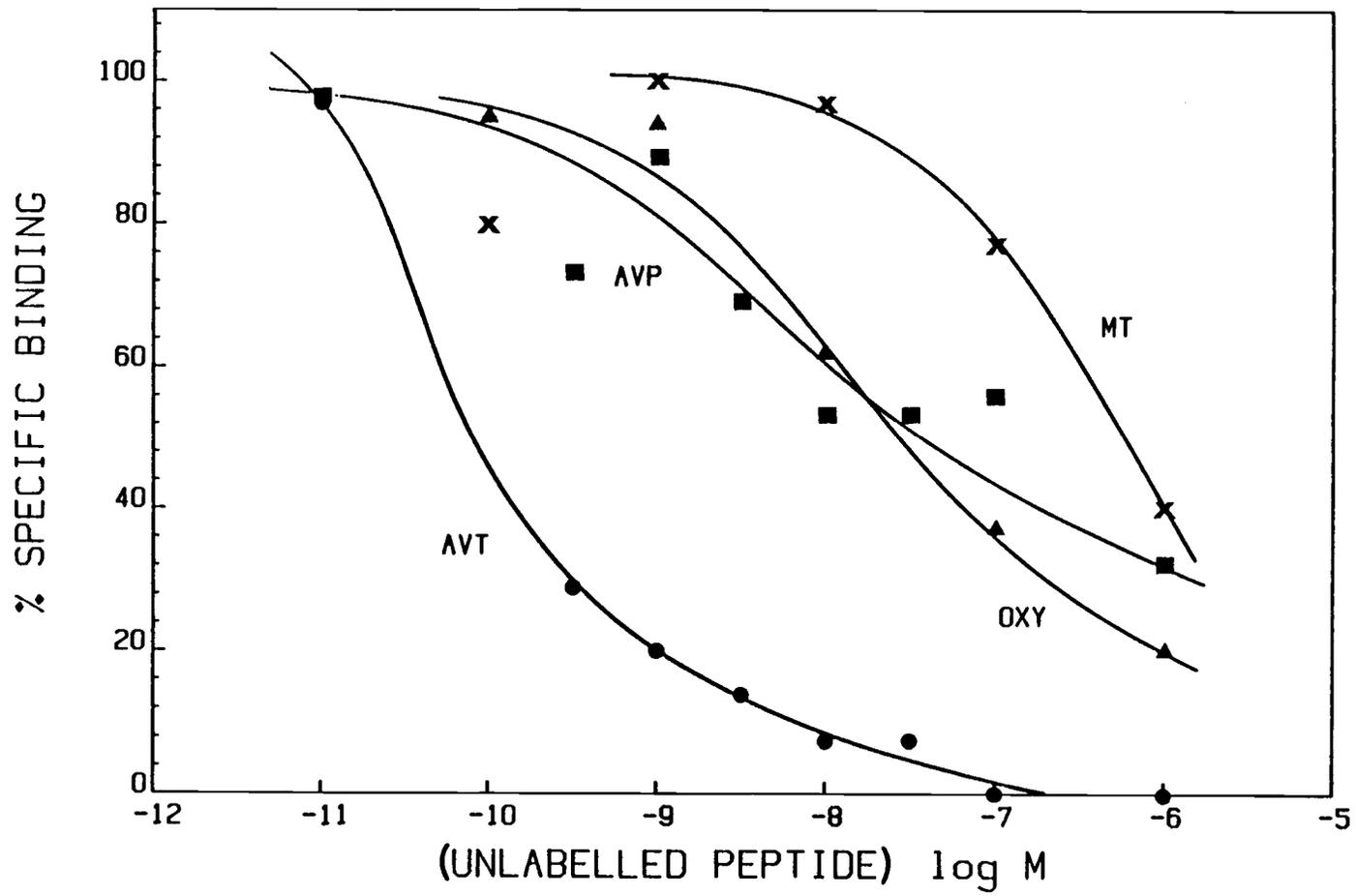


FIGURE 6A

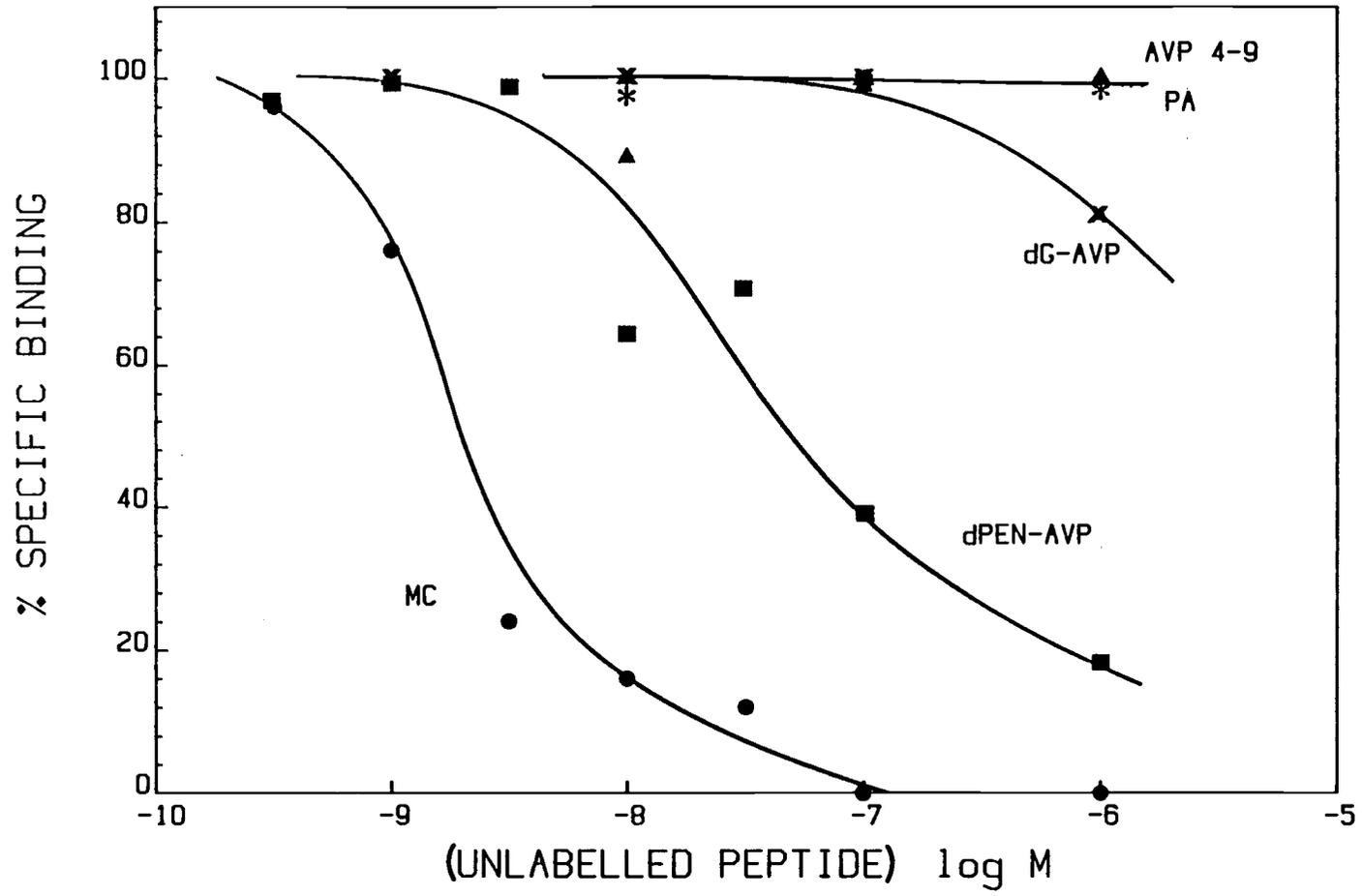


FIGURE 6B

TABLE 1

Inhibition Constants ( $K_I$ ) for Vasotocin and Related Peptides in Newt Medial Pallium.

Peptide*	Inhibition Constant (nM) Medial Pallium
Arginine Vasotocin	0.1
Arginine Vasopressin	74.2
Oxytocin	33.4
Mesotocin	491.2
d(CH <sub>2</sub> ) <sub>5</sub> [Tyr(Me) <sup>2</sup> ]AVP	2.2
[dPen <sup>1</sup> Tyr(Me) <sup>2</sup> ]AVP	73.9
desGly(NH <sub>2</sub> )AVP	>1000
AVP fragment 4-9	>1000
Pressinoic Acid	>1000

\* See Methods for explanation of abbreviations.

DISTRIBUTION OF PUTATIVE ARGININE VASOTOCIN  
RECEPTORS IN THE BRAIN OF MALE AND FEMALE NEWTS

Chapter III

INTRODUCTION

Arginine vasopressin (AVP) and vasotocin (AVT) influence reproductive behaviors in a variety of vertebrates, including certain mammals (Sodersten et al., 1983), birds (Kihlstrom and Danninge, 1972), amphibians (Diakow, 1978; Zoeller and Moore, 1982), and fish (Pickford and Strecker, 1977). In the urodele amphibian Taricha granulosa (rough-skinned newt), the effects of central injection of AVT, AVT antagonists, or AVT antisera indicate that this neuropeptide stimulates male sexual behaviors at the level of the brain (Moore and Miller, 1983).

The precise neural site of action for these behavioral effects of AVT and AVP is unknown. AVP receptors in the rat brain have been localized in many hypothalamic and extra-hypothalamic sites (Audigier and Barberis, 1985; Brinton et al., 1984; Cornett and Dorsa, 1985; DeKloet et al., 1984; Dorsa et al., 1984), however the distribution of AVT receptors in the brains of non-mammalian vertebrates has not been reported.

Consequently, we used computer-assisted quantitative autoradiography to determine the distribution and density of putative AVT receptors in the brain of rough-skinned newts. In addition, because AVP and AVT neurotransmitter systems in rat and reptile brains are sexually dimorphic (DeVries et al., 1984; Stoll and Voorn, 1985), we compared AVT receptor concentrations in male and female newts.

## METHODS AND MATERIALS

Adult male and female rough-skinned newts were collected locally at the height of the breeding season (March). Ten pairs that were showing sexual behaviors (amplectic clasping) on return to the laboratory were immediately sacrificed by decapitation. Brains were stored at  $-80^{\circ}\text{C}$  and serial coronal sections ( $48\ \mu\text{m}$ ) were made using a microtome at  $-20^{\circ}\text{C}$ . Alternate sections were placed on separate slides for determination of nonspecific binding. These pairs of slides were then used for quantitative in vitro autoradiography for  $^3\text{H}$ -AVP binding sites, using techniques we have previously described and validated for newt brains (Chapter II).

Briefly, slide-mounted sections were incubated with  $10 \times 10^{-9}\text{M}$   $^3\text{H}$ -AVP (New England Nuclear; 40 Ci/mmol specific activity) in 2 ml Tris-HCl buffer for 30 min at  $23^{\circ}\text{C}$ . Sections for nonspecific binding determination were incubated under the same conditions except for the addition of  $10\ \mu\text{M}$  unlabelled AVT (Sigma). Sections were rinsed, then dried on a slide warmer. Slides were exposed to tritium-sensitive film (LKB Ultrafilm) for two months at room temperature. Films were developed in Kodak D-19 at  $21^{\circ}\text{C}$  for 4 min. Tissue sections were lightly stained with cresyl violet for identification of neuroanatomy (Herrick,

1948; Northcutt and Kicliter, 1980). Autoradiograms were analyzed for differences in optical density using an IBM PC/AT-based image analyzer (Gallistel and Tretiak, 1985). These optical density values were converted to fmol/mg protein, using brain-mash standards prepared from newts and exposed in each cassette along with serial sections (Boyd and Moore, 1987; Rainbow et al., 1984).

## RESULTS

Measurements of optical density revealed several discrete areas in the central nervous system of T. granulosa with high concentrations of  $^3\text{H}$ -AVP binding sites (Fig. 7). Binding in these areas was specific; addition of unlabelled AVT to the incubation medium abolished binding of  $^3\text{H}$ -AVP and resulted in homogeneous background binding across the sections. Dense concentrations of binding sites were observed in the olfactory nerve as it entered the olfactory bulb. This binding disappeared rostral to the accessory olfactory bulb. Within the telencephalon, the medial (hippocampal) pallium, dorsal pallium, and amygdala pars lateralis contained specific binding sites for  $^3\text{H}$ -AVP. Binding in the medial pallium extended for the entire length of the telencephalon, whereas binding in the dorsal pallium was best-developed at mid-hemispheric levels and was not present at the rostral or caudal extremities of the telencephalon. Binding in the amygdala was solely in the pars lateralis in the caudal telencephalon. Finally, dense binding was observed in the lateral hindbrain in the tegmental region of the medulla oblongata. These binding sites were localized to the tegmental root region extending from roots of cranial nerves V to VIII. The location of receptors in all these areas was

almost exclusively over the neuropil.

