AN ABSTRACT OF THE THESIS OF

Christopher A. Lowry for the degree of Doctor of Philosophy in Zoology presented on June 2, 1995. Title: Neurobiology of Stress: Central Actions of Corticotropin-Releasing Factor in an Amphibian.

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Frank L. Moore

Corticotropin-releasing factor (CRF) is a 41-amino acid peptide that elicits a broad array of neuroendocrine, autonomic and behavioral responses that are also observed in the adaptive response to stress. The studies in this thesis characterize the effects of CRF on locomotor activity in a urodele amphibian, Taricha granulosa, as well as the neurochemical and electrophysiological correlates of this behavior. These studies establish that the effect of CRF on locomotor activity in T. granulosa is dose- and time-dependent and is completely blocked by administration of the specific CRF receptor antagonist, $\alpha$-helical CRF$_{9-41}$. In addition, $\alpha$-helical CRF$_{9-41}$ suppresses stress-induced locomotor activity suggesting that an endogenous CRF-like neuropeptide may contribute to the initiation and expression of stress-induced locomotor activity. This conclusion is supported by the observation that CRF infused directly into the lateral ventricle of T. granulosa rapidly (within 2-4 minutes) alters medullary neuronal activity associated with increased locomotion.

Interactions between CRF, opioidergic, and monoaminergic systems in the control of locomotor activity were investigated using behavioral, pharmacological, and biochemical approaches. These studies demonstrated that endogenous opioid peptides
can suppress CRF-induced locomotor activity through activation of the mu-type opioid receptor. Microdissection and high performance liquid chromatography with electrochemical detection techniques were used to characterize the distribution of catecholamines and indoleamines in the central nervous system of T. granulosa; other studies using these techniques determined that CRF-induced locomotor activity is associated with site-specific changes in the tissue concentrations of serotonin, dopamine, and 5-hydroxyindoleacetic acid -- monoaminergic neurotransmitters and a serotonin metabolite. These changes in monoamines and 5-hydroxyindoleacetic acid were restricted to the striatum and dorsomedial hypothalamus. The dorsomedial hypothalamus in vertebrates is an important structure for the coordination of the autonomic and behavioral responses to stress and therefore the CRF-induced neurochemical changes in this region may have important consequences for the physiological and behavioral state of the organism. Together, these studies support a role for CRF as a neuromodulator or neurotransmitter in the regulation of locomotor responses to stressful stimuli in T. granulosa.
Neurobiology of Stress: Central Actions of Corticotropin-Releasing Factor in an Amphibian

by

Christopher A. Lowry

A THESIS

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.
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PREFACE


CHAPTER I: GENERAL INTRODUCTION

In mammals CRF elicits neuroendocrine, autonomic, and behavioral events that are also observed in the adaptive response to stress (reviewed by Taylor and Fishman, 1988). Immunocytochemical studies suggest a homologous distribution of CRF perikarya and fibers among vertebrates including a wide distribution of CRF-like immunoreactivity in hypothalamic and extrahypothalamic areas (reviewed by Peter, 1986). These studies, in addition to the widespread distribution of CRF receptors in the rat brain (De Souza, 1987), are consistent with possible neuromodulator functions for CRF in addition to hypophysiotrophic functions.

To our knowledge only one nonmammalian species, the rough-skinned newt (Taricha granulosa), has been used to study the behavioral effects of CRF. In this species CRF elicits behavioral responses that are similar to responses observed in rats. In rats and T. granulosa CRF suppresses male sex behavior (Sirinathsinghji, 1987; Moore and Miller, 1984) and stimulates locomotor activity (Sutton et al., 1982; Moore et al., 1984). The stimulatory effects of CRF on locomotor activity persist in hypophysectomized animals indicating a mechanism independent of the hypothalamic-pituitary axis (Eaves et al., 1985; Moore et al., 1984).

Several studies have examined interactions between CRF and other neurochemical systems. For example, the opioid system can modulate CRF-induced locomotor activity, however, CRF-induced locomotor activity is not dependent on the activation of opioid receptors (Koob et al., 1984; Saunders and Thornhill, 1986;
Lowry et al., 1990). Monoaminergic systems also can modulate CRF-induced locomotor activity (Koob et al., 1984; Imaki et al., 1985, 1987), however, the conclusion from these studies and others is that CRF-induced locomotor activity is not dependent on the activation of dopaminergic or noradrenergic receptors (Swerdlow and Koob, 1985; Butler et al., 1990; Cole and Koob, 1988; reviewed by Swerdlow et al., 1986). Although administration of serotonergic 5-HT₁A agonists does not alter CRF-induced locomotor activity in rats (Lazosky and Britton, 1991), the possibility that CRF-induced locomotor activity is dependent on the activation of other serotonergic receptor systems has not been thoroughly addressed.

Recent studies suggest that CRF stimulates 5-HT release and tryptophan hydroxylase activity in the rat (Corley et al., 1990; Singh et al., 1992; Lavicky and Dunn, 1993). These findings are consistent with recent work in our laboratory in which the 5-HT uptake inhibitor fluoxetine had no effect on locomotor activity of saline-injected animals but greatly enhanced locomotor activity in CRF-injected animals (Lowry et al., 1993). The most parsimonious interpretation of the behavioral data is that CRF stimulated 5-HT release in newt brain and fluoxetine enhanced serotonergic neurotransmission by preventing reuptake from the synaptic cleft.