The concentration of binding sites differed significantly between various brain regions (Fig. 8). Highest concentrations were observed in the telencephalon and lowest in the olfactory nerve. The concentration of receptors in each brain area did not differ significantly between male and female newts (Fig. 8).

## DISCUSSION

These specific and discretely-distributed  $^3\text{H}$ -AVP binding sites within the brain of a urodele amphibian may represent the precise sites of action of AVT in controlling reproductive behaviors in this species. Alternatively, these sites may have another, yet unidentified, function. We have presented evidence that the binding sites in the medial pallium of newts are authentic AVT receptors (Chapter II). All regions with high binding contain detectable concentrations of immunoreactive AVT in T. granulosa, except the olfactory nerve where no data have been reported (Zoeller and Moore, 1986). There is good correlation, therefore, between the distribution of this peptide and its receptors in the brain of newts.

The neuroanatomical distribution of AVT receptors in newts is similar to that described for central AVP receptors in rats (Audigier and Barberis, 1985; Brinton et al., 1984; Cornett and Dorsa, 1985; DeKloet et al., 1984; Dorsa et al., 1984). AVP receptors have been reported in the olfactory nucleus, hippocampus, amygdala and dorsal hindbrain. These foci apparently correspond in this amphibian to the olfactory bulb, hippocampal pallium, amygdala pars lateralis, and dorsal tegmental region of the medulla, areas which contain AVT receptors in newts (Herrick, 1948; Kicliter

and Ebbesson, 1976; Northcutt and Kicliter, 1980). These consistencies in location of AVP binding sites in amphibian and mammalian brains suggest functional homology across these areas.

Immunocytochemistry shows a sexual dimorphism in density of AVT and AVP neuronal fibers in specific regions of the lizard and rat brain, respectively (DeVries et al., 1984; Stoll and Voorn, 1985). AVT receptors were not observed in any of these regions in newt brains. Likewise, the distribution and density of AVT receptors did not differ between male and female newts in this study. This is not unexpected since AVT has been shown to stimulate both female-typical and male-typical reproductive behaviors in amphibians (Diakow, 1978; Moore and Miller, 1983).

The presence of  $^3\text{H}$ -AVP binding sites in these extra-hypothalamic regions suggests that AVT receptors may be involved in the integration of complex behaviors. In particular, the medial pallium and amygdala of amphibians receive input primarily from visual and olfactory systems and both have fibers which project to the preoptic area and other hypothalamic areas that control reproduction and reproductive behaviors (Kicliter and Ebbesson, 1976; Moore and Deviche, 1987; Northcutt, 1981; Northcutt and Kicliter, 1980; Wicht and Himstedt, 1986). AVT receptors in the olfactory bulb are likely involved in the sensory

functions of the olfactory nerve (Northcutt and Kicliter, 1980). Receptors in the medulla oblongata, on the other hand, were localized over regions with primarily motor functions (Herrick, 1948). The fact that cells in the amygdala and dorsal tegmental region of the hindbrain of anuran amphibians concentrate sex steroids also supports the idea that these areas are important in sexual physiology or behavior (Kelley, 1980; Kelley et al., 1978). AVT could be altering sexual behavior of amphibians by acting at all levels -- sensory, motor, and integrative -- along the neural pathways controlling this behavior (Kelley, 1980).

## FIGURE 7

Schematic diagram of the newt brain with regions containing putative AVT receptors indicated by closed triangles ( ▲ ). The top figure is an exterior side view. Figures labelled A-E are typical frontal sections at the levels indicated on the side view. Abbreviations: amy, amygdala pars lateralis; b.ol., olfactory bulb; cb, cerebellum; dp, dorsal pallium; gl, glomerular layer; hyth, hypothalamus; inp, interpeduncular nucleus; lp, lateral pallium; mot, medial olfactory tract; mp, medial pallium; na, nucleus accumbens; pit, anterior pituitary; poa, preoptic area; sep, septal nucleus; teg, tegmentum V and VII; II-X, cranial nerves.

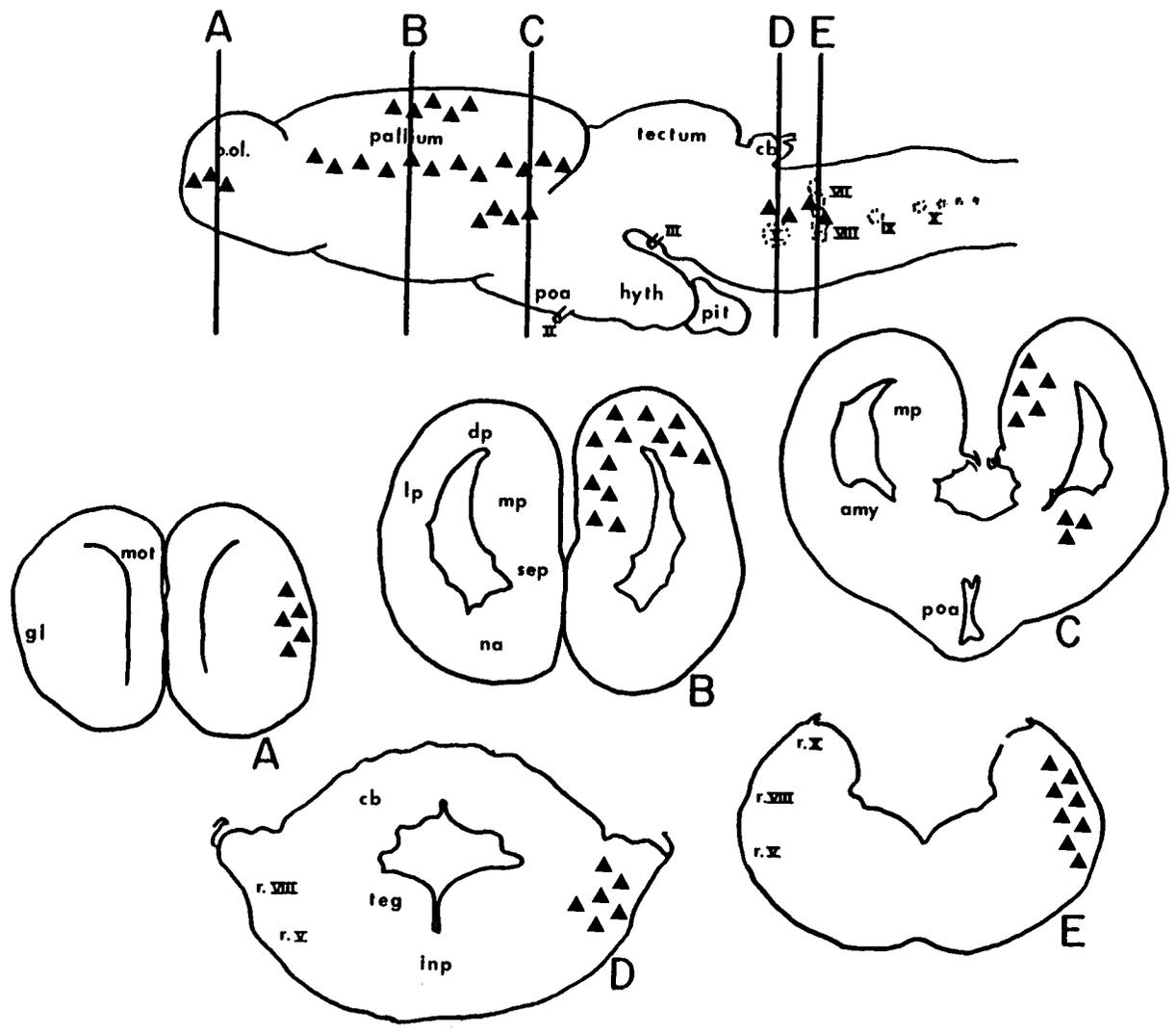


FIGURE 7

## FIGURE 8

Concentration of  $^3\text{H}$ -AVP binding sites in five regions of the brain in male and female Taricha granulosa. Two-way analysis of variance for these data showed that concentrations varied significantly across brain areas ( $F=11.69$ ;  $p<0.001$ ) but did not differ between males and females ( $F=1.95$ ;  $p>0.16$ ). There was no interaction of sex and brain area ( $F=0.67$ ;  $p>0.62$ ). Fisher's protected least significant difference test was used to compare means across brain areas. Receptor concentration in the olfactory nerve was significantly different from all other brain areas. The hippocampal (medial) pallium was similar to the amygdala and differed significantly from other areas. Binding in the dorsal pallium was significantly different from the olfactory nerve and hippocampal pallium only. The amygdala differed significantly from the olfactory nerve and tegmentum and, finally, the tegmentum was significantly different from all areas except the dorsal pallium.

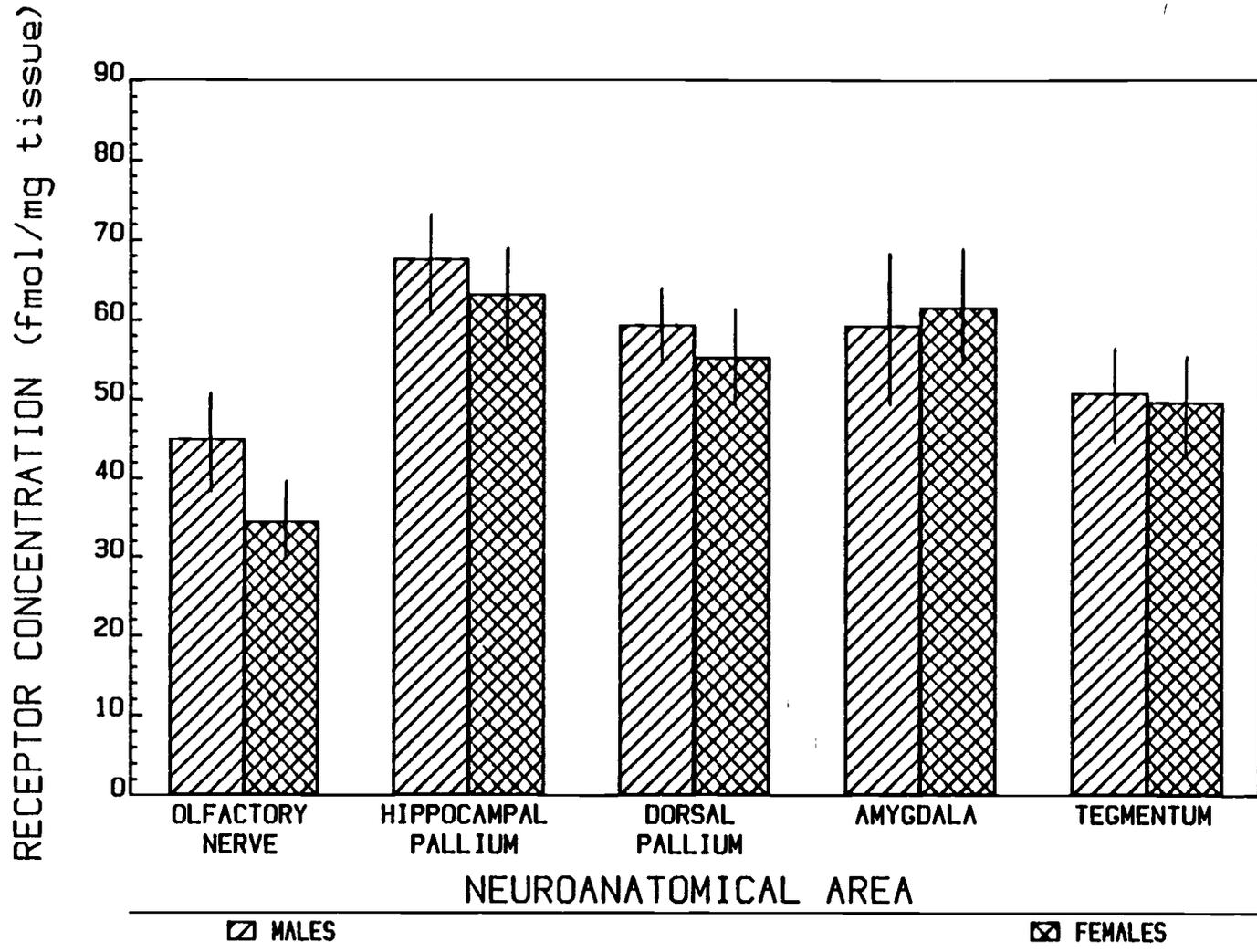


FIGURE 8

GONADECTOMY REDUCES THE CONCENTRATION OF ARGININE  
VASOTOCIN RECEPTORS IN THE BRAIN OF AN AMPHIBIAN

Chapter IV

INTRODUCTION

Gonadal steroid hormones are involved in activating reproductive behaviors in almost all species of vertebrate studied (Kelley and Pfaff, 1978; Crews, 1979; Larsson, 1979). For example, castration abolishes masculine sexual behaviors of the rough-skinned newt (Taricha granulosa) and administration of androgen can maintain or restore these behaviors (Moore, 1978). In addition, a variety of small peptide hormones are also potent modulators of vertebrate behaviors (Mauk et al., 1980; van Wimersma Greidanus et al., 1983a; Veldhuis and DeWied, 1984). The neuropeptides luteinizing hormone-releasing hormone (LHRH) and arginine vasotocin (or vasopressin) are key regulators of reproduction and reproductive behavior in both rats and newts (Mauk et al., 1980; Moore and Deviche, 1987; Moss and McCann, 1976; Pfaff, 1976).

There is a complex interaction between peptides and steroids in the control of reproductive behavior. Low levels of gonadal steroids are required for the behavioral effects of the peptide to be observed. LHRH

stimulates lordosis behavior in female rats only when estradiol is present (Moss and McCann, 1976; Pfaff, 1976). Likewise, copulation in male voles is facilitated by LHRH when testosterone is circulating in baseline amounts (too low to maintain behavior alone in castrates; Boyd and Moore, 1985). In the male newt, arginine vasotocin (AVT) stimulates amplexic clasping behavior in intact males and those castrated for only a few days (Moore and Zoeller, 1979; Zoeller and Moore, 1982). AVT is ineffective at stimulating sexual behavior in males that have been castrated for 30 days or more. Therefore, gonadal steroid hormones are maintaining the behavioral responses to these peptides.

One poorly understood phenomenon in vertebrates, however, is that masculine sexual behaviors decline gradually after castration; these behaviors can persist for several weeks or months after the testes are removed and circulating androgens are undetectable (Bloch and Davidson, 1968; Carpenter, 1933; Kelley and Pfaff, 1976). This slow decrease in spontaneous sexual behavior following castration is also observed for neuropeptide-induced sexual behaviors in newts (Zoeller and Moore, 1982). In female newts as well, ovarian steroids are required for AVT's induction of reproductive behaviors (Boyd, unpublished data). From these observations, I developed the hypothesis that gonadal steroid hormones maintain receptors for the

behaviorally-active neurohypophyseal peptides in the brain and that levels of these receptors decline gradually following gonad removal. To test this hypothesis, I used in vitro quantitative autoradiography to measure the concentration and dissociation constants of AVT receptors in the brain of newts. We have previously described the neuroanatomical location and binding characteristics of AVT receptors in the brain of rough-skinned newts, Taricha granulosa (Chapter II and III). Some of these regions where receptors are localized also concentrate gonadal steroid hormones in other amphibians and are involved in the display of sexual behavior (Kelley, 1980). Therefore, I examined whether lowering circulating gonadal steroid hormone levels would alter AVT receptors in any brain area. Specifically, we compared receptors in sham-operated and gonadectomized male and female newts collected during the breeding season.

## METHODS

Animals. Adult male and female rough-skinned newts were collected locally at the height of the breeding season (March). Under benzocaine anesthesia (Sigma Chemical Co, immersed in 1% solution), newts were either sham-operated or gonads were removed through a single, abdominal incision. Incisions were closed with 9-mm autoclips (Clay Adams). Animals were maintained at 10°C in dechlorinated water on a photoperiod of 12L:12D (lights out at 2200 hr). Newts were treated with amphibian antibiotic for 2 hr 3 times per week and were fed chopped beef heart once a week.

After 30 days, newts were decapitated and their brains immediately removed. Brains were stored at -80°C for several months. Using a cryostat at -20°C, serial coronal sections were made at 48 µm thickness, except in the caudal telencephalon where sections were taken at 16 µm. Alternate sections were placed on separate slides for determination of nonspecific binding. These pairs of slides were then used for quantitative in vitro autoradiography for <sup>3</sup>H-AVP binding sites, using techniques we have previously described and validated for newt brains (Chapter II).

Serially sectioned brains from each newt were incubated with  $10 \times 10^{-9}M$  <sup>3</sup>H-AVP (New England

Nuclear; 40 Ci/mmol specific activity) in 2 ml Tris-HCl buffer for 30 min at 23°C. Sections for nonspecific binding determination were incubated under the same conditions except for the addition of 10 µM unlabelled AVT (Sigma). Thin (16 µm) sections through the caudal telencephalon were distributed across separate slides for inclusion in saturation experiments. Therefore, duplicate sections from each animal were included at each concentration of <sup>3</sup>H-AVP. Following rinsing and drying, sections were exposed to tritium-sensitive film (LKB Ultrafilm) for two months at room temperature. Newt brain mash standards were included on each film. Autoradiograms were analyzed for differences in optical density using an IBM PC/AT-based image analyzer (Gallistel and Tretiak, 1985). Data were analyzed using LIGAND (Munson and Rodbard, 1980).

## RESULTS

When serial sections through newt brain were incubated with a single saturating concentration of  $^3\text{H}$ -AVP, the concentration of binding sites in the amygdala pars lateralis was significantly lower in gonadectomized animals than sham-operated controls for both males (Fig. 9) and females (Fig. 10). Likewise, similar results were obtained when concentrations in the amygdala were determined from the  $B_{\text{max}}$  (Table 2). Analysis of saturation binding isotherms showed that the dissociation constant ( $K_d$ ) of receptors in the amygdala was not altered by this treatment (Table 2). Castrated male newts had a 40% reduction in receptor concentration in the amygdala, compared with control males. Likewise, ovariectomized female newts had a 20% decrease in this areas, compared with sham-operated females.

This effect of gonadectomy on AVT receptor concentrations was specific to the amygdala; the concentrations of AVT receptors in the olfactory bulb, medial and dorsal pallium, and tegmentum were not affected by gonadectomy (Fig. 9, 10). There was no sexual dimorphism in receptor concentration in any area in controls or treated animals (Fig. 9, 10, Chapter III).

## DISCUSSION

Concentration of AVT receptors in the amygdala pars lateralis of gonadectomized male and female newts was significantly reduced compared with sham-operated controls. These results support the hypothesis that gonadal steroid hormones may maintain sexual behaviors in this amphibian by maintaining AVT receptors in the amygdala. These findings support the hypothesis that, in vertebrates in general, one action of gonadal steroid hormones on neural substrates is to maintain receptors to behaviorally active neuropeptides.

Of the five neuroanatomical areas examined, the amygdala was the only one which exhibited changes in AVT receptor concentration following castration or ovariectomy. Interestingly, the amygdala is the only brain area that has been reported to be sexually dimorphic in amphibians (Takami and Urano, 1984). Furthermore, the amygdala contains cells which concentrate radiolabelled estradiol in frogs (Kelley, 1980). Our finding that gonad removal changes AVT receptor concentration in this area, therefore, may represent a direct effect of estradiol (whether ovarian in origin or produced following aromatization of testosterone in the brain) on these receptors. This hypothesis is also supported by behavioral findings: estradiol treatment of male or female newts can

maintain behavioral responses to AVT in gonadectomized animals (Moore and Miller, 1982; Boyd, Moore, and Wood, unpublished observations).

There is indirect evidence that the amygdala may function in integrating olfactory information for the coordinated control of sexual behavior. The amygdala of amphibians has been implicated in the control of reproductive behavior using electrophysiological methods (Urano, 1984). The pars lateralis portion of this nucleus in salamanders receives projections from the vomeronasal organ and has projections to the dorsal and ventral thalamus and hypothalamus (Northcutt and Kicliter, 1980).

It is known that, following 30 days of gonadectomy, the behavioral responses to AVT disappear (Boyd, Moore, and Wood, unpublished data; Zoeller and Moore, 1982). Gonadectomy of male and female newts resulted in only a 20-40% decrease in number of AVT receptors in the amygdala, not a complete disappearance. This suggests that effects of steroids on AVT receptors is only one part of the chain of events leading to the display of reproductive behavior. For example, gonadal steroids may enhance the synthesis and release of AVT and other neurochemicals that act "down stream" of AVT. It is also important to note that these effects of gonadectomy on amygdala receptors may be indirect since

we have not yet shown that steroid replacement therapy will restore receptor concentration in this area. Alternatively, the 20-40% decrease in AVT receptors may reflect subpopulations of neurons in the amygdala that are specifically involved in sexual behavior.

## FIGURE 9

Receptor concentration in five brain areas from either sham-operated male newts or males that had been castrated for 30 days. Concentrations differed significantly only in the amygdala pars lateralis (ANOVA  $F=8.41$ ;  $p<0.0001$ ).

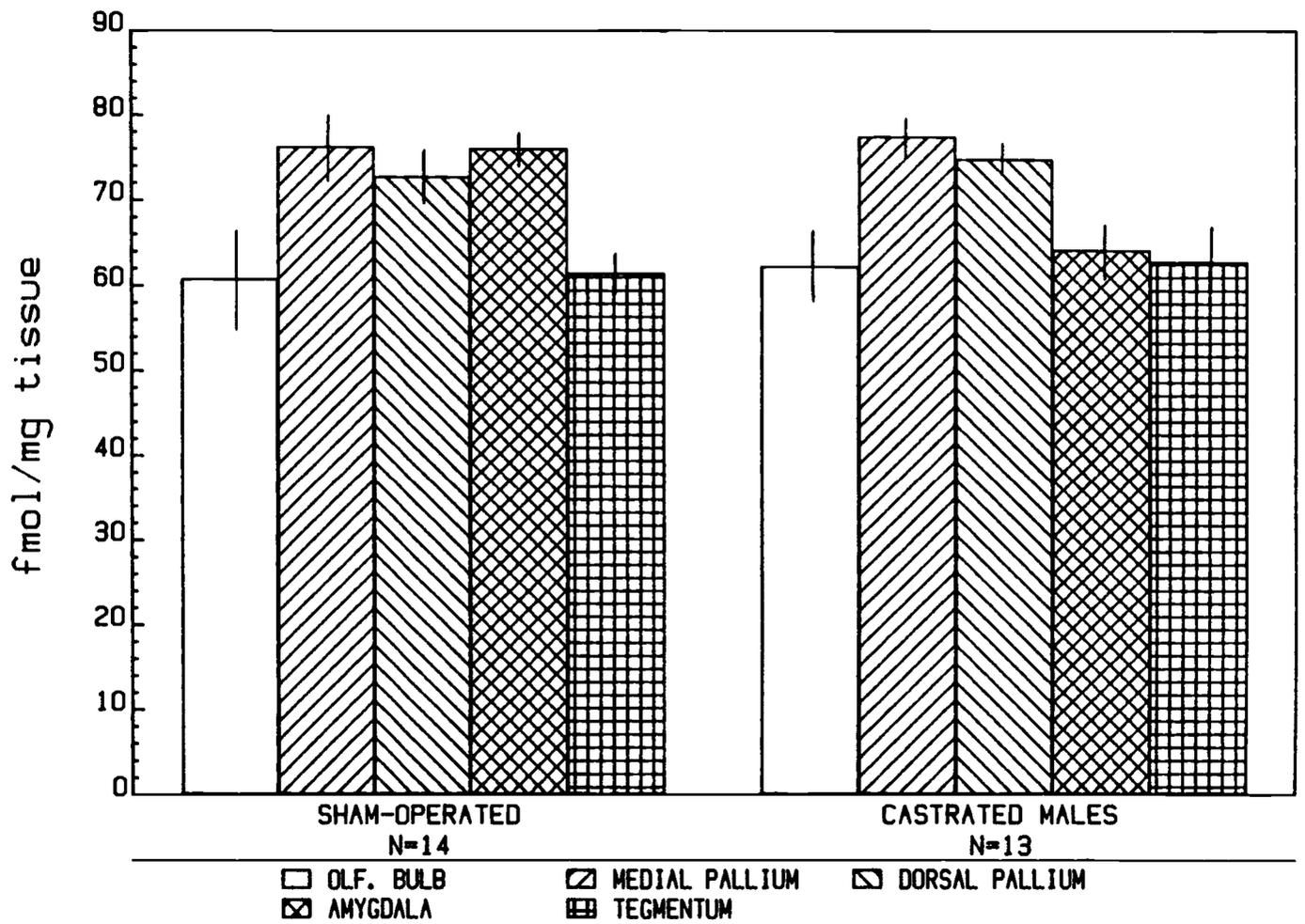


FIGURE 9

## FIGURE 10

AVT receptor concentrations in five neuroanatomical regions for sham-operated female newts and females ovariectomized for 30 days. Significant differences between control and ovariectomized females were only observed in the amygdala pars lateralis (ANOVA results in legend for Fig. 9).