Neurochemical studies have described associations between CRF-induced locomotor activity and site-specific changes in monoaminergic systems (Dunn and Berridge, 1987; Kalivas et al., 1987; Barrett et al., 1989; Matsuzaki et al., 1989; Shimizu and Bray, 1989; Emoto et al., 1993; Lavicky and Dunn, 1993; Lee et al., 1994; reviewed by Dunn and Berridge, 1990). These studies demonstrated that CRF
alters catecholamine utilization in several brain areas. A few of these studies also
have described CRF-induced effects on serotonin metabolism (Barrett et al., 1989;
Shimizu and Bray, 1989; Lavicky and Dunn, 1993). In summary, although changes
in monoamine metabolism have been associated with CRF-induced locomotor activity,
the neurochemical mechanisms by which CRF stimulates locomotor activity are
unclear.

In addition to studies of the potential neurochemical mechanisms of CRF-
induced effects on behavior, several studies have investigated the potential
neuroanatomical substrates for initiation of CRF-induced effects. In studies using
rodents CRF effectively stimulates locomotor activity when injected into the
hippocampus (Lee and Tsai, 1989), amygdala (Lee and Tsai, 1989), ventral
tegmental area (Kalivas et al., 1987), paraventricular hypothalamic nucleus (Krahn et
al., 1988), and cerebral ventricle (Tazi et al., 1987b). The multiplicity of sites in
which CRF administration effectively stimulates locomotor activity in rodents suggests
an anatomical redundancy in the substrate for CRF action. Anatomical redundancy in
the substrate for CRF action was also suggested based on data from a study of CRF
activation of the autonomic nervous system in the rat (Brown, 1986). In this study,
Brown made site-specific injections of CRF throughout the rostrocaudal extent of the
rat brain. Although the injections were most effective when given in hypothalamic
areas and the third ventricle, injections in many other areas were also effective. In
the study by Brown, no site was more effective than the third ventricle for increasing
plasma norepinephrine levels. Other studies also suggest that the third ventricle and
the ventral forebrain are important neuroanatomical substrates for CRF-induced effects (Tazi et al., 1987b; Spadaro et al., 1990).

In summary, the neuropeptide corticotropin-releasing factor (CRF) initiates physiological and behavioral responses to stress. The mechanisms by which CRF affects brain function and the neuroanatomical sites involved, however, are not clear. The studies presented in this thesis use behavioral, pharmacological, electrophysiological, and biochemical approaches to characterize the effect of CRF on locomotor activity, with the ultimate aim of providing new information on possible neurochemical mechanisms and neuroanatomical sites involved in this behavior. This research involves the use of an amphibian model with the advantages of a structurally simple brain and a behavioral response that is robust and easily quantified. The neuroendocrine and behavioral effects of CRF are similar in amphibians and mammals, suggesting that an understanding of CRF’s effects in amphibians will contribute to a general understanding of CRF actions in other animals, including humans.
CHAPTER II: EFFECTS OF CORTICOTROPIN-RELEASING FACTOR (CRF) AND OPIOIDES ON AMPHIBIAN LOCOMOTION

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ABSTRACT

Male rough-skinned newts (*Taricha granulosa*) were used as a model for the study of the neuroendocrine regulation of locomotion. Intracerebroventricular (icv) injections of nanogram quantities of corticotropin-releasing factor (CRF) dose-dependently increased locomotion as measured in a circular open-field test arena. In other studies animals received intraperitoneal (ip) injections of saline or naloxone, a synthetic opioid antagonist, followed by icv injections of saline or CRF. With 1-minute intervals between injections, neither ip saline nor naloxone injections modified the stimulatory effects of CRF injections on locomotor activity. In contrast, with 20-minute intervals between injections, the naloxone-plus-CRF injected newts displayed more locomotor activity than the saline-plus-CRF injected newts, suggesting that the opioid system modulated the behavioral effects of CRF. An ip injection of bremazocine, an opiate k-receptor agonist, suppressed spontaneous locomotion but not CRF-induced locomotion. In contrast, an ip injection of morphine, an opiate μ-receptor agonist, did not affect spontaneous locomotion but reduced CRF-induced locomotion, indicating further that the opioid system may modulate the behavioral effects of CRF in this amphibian. The present study provides the first evidence that both CRF and opioids may be involved in the regulation of amphibian locomotor activity.
INTRODUCTION

Corticotropin-releasing factor (CRF), a 41-amino acid peptide originally isolated from the ovine hypothalamus (Spiess et al., 1981; Vale et al., 1981), stimulates the release of adrenocorticotropic hormone (ACTH) and β-endorphin from the adenohypophysis (Fryer et al., 1983; Rivier et al., 1982; Tonon et al., 1986; Vale et al., 1981). In addition to these neuroendocrine effects, CRF elicits autonomic (Brown et al., 1982) and behavioral (Berridge and Dunn, 1986; Britton et al., 1982) events associated with the 'stress response'. Behavioral effects of CRF include the inhibition of sexual behaviors and the stimulation of locomotor activity in amphibians and mammals (Koob and Bloom, 1985; Moore and Miller, 1984; Moore et al., 1984; Rivier and Vale, 1984; Sutton et al., 1982).

Studies of the effects of intracerebroventricular (icv) injections of CRF on locomotor activity of nonmammalian vertebrates are limited to one species, the rough-skinned newt (Taricha granulosa; Moore et al., 1984). In this species, injections of synthetic ovine