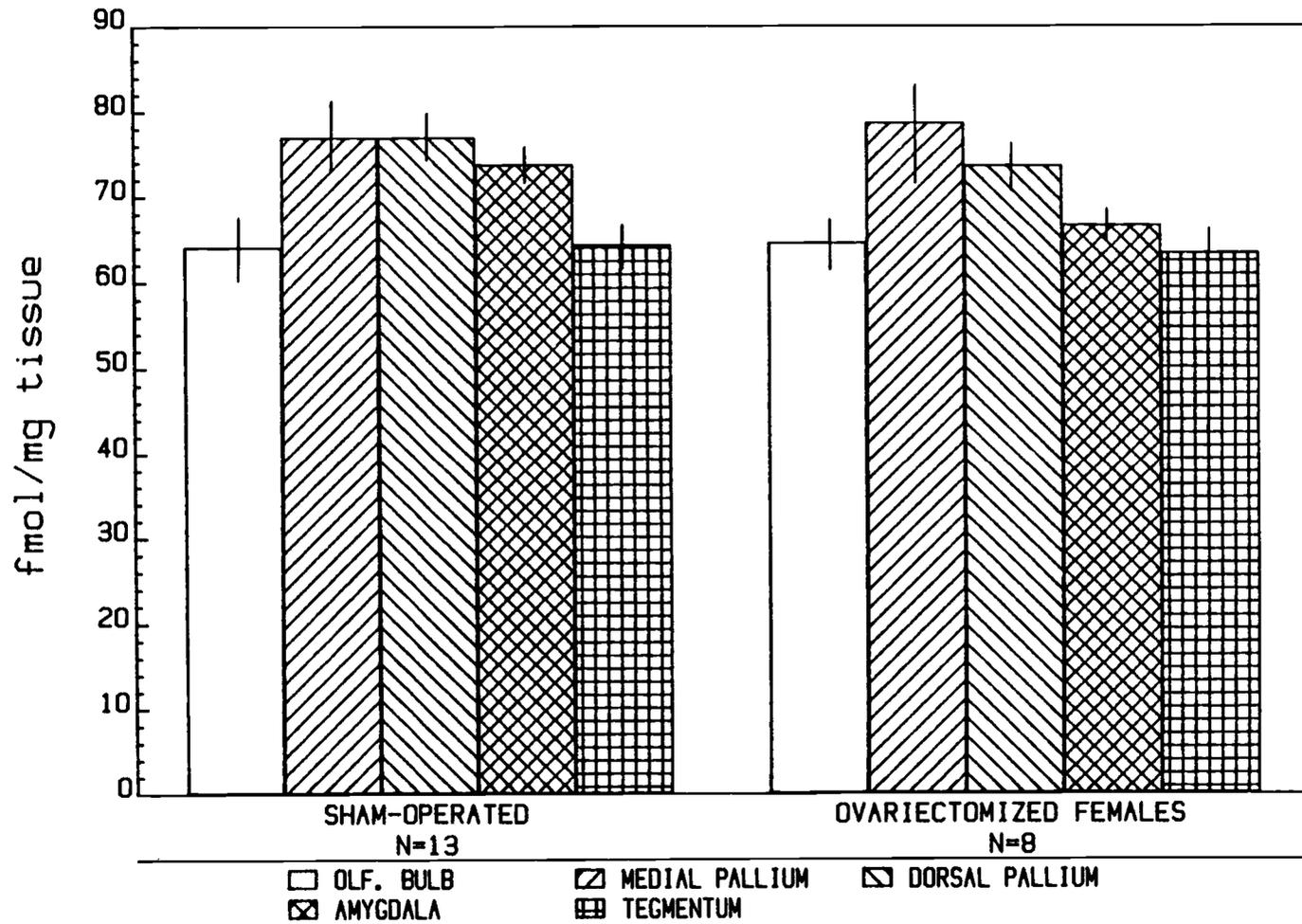


FIGURE 10

TABLE 2

AVT receptor dissociation constants and concentrations within the amygdala pars lateralis of sham-operated or gonadectomized male and female newts.

GROUP	N	$K_d^1$ (nM)	$B_{max}^2$ (fmol/mg tissue)
SHAM MALES	14	0.25 ± 0.02	75.3 ± 6.1
SHAM FEMALES	13	0.25 ± 0.02	87.9 ± 7.3
CASTRATED MALES	13	0.20 ± 0.03	46.1 ± 5.8
OVARIECTOMIZED	8	0.23 ± 0.01	69.4 ± 6.6

<sup>1</sup> Data presented are means + standard errors of the estimates. The 95% confidence intervals for all four groups overlapped and therefore  $K_d$ 's were not significantly different.

<sup>2</sup> Data presented are means + standard errors. The 95% confidence intervals for sham-operated males and females overlapped, as did those for gonadectomized males and females. However, sham-operated animals differed significantly from gonadectomized newts.

AUTORADIOGRAPHIC LOCALIZATION AND CHARACTERIZATION  
OF PUTATIVE ARGININE VASOTOCIN RECEPTORS IN THE  
KIDNEY OF A URODELE AMPHIBIAN

Chapter V

INTRODUCTION

The peptide hormone arginine vasotocin (AVT) has been found in species representing every major vertebrate class (Sawyer, 1977a). It has been proposed that the renal responses to AVT in fish and amphibians can be explained by the vascular responses (Nouwen and Kuhn, 1981; Pang et al., 1983). This hypothesis relates to the evolution of vasotocin function and implies that changes in the distribution and characteristics of the renal receptors for AVT occurred during vertebrate evolution. In particular, Pang et al. (1983) proposes that AVT is antidiuretic in urodele amphibians because of vasopressor actions on the renal preglomerular arterioles, rather than because of water permeability actions on renal tubules. Effects of AVT on renal tubular water reabsorption appear only among anuran amphibians and the amniotes (Sawyer, 1977b). In the rat, distinctly different vascular pressor (V1) and

tubular antidiuretic (V2) receptors for arginine vasopressin (AVP) have been described (Sawyer et al., 1981).

Although this hypothesis is supported by pharmacological data, no studies have been conducted to determine whether AVT receptors are, in fact, located only on glomeruli of urodele kidneys and whether AVT receptors in urodeles resemble mammalian pressor or tubular receptor subtypes. We therefore used the technique of in vitro quantitative autoradiography to determine the distribution and characteristics of AVT receptors in the kidney of a urodele amphibian, the rough-skinned newt, Taricha granulosa.

## METHODS

Tissue Preparation. Forty adult male newts were collected locally during October and maintained in large laboratory tanks of dechlorinated water at 20°C overnight. The next day they were killed by decapitation; kidneys were removed, frozen on dry ice, and embedded in Tissue Tek O.C.T. compound. Kidneys were stored at -80°C.

Tissues were sectioned (16  $\mu\text{m}$ ; horizontal orientation) in a cryostat at -20°C. Two sections were thaw-mounted onto a gelatin-chrom alum coated slide to permit "duplicate" determinations of total binding for each animal. Nonspecific binding was determined on two anatomically adjacent sections placed on another slide. Sections were dried for 2 hr in a vacuum desiccator at 0 to -5°C. Slides were stored overnight at -20°C. Pairs of slides (total and nonspecific binding slides) were assigned randomly to treatment groups to control for differences among animals. Data reported are means of determinations from three different animals.

Binding Procedures. Incubation procedures for kidney slices were identical to those that were previously described and validated for AVT receptors in the central nervous system of this species (Chapter II). Briefly, tissue sections were incubated with 10 x

$10^{-9}$  M  $^3\text{H}$ -AVP (New England Nuclear, 40 Ci/mmol; this concentration was varied for saturation experiments as described in Results) in 200  $\mu\text{l}$  of 50 mM Tris-HCl (pH 7.4) containing 5 mM  $\text{MgCl}_2$ , 2 mg/ml bovine serum albumin, 0.5 mg/ml bacitracin, and 10  $\mu\text{g}/\text{ml}$  aprotinin (Sigma Chemical Co.). Blanks (nonspecific binding sections) were incubated in the same medium with the addition of 1  $\mu\text{M}$  unlabelled AVT (Sigma). Following 30 min of incubation at 23°C, slides were washed in two 30-sec rinses of ice cold buffer (10 mM Tris-HCl, 1 mM  $\text{MgCl}_2$ , and 1 mg/ml bovine serum albumin) followed by brief dipping in double distilled water and drying on a slide warmer at 40°C. Slides were stored desiccated overnight then apposed to tritium-sensitive Ultrofilm (LKB Instruments) in X-ray cassettes and exposed for 2 months at room temperature. Films were developed in Kodak D-19 for 4 min at 21°C. Tissue sections were stained with hematoxylin and eosin.

Displacement studies used AVP, AVT, oxytocin (OXY), AVP fragment 4-9, desGly(NH<sub>2</sub>)AVP (all from Sigma), mesotocin (MT), [1-deaminopenicillamine, 2- $\theta$ -methyltyrosine]AVP (or [dPen<sup>1</sup>Tyr(Me)<sup>2</sup>]AVP), d(CH<sub>2</sub>)<sub>5</sub>[Tyr(Me)<sup>2</sup>]AVP, and pressinoic acid (all from Bachem Chemical).

Autoradiogram Analysis. Differences in optical densities generated by binding of tritiated AVP to the

kidney of newts were determined using the IBM PC/AT-based DUMAS system (Circon microvideo camera) and BRAIN software developed at the Drexel Image Processing Center (Gallistel and Tretiak, 1985). Density data were taken for two separate regions of binding on both right and left sides of the kidney and converted to  $\mu\text{Ci/gm}$  using tritium plastic standards developed on each film (ARC). Data were analyzed using LIGAND (Munson and Rodbard, 1980).

## RESULTS

Measurements of optical density revealed discrete areas in the kidney of T. granulosa with high concentrations of  $^3\text{H}$ -AVP binding sites (Fig. 11). These sites were located over the glomeruli and not over the tubules or collecting ducts (Chase, 1923). This binding was specific; addition of unlabelled AVT to the incubation medium abolished binding of  $^3\text{H}$ -AVP in these areas and resulted in homogeneous background binding across the sections.

Binding of  $^3\text{H}$ -AVP in the newt kidney was saturable and of high affinity. A direct plot of binding isotherm data shows saturation of specific binding at a concentration of approximately 12 nM (Fig. 12). Nonspecific binding was linear. Scatchard analysis of specific binding showed that, in the range of concentrations tested (0.2-22 nM),  $^3\text{H}$ -AVP bound to a single class of binding site with a dissociation constant ( $K_d$ ) of  $0.77 \pm 0.04$  nM and a binding site concentration of  $35 \pm 7$  fmol/mg protein (Fig. 12, inset). The Hill coefficient was 0.96.

The rank order of potency of peptides competing for  $^3\text{H}$ -AVP binding sites in the newt kidney was AVT =  $\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$  > AVP =  $[\text{dPen}^1\text{Tyr}(\text{Me})^2]\text{AVP}$  (Fig. 13). Binding was not altered by oxytocin, mesotocin, desGly(NH<sub>2</sub>)AVP, AVP

fragment 4-9, or pressinoic acid. Calculated inhibition constants ( $K_I$ ) for these peptides are in Table 1.

## DISCUSSION

Localization of  $^3\text{H}$ -AVP binding sites over newt kidney glomeruli and the ligand specificity of these sites support the hypothesis that AVT causes antidiuresis in urodele amphibians via a vasoconstricting (pressor) action rather than a tubular antidiuretic action. In both anuran and urodele amphibians, AVT has potent vasoconstricting effects on glomerular blood vessels as measured in perfusion experiments (Pang et al, 1980; Pang et al, 1982). On the other hand, neither AVT nor AVP can cause antidiuresis when administered specifically to the kidney tubules of the urodele Necturus maculosus (Pang et al, 1982). These peptides also do not alter water permeability of either the late distal tubule or the collecting duct of Ambystoma (Stoner, 1977). These physiological and pharmacological studies are consistent with our observation that in this urodele amphibian AVT receptors are located over kidney glomeruli and not over tubules or collecting ducts. The resolution of the quantitative autoradiographic technique is not sufficient to determine whether these receptors are located exclusively over preglomerular arterioles.

The  $^3\text{H}$ -AVP binding sites on newt kidney glomeruli differ in several important respects from

binding sites previously described in toad skin and bladder as well as in mammalian kidney medulla. The synthetic analogs  $d(CH_2)_5[Tyr(Me)^2]AVP$  and  $[dPen^1Tyr(Me)^2]AVP$  are specific antagonists for the pressor subtype ( $V_1$ ) of AVP receptor found in mammalian hepatocytes and vascular smooth muscle (Sawyer et al., 1981; Sawyer and Manning, 1984). These peptides have very little affinity for the antidiuretic ( $V_2$ ) subtype of receptor found in rat kidneys, toad bladder and toad skin (Sawyer et al., 1981; Sawyer and Manning, 1984). That these analogs compete well for  $^3H$ -AVP binding sites in newt kidneys suggests these binding sites resemble the pressor,  $V_1$ , receptor subtype.

Physiological studies with other species also indicate that the pressor receptor subtype is present in kidneys of urodeles. In Necturus, pretreatment with a pressor receptor antagonist has been shown to block AVT-induced antidiuresis (Pang et al., 1983). Also, AVT has no effect on the renal cAMP content of Necturus, which is as would be predicted from studies of  $V_1$  receptors in mammals (Pang and Yen, cited in Pang et al, 1983; Cantau et al, 1980; Rajerison et al, 1974). Therefore, the kidney of these urodele amphibians appears to contain only the pressor,  $V_1$ , subtype of receptor, indicating that antidiuresis is a consequence of vascular constriction in the glomeruli.

The dissociation constant and maximal binding capacity observed in newt kidneys are similar to those previously reported for central nervous system AVT receptors in this species (Chapter II). These values are also within the range reported for AVP receptors in various mammalian tissues (Butlen et al., 1978; Cantau et al., 1980). Although both brain and kidney receptors in T. granulosa resemble the  $V_1$  receptor subtype, this does not preclude the possibility that the  $V_2$  receptor subtype exists in other anatomical sites or in other urodele amphibians. The site of antidiuresis varies in this class depending on whether the species is relatively more aquatic or terrestrial (Bentley and Baldwin, 1980; Bentley and Heller, 1965). In frogs, toads and terrestrial salamanders (Salamandra maculosa), AVT acts on the skin, bladder, and kidney to alter water retention. In more aquatic salamanders such as Triturus (closely related to Taricha), Ambystoma, and Necturus, AVT causes antidiuresis through primarily a renal effect. Terrestrial urodeles may have tubular antidiuretic AVT receptors similar to frogs and toads. As well, T. granulosa may have  $V_2$  renal receptors during terrestrial stages of its life cycle (newts studied here were fully aquatic adults). This receptor subtype may also be present on other effector sites, the skin or bladder, in urodele amphibians.

The ubiquitous distribution of AVT and its vascular actions in vertebrates suggests that the most primitive functions of this peptide are associated with its pressor effects on smooth muscle (Sawyer, 1977b). We have directly demonstrated that the kidney glomerulus, and not the tubules, contain specific binding sites for  $^3\text{H}$ -AVP and therefore are the likely site of action for AVT in the antidiuretic response of this species.

## FIGURE 11

Photomicrograph of an autoradiogram showing  $^3\text{H}$ -AVP binding to discrete regions corresponding to glomeruli along the midline in a horizontal section through the newt kidney.

FIGURE 11



## FIGURE 12

Direct plot of results from saturation binding experiment. Each point represents the mean of determinations from 3 different kidneys (SEM was less than 10% of the mean). Specific binding (SB) could best be expressed as a log function ( $r=0.94$ ). Nonspecific binding (NSB) was linear ( $r=0.98$ ).

Inset: Scatchard replot of specific binding data which was linear ( $r=0.95$ ). Equation for the line yielded a  $K_d$  of 0.77 nM and a  $B_{max}$  of 35 fmol/mg protein.

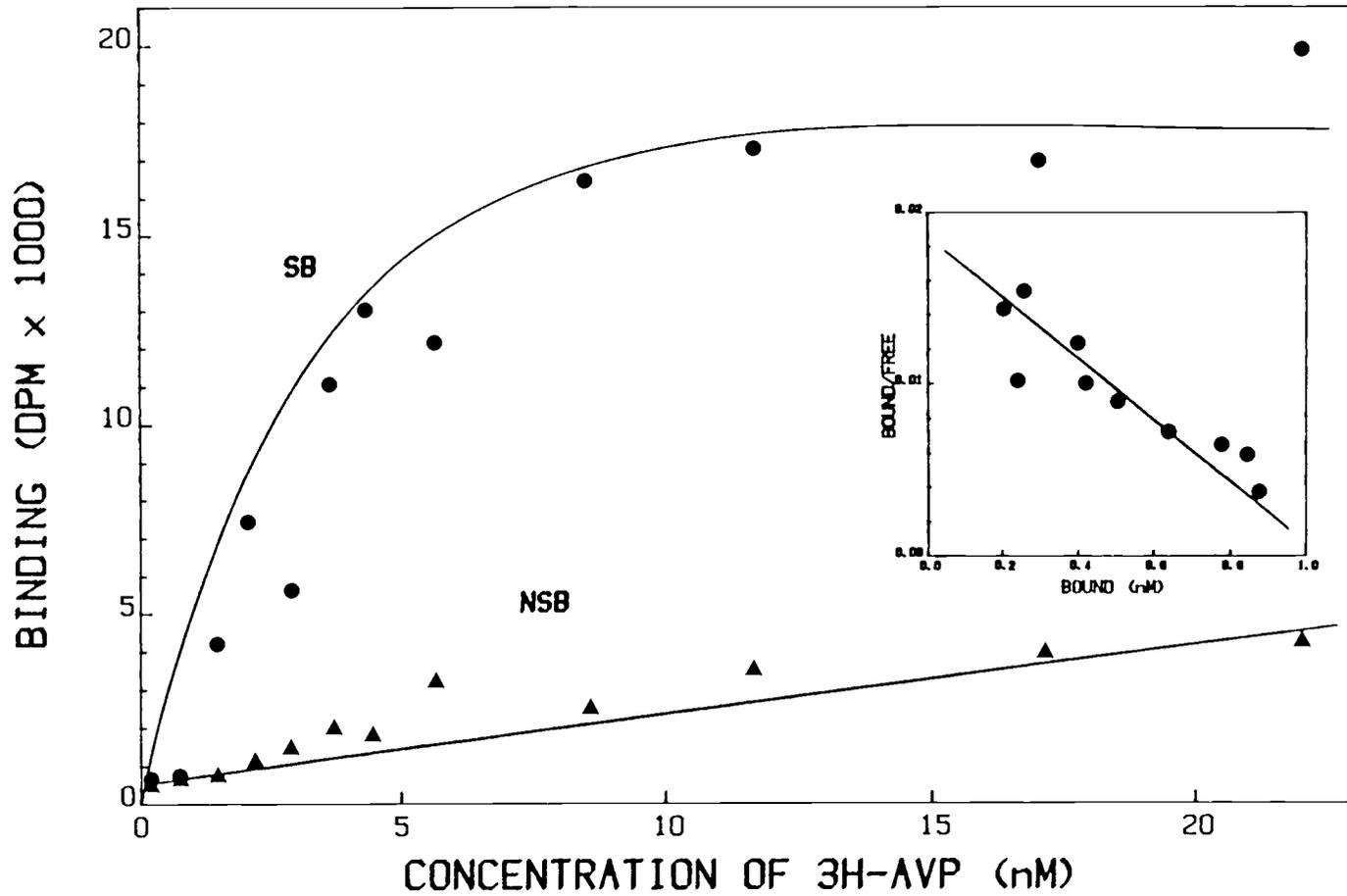


FIGURE 12

## FIGURE 13

Dose-dependent inhibition of  $^3\text{H}$ -AVP binding to newt kidney glomeruli by unlabelled peptides. Kidney slices were incubated in the presence of 10 nM  $^3\text{H}$ -AVP and increasing amounts of unlabelled peptides. Values measured in the presence of unlabelled peptide were expressed as a percentage of binding in the absence of competitor (0% was defined in the presence of 1  $\mu\text{M}$  AVT). Filled circles, AVT; filled triangles, AVP; filled squares,  $\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$ ; open circles,  $[\text{dPen}^1\text{Tyr}(\text{Me})^2]\text{AVP}$ .

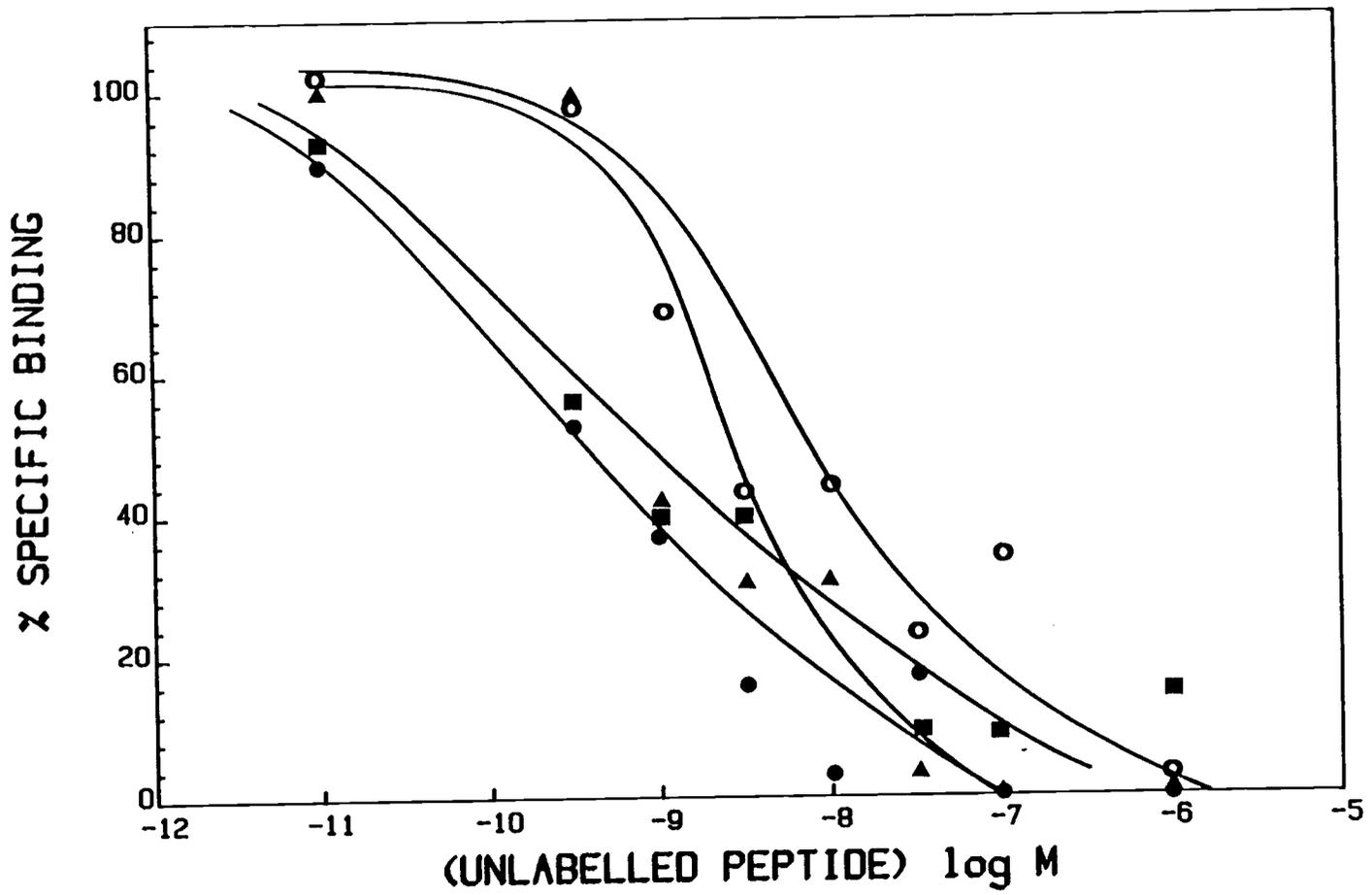


FIGURE 13

TABLE 3

Inhibition Constants ( $K_I$ ) for AVT and Related Peptides Competing for  $^3\text{H}$ -AVP Binding Sites in Newt Kidneys.

Peptide	$K_I$ (nM)
Arginine Vasotocin	0.09
Arginine Vasopressin	2.26
$\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$	0.20
$[\text{dPen}^1\text{Tyr}(\text{Me})^2]\text{AVP}$	7.59
Oxytocin	>1000
Mesotocin	>1000
desGly(NH <sub>2</sub> )AVP	>1000
AVP fragment 4-9	>1000
Pressinoic Acid	>1000

## GENERAL DISCUSSION

## Chaper VI

The present findings on arginine vasotocin receptors provide important insights into the target tissues and mechanism of action of this peptide in a non-mammalian vertebrate. The central nervous system of the newt contains AVT receptors in specific neuroanatomical regions. The function of AVT in these areas remains to be determined. It is an intriguing possibility that AVT alters newt sexual behavior by acting at all these sites -- perhaps modulating neurons at several stations along the pathway that controls behavior. It is also possible that receptors in these regions have diverse functions and only a subset are involved in behavior modulation. Further studies should not only address this question but also determine which specific cell types in each region express AVT receptors. For example, are receptors in the medial pallium located on cell processes of neurons intrinsic to that area or, perhaps, on axonal projections from cell bodies in a distant brain region? The chemical identity of cells with AVT receptors is also critical in uncovering the chain of events following the binding of AVT to a receptor. Are

AVT receptors in the medial pallium present on GABAergic cells, dopaminergic cells, or others?

The finding that the newt brain receptor is similar to the  $V_1$  receptor subtype, previously characterized in mammals, suggests that the newt brain and mammalian  $V_1$  receptor may also function similarly. For example, the mammalian  $V_1$  receptor does not use cAMP as a second messenger like the  $V_2$  receptors but instead acts through phosphatidylinositol turnover and changes in intracellular calcium. Whether the central nervous system receptor for AVT (or AVP) actually functions in this fashion is unknown. It is also worthwhile to note that the characterization data reported here are for one brain region only -- the medial pallium. Characteristics of receptors in other regions may vary and, indeed, this sort of variation could be an important mechanism for separating diverse roles of AVT within the brain.

Despite subtle differences in receptor specificity, the AVT receptor present in the newt kidney is also similar to the  $V_1$  subtype of mammals and the newt brain receptor subtype. Results of the kidney binding studies also provide independent validation and confirmation of in vitro autoradiography, which had never before been applied to a non-mammalian vertebrate. These observations on kidney and brain receptors lead to the interesting

hypothesis that this receptor subtype is the more primitive form. Although this hypothesis cannot be specifically tested, a thorough comparison of AVT receptors in different tissues (vas deferens and oviduct smooth muscle, especially) across a variety of vertebrates may point to common themes. Although no information is available on the newt glomerular receptor, the mammalian receptor in the same location (also  $V_1$ ) significantly alters prostaglandin synthesis in this tissue. Since prostaglandins have also been shown to affect amphibian sexual behavior, the possibility exists that AVT in the brain also influences prostaglandin synthesis and, thus, the display of reproductive behaviors.

Although gonadal steroids are required for AVT to induce sexual behavior in newts, information on the effect of such steroids on AVT receptors is still preliminary. One might predict that the distribution or density of AVT receptors would be sexually dimorphic since males and females have different circulating gonadal steroid hormones. This is not, in fact, the case in the five neuroanatomical regions reported here. These findings are consistent with the recent findings that AVT induces reproductive behaviors in both male and female newts. One interesting possibility is that AVT alters arousal levels in newts and the display of either male-typical or

female-typical behavior is under separate control. Another possibility is that AVT actually turns on a motor program in newts. This is supported by the finding that AVT-induced amplexic clasping in males is topographically similar to AVT-induced egg laying behavior in female newts.

The finding that gonadectomy is associated with a decrease in AVT receptor levels in the amygdala has exciting consequences for the field of vertebrate sexual behavior as a whole. These results suggest that gonadal steroids are required for the display of reproductive behaviors because they maintain central nervous system receptors for a neuroactive peptide. This is supported by behavioral studies where the presence of the gonads, or gonadal steroids, is required for exogenous AVT to induce either male-typical or female-typical behavior. The data reported here demonstrate that gonad removal alters brain AVT receptor concentration. Future experiments should determine whether steroid replacement therapy will reverse this effect.

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## APPENDICES

## APPENDIX A

IN VITRO <sup>3</sup>H-AVP AUTORADIOGRAPHY PROTOCOL

## A. Tissue Preparation

1. Decapitate newt and immediately remove brain from skull. Freeze brain on dry ice.
2. Plunge brain, nose down, into a 500  $\mu$ l microfuge tube filled with room temperature O.C.T. compound (Tissue Tek). Orientation is critical to the success of sectioning - brain must be in the middle of the tube, surrounded on all sides with OCT. Close tube and re-freeze in liquid nitrogen.
3. Prepare subbed slides before starting sectioning.
4. To mount brain for sectioning, cut both ends off microfuge tube with a razor blade. Use forceps to push the plug of OCT + brain out on to dry ice. Trim this cylinder back to 1.5 cm or less by removing excess OCT from both ends with a razor blade. Put some room temperature OCT onto a microtome chuck, put chuck in depression on dry ice and immediately set brain upright in center of chuck. Tap brain as it freezes to bring it back in to an upright position if it tips.
5. Mount chuck with brain into cryostat set at  $-20^{\circ}\text{C}$ . Slice brain at 16, 32, or 48  $\mu\text{m}$ , and move frozen sections to a cold slide with

watchmaker's forceps kept frozen on dry ice.

Alternate sections should be placed on another slide.

6. When slide is full, carefully pick it up and place it on the palm of your hand to melt the sections onto the slide. Dry the end of the slide and label it. (I recommend "animal number-slide pair number-a or b." The "a" for total binding slide and "b" for the slide destined for nonspecific binding.) Use a water/alcohol proof pen.
7. Place slides flat in vacuum desiccator and apply vacuum until mostly dry (5-15 min). Then close valve to maintain vacuum and put desiccator in freezer propped open to maintain temperature of 5°C. Slides should be dried at this temperature for a least 2 hours. As you finish additional pairs of slides, put previously dried pair in a slide box within the same desiccator. Complete drying is critical -- sections do not adhere to slides through incubation otherwise. After all slides have been dried thoroughly put a lid on the slide box and put it into a plastic bag containing Dri-Rite. Freeze boxes of slides at -20°C.

## B. Incubation

1. Thaw slides to room temperature. Place flat on absorbent paper in trays.

2. Place 200  $\mu$ l (for 2 sections, more for full slides) of incubation buffer over sections. One slide of each pair (the "a" slide) should receive buffer with  $^3\text{H}$ -AVP and the other ("b") slide should receive buffer with both  $^3\text{H}$ -AVP and cold AVT.
3. Incubate for 30 min at room temperature.
4. Drain excess media into kimwipe in beaker and immediately place slides in rinse buffer in coplin jars. There should be two sets of rinses (one for total binding slides and separate one for non-specific binding slides). Each set should contain two coplin jars with rinse buffer and a third with double distilled water. All rinses should be on ice.
5. Transfer slides through two rinse buffer rinses of 30 sec each. Dip briefly in double distilled water rinse to remove buffer salts.
6. Dry slides on a 40°C slide warmer tilted so moisture on slides runs away from end with sections.
7. When slides are dry, place in a slide box inside a plastic bag containing Dri-Rite and continue to desiccate overnight.

#### C. Generating Autoradiograms

1. Using double-sided tape, tape slides to a piece of cardboard cut the same size as the film. Cut

- slides down with a glass cutter if necessary (i.e. there is a lot of space without any sections).
2. Include on each film one set of plastic standards (ARC #ART-123) and one set of newt brain mash standards.
  3. In a darkroom (in the dark!), place a sheet of LKB Ultrofilm emulsion side down against the slides. Place in X-ray cassette and put another piece of cardboard on top to ensure a tight fit. Close the cassette carefully.
  4. Expose film for 8 weeks at room temperature. Keep cassette in a dark drawer away from other sources of radiation.

#### D. Developing Autoradiograms

1. Open cassette in dark and develop film in total darkness keeping film emulsion side up at all times. Make sure trays are large enough for the size film you are developing.
2. Develop in Kodak D-19 at 21°C for 4 min.
3. Rinse in tap water at 21°C for 1 min.
4. Fix in Kodak Rapid Fixer at 21°C for 5 min.
5. Rinse in running tap water for 30 min. Do not rinse more than one film at a time.
6. Rinse in Photo-Flo for 30 sec. Shake film to remove as much water as possible and hang to dry.
7. When dry, place film in a plastic protector to prevent scratches which will interfere with quantification.

## E. Preparing Brain Mash Standards

1. Dissect out at least five newt brains for every concentration desired (i.e., 25 brains for 5 concentrations used here). Put all 25 into a glass homogenizer together and mix as thoroughly as possible.
2. Divide equally (by weight) into 5 400  $\mu$ l microfuge tubes.
3. Add tritiated leucine to each tube as shown below.  
 $^3\text{H}$ -Leu (NET-135H from NEN) comes as a stock solution of 1  $\mu\text{Ci}/\mu\text{l}$  and should be used as is.

Tube 1	.5 $\mu$ l
2	1.0 $\mu$ l
3	2.0 $\mu$ l
4	4.0 $\mu$ l
5	8.0 $\mu$ l

4. Mix thoroughly on vortex. Freeze tube on dry ice.
5. Fill 5 1.5 ml microfuge tubes with OCT compound. Punch plug of frozen brain mash from small microfuge tubes onto dry ice. Using frozen tips of forceps plunge this plug of tissue into the OCT to "embedd" it for future sectioning. Re-freeze in liquid nitrogen or dry ice. Store at  $-80^{\circ}\text{C}$ .
6. Mount these and section at the same thickness as used for actual brains. Put two sections per standard on a single slide to be exposed along with each film. Also place one section each in 2 500  $\mu$ l

microfuge tubes for every standard.

8. Dissolve these sections in microfuge tubes in 200  $\mu$ l of double distilled water. Vortex 15 sec.
9. Sonicate for 20 sec (Tune 4, Power 5). Vortex for 5 sec at setting #3.
10. Pull off 50  $\mu$ l and put in scintillation vials. Dry and fill vial with scintillation fluid. Count.
11. Pull off two more 50  $\mu$ l aliquots and put into two plastic test tubes. Dry in 40°C water bath. Store at -20°C until assayed for protein.

#### F. Quantification of Images

##### Set-Up

1. Work with room lights off and make sure all hardware is hooked up and turned on. Read image analyzer manual before starting this section!
2. Boot Xenix. Load program by typing "brain" when the 1% prompt appears.
3. Configure program if desired (generally, use defaults except enable contrast enhancement and set calibration bar to read in concentration units).

##### Calibration

1. Choose "K--calibrate system" from main menu.
2. Choose "C--video, ODs, CONC" from calibration menu.
3. Video is done first, set magnification and f-stop (usually 4.5). If you later change magnification or any other parameter, you will need to completely

redo the calibration.

4. Do OD calibration using Kodak Neutral Density filters (photographic step tablet no. 3, calibrated, #152-3422). Enter steps 1 through 10. Enlarge the window size to about 2 times the default setting.
5. Isotope concentration calibration is done using the image of the plastic standards that should be on your film. Enlarge window to 2 times default. Enter 10 standards (0, 0.29, 1.04, 2.0, 3.7, 8.0, 16.6, 36.3, 63.1, and 138.1 uCi/g).

#### Quantification

1. Using the "fixed scans" option from the main menu. Set window size to at least 20 x 20 pixels. Set this window in the center of your mash standards also present on the same film and quantify the OD. This will be used to generate your standard curve and accuracy is therefore very important. Try to avoid scratches and inconsistencies.
2. If your entire experiment is present on one film, proceed to step 3. If sections are present on more than one film, you will need some measure of the variability across those films. At this point, therefore, you should use this same fixed scan option to measure the OD of all concentrations of plastic standards on all films and all concentrations of mash standards on all films. With these figures you can compute the coefficient of

variation for plastic and mash standards across films.

3. Quantify binding in specific brain regions using the fixed scans option and a window size that is the same for all brains and will fit well inside the area in question. Remember to do both right and left sides of the section.
4. Quantify all sections on a film using the calibration done for that film. If you have more than one film you will need to re-calibrate for each because the density of the plastic standards will vary slightly from film to film.

#### Exit from Program

1. You CANNOT simply turn computer off. Chose "X--exit" option from main menu, followed by exit to operating system.
2. When you see the 2% prompt, type "shut up." NOW you can turn the PC off.

#### G. Recipes

1. Tris-HCl Buffer Stock Solution.  
50 mM Trizma Base (Sigma #T-1503)=6.055 g/l ddH<sub>2</sub>O  
pH to 7.4 with HCl  
add 5 mM MgCl<sub>2</sub>-6H<sub>2</sub>O (Sigma #M-2393)=1.0165 g/l  
recheck pH = 7.4 (at room temperature)  
store refrigerated up to 6 months
2. Incubation Medium.

Add to Tris-HCl stock solution:

2 mg/ml BSA (0.2%, Sigma #A-7906)

0.5 mg/ml bacitracin (Sigma #B-0125)

10  $\mu$ g/ml aprotinin (Sigma #A-4529; or 10  $\mu$ l/ml)

Add  $^3$ H-AVP to this solution:

Stock of \*AVP is 2.09  $\mu$ M therefore, for a 10 nM solution add 10  $\mu$ l \*AVP/2.09 ml buffer and for a

5 nM solution add 5  $\mu$ l \*AVP/2.09 ml buffer

When doing saturation isotherms, start with about 50 nM solution and do 8-10 serial dilutions.

Divide this \*AVP into 2 equal portions. One will be used as-is for incubation of total binding slides. Add unlabelled AVT to the other portion (Sigma #V-4252).

For usual 1000-fold excess (10  $\mu$ M solution) add 0.011 mg AVT/ml buffer. For large number of solutions to be made at a single time, it is easier to make a very concentrated (for example, 1 mM) solution and add small aliquots to each individual buffer solution to reach desired conc. These solutions must be made fresh immediately before use (may be frozen for short periods). Use polypropylene or polyethylene only.

3. Rinse Buffer.

Take 50 ml of Tris-HCl stock solution and dilute out to 250 ml with ddH<sub>2</sub>O. This produces a 10 mM Tris-HCl buffer with 1 mM MgCl<sub>2</sub>. Add 1 mg/ml BSA

to this solution (0.1%). Refrigerate, then pH to 7.4 at 4°C (solution will be used at this temp). Rinse buffer may be stored in refrigerator for up to 1 week.

4. Kodak D-19 Developer.

Use Kodak D-19 powder (#146-4593) and dissolve in hot tap water according to package instructions. Make fresh and use only once for autoradiograms.

5. Kodak Fixer.

Use Kodak Rapid Fixer solutions (#146-4106). Mix according to package instructions for films and plates. May be saved and reused.

6. Photo-Flo.

Kodak Photo-Flo 200 (#146-4510) diluted in cold tap water at 1 cap/l.

7. Subbing Solution.

Mix 5.0 g gelatin (Knox) and 0.5 g chrome alum (also called chromium potassium sulfate; Baker #1624-1 - 12 hydrate, crystal) in hot ddH<sub>2</sub>O to make 1 liter. Cool. Filter solution immediately before use. Dip clean slides once in subbing solution at room temperature. Drain and dry in dust-free atmosphere. Store in clean, covered containers of plastic or glass. Do not keep the solution for more than 48 hr.

## APPENDIX B

## MICRODENSITOMETRY CALIBRATION AND VALIDATION

## FIGURE 14

Plot of calibration of camera gray scale against optical density using Kodak Neutral Density filters. Gray scale values plotted represent means of 2500 pixels. Standard errors were less than 10% of the mean. This curve is linear ( $r=0.97$ ) between optical densities of 0.05 and 0.94.

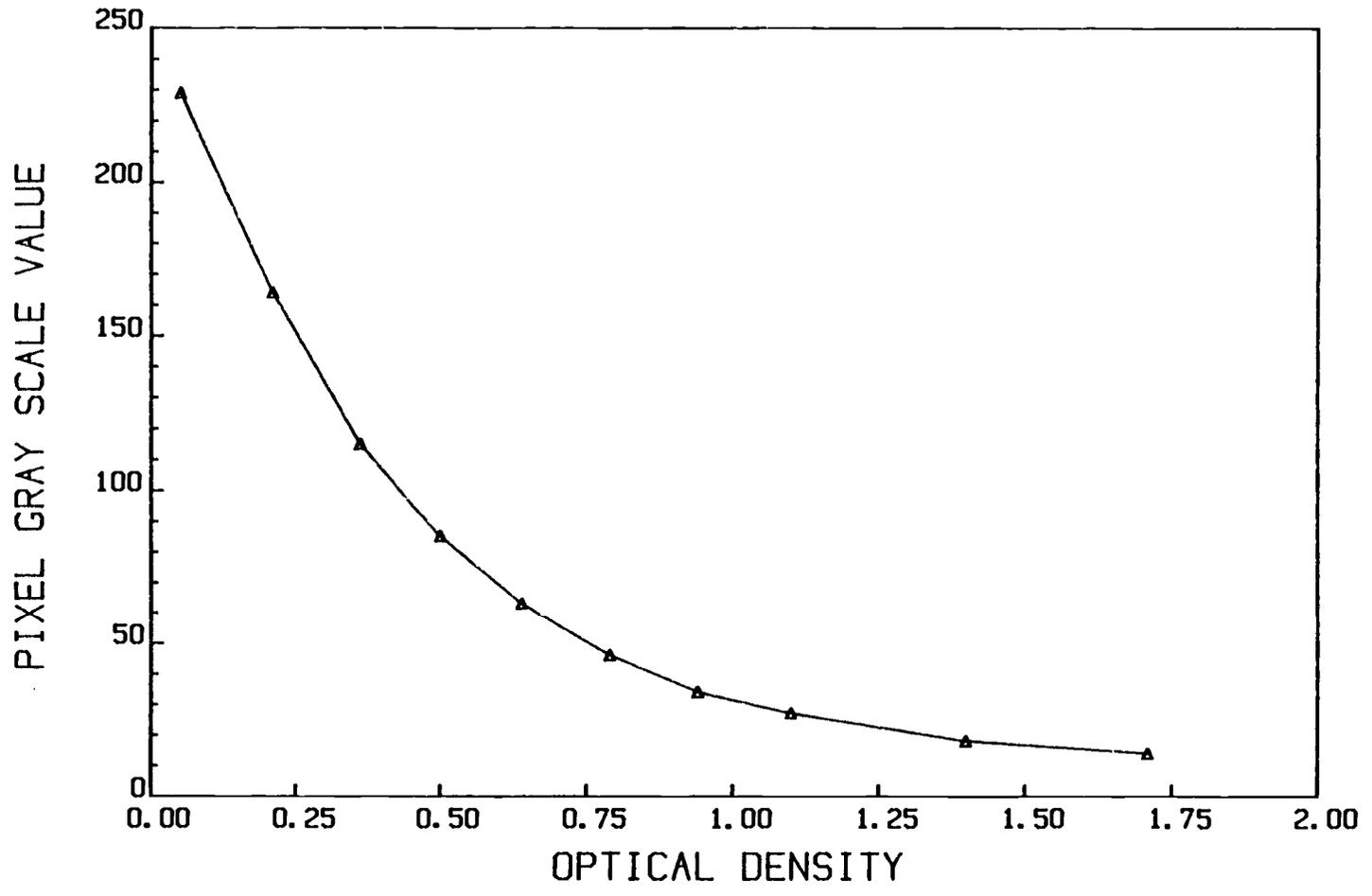


FIGURE 14

## FIGURE 15

Plot of calibration of camera gray scale against tritium concentration in plastic standards. Gray scale values represent means of gray level in 2500 pixels. Standard errors were less than 10% of the mean. This curve is linear between concentrations of 0 and 16.6  $\mu\text{Ci/gm}$ . All values are minus film background.

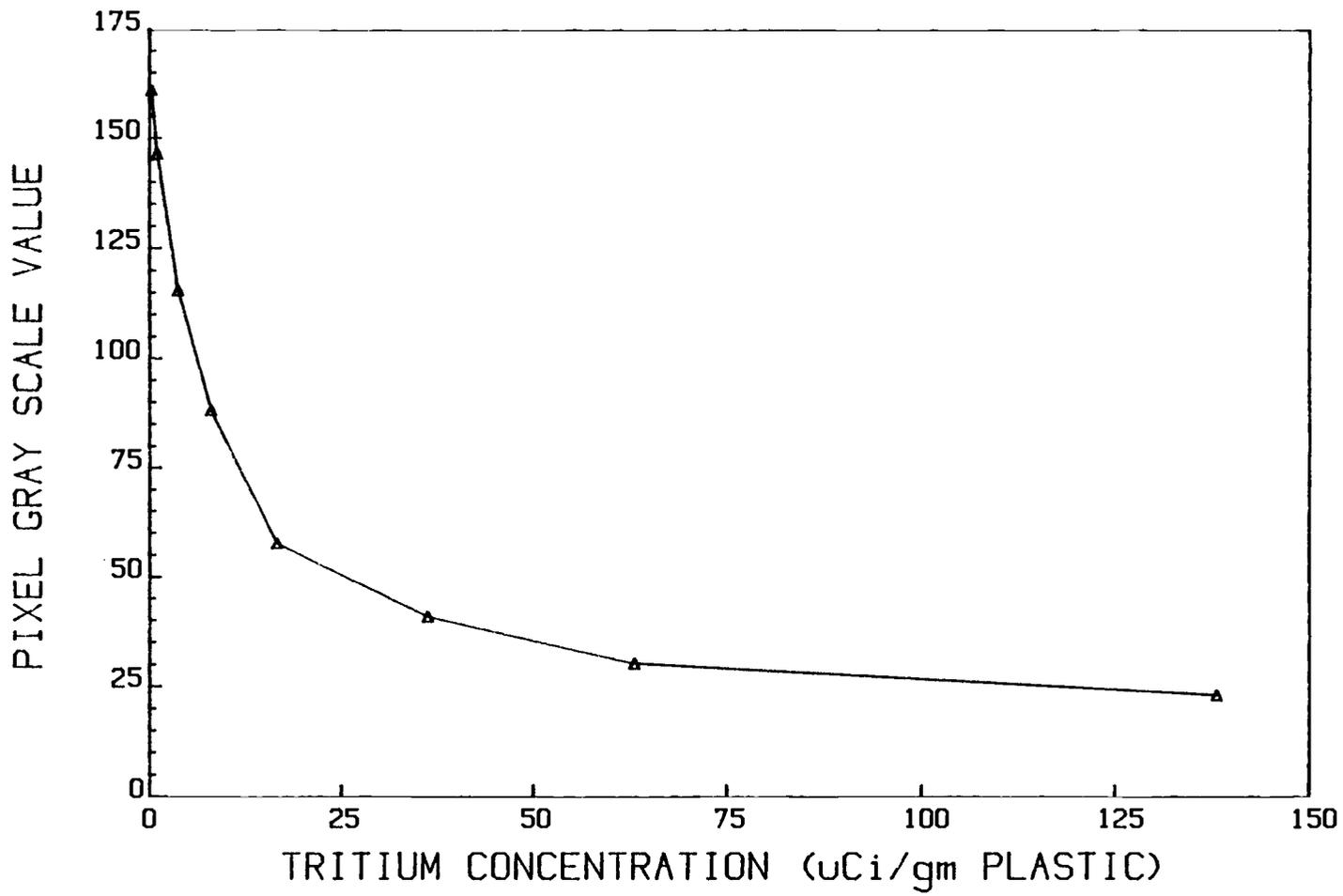


FIGURE 15

FIGURE 16

Plot of standard optical density against tritium concentration in plastic standards after 60 days exposure. Optical density (OD) was defined as the log (incident light/transmitted light) and was measured as described in the text. OD values are the means of 13 to 16 exposures of separate standards, using 2 different lots of LKB Ultrofilm. All values are minus film background OD. Standard errors were less than 5 % of the mean. Curve fitting analysis indicated that the relationship between mean OD and tritium concentration could best be expressed as a linear function,  $y=aX+b$  by substituting  $\ln$  tritium concentration for its actual value. The slope of this line was 0.01 and its y-intercept was 0.19,  $r=0.97$ .

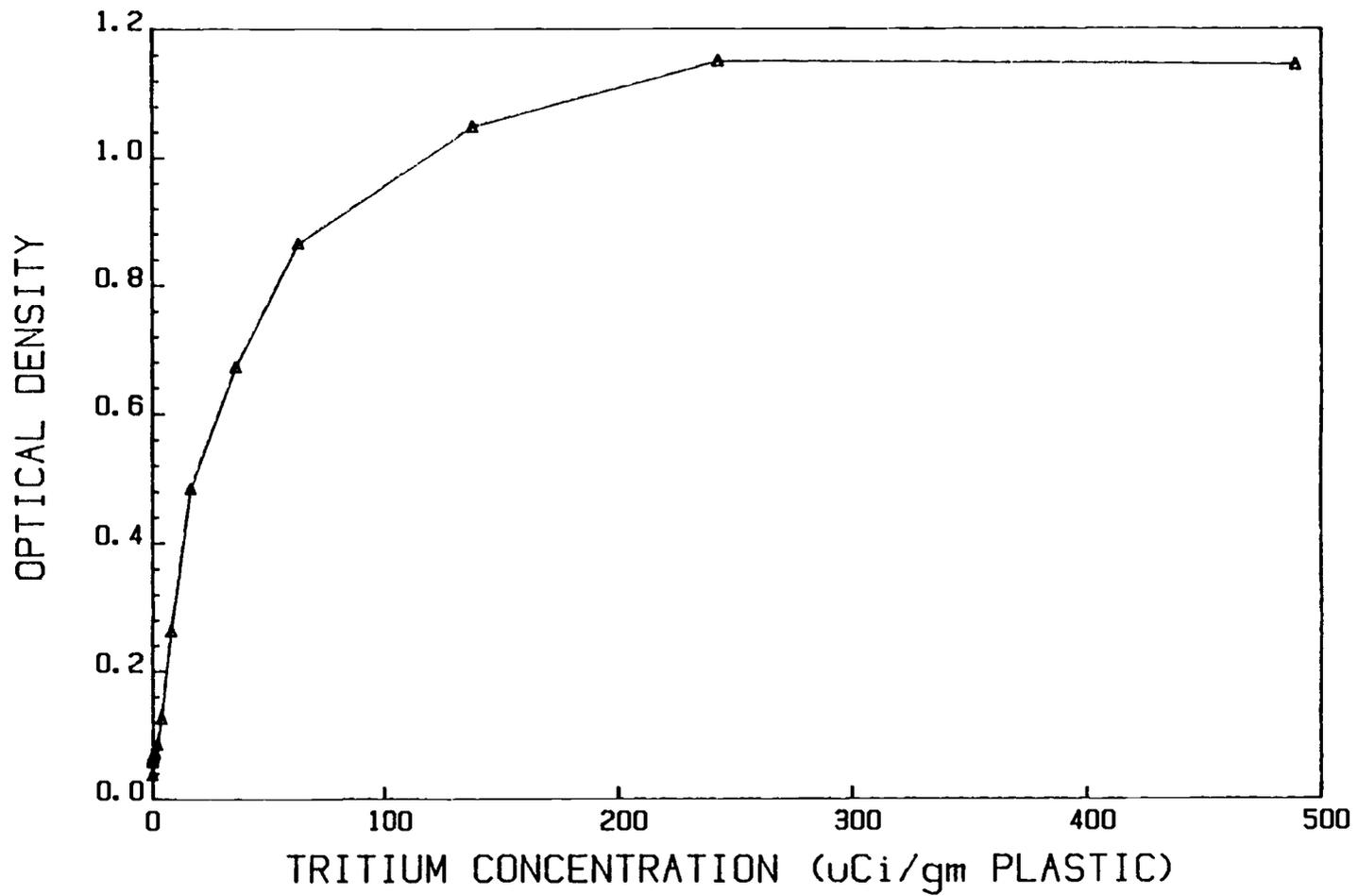


FIGURE 16

## FIGURE 17

Calibration of concentration of tritium in  $\mu\text{Ci/gm}$  in plastic standards against  $\text{nCi/mg}$  wet weight of brain tissue (at infinite thickness). Values plotted are means. Standard errors were less than 5% of the mean. This curve is linear ( $r=0.99$ ) with a slope of 0.21 and y-intercept of 3.01.

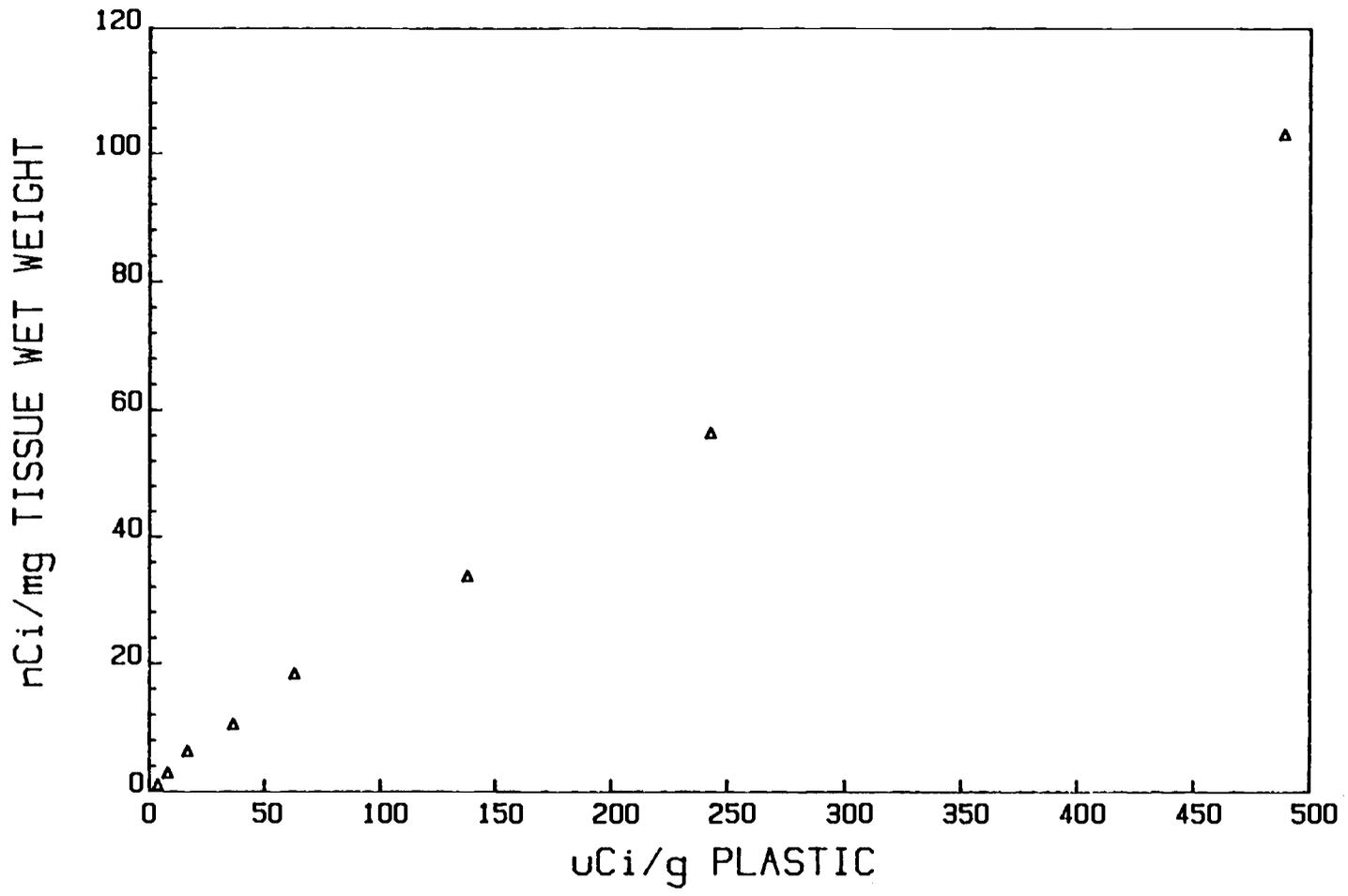


FIGURE 17

## FIGURE 18

Plot of the linear relationship between the optical density of newt brain mash standards on film (expressed as  $\mu\text{Ci/g}$  plastic) and the concentration of tritium in those standards in  $\text{fmol/mg}$  protein (determined from liquid scintillation counting and protein assay of standard sections). The slope of this line was 340 and the y-intercept was 105,  $r=0.99$ .

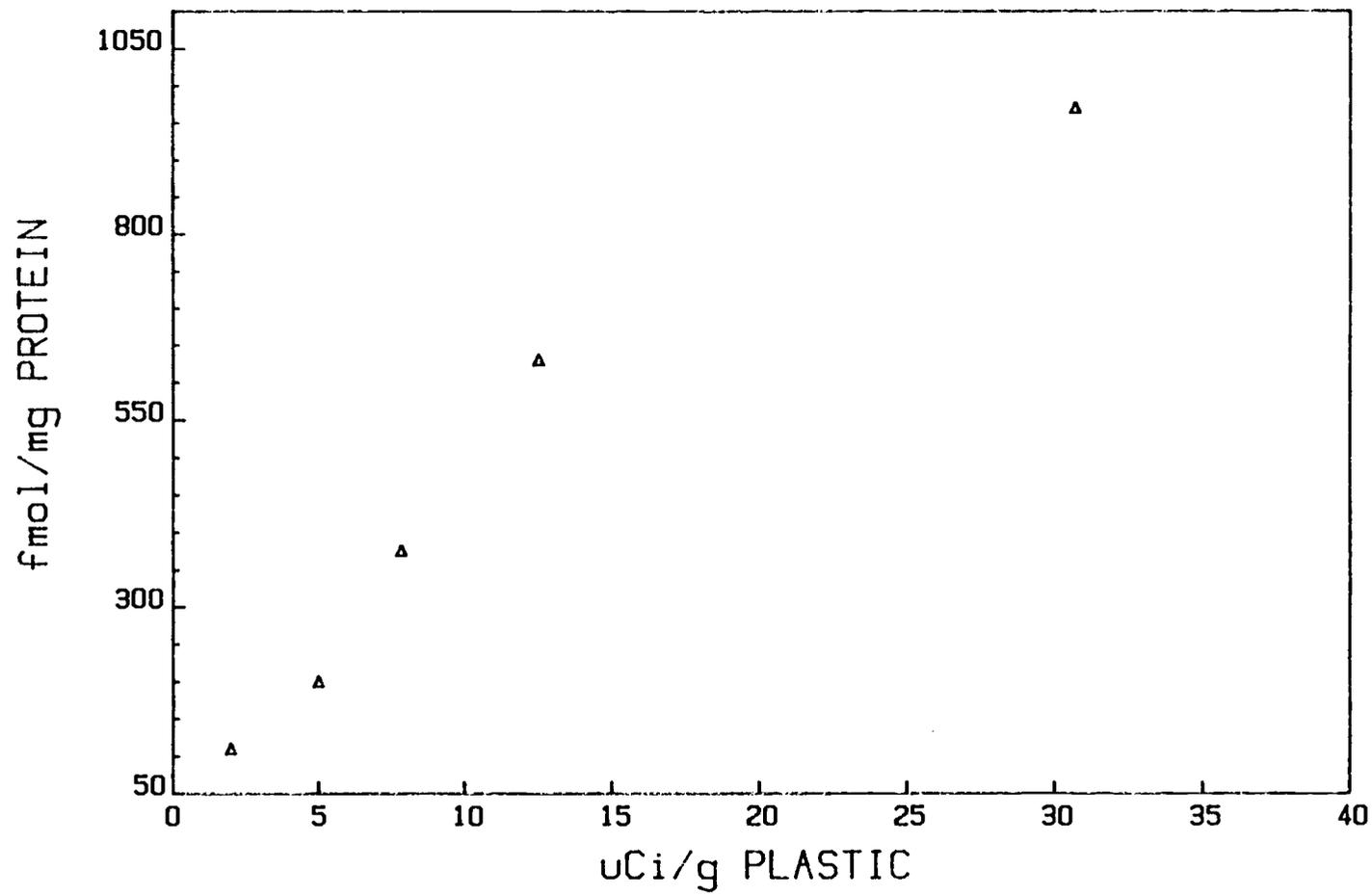


FIGURE 18

## FIGURE 19

Rostro-caudal changes in tritium concentration within the medial pallium, ranging from mid-telencephalon through caudal telencephalon (to level of preoptic area). Points represent means  $\pm$  SEM. A t-test comparison of tritium concentrations on right and left sides of the brain revealed no significant differences ( $t=-0.30$ ;  $p=0.77$ ). Therefore, right and left medial pallia values were included in the same analysis. One-way ANOVA yielded  $F=1.92$ ;  $p=0.09$ .

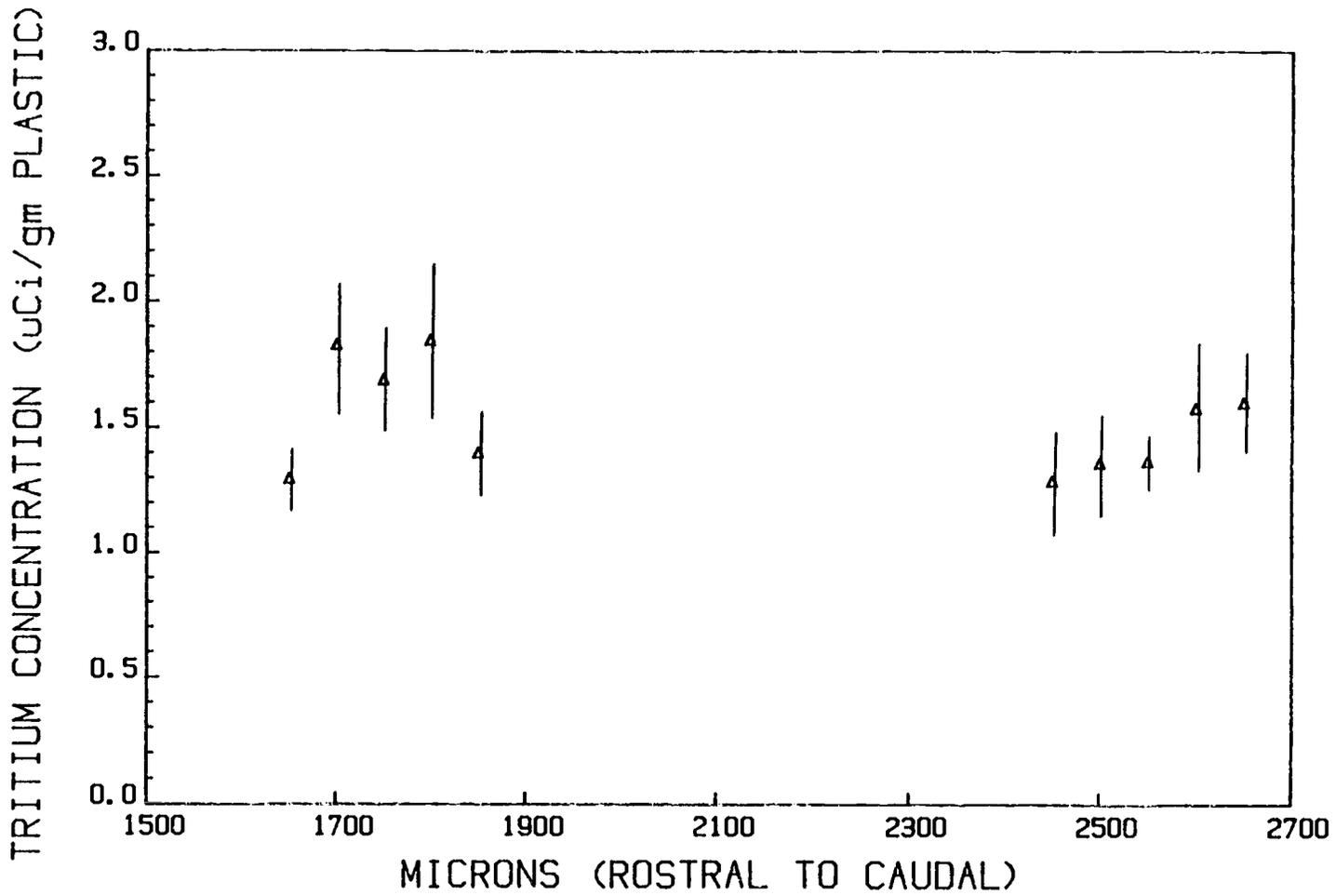


FIGURE 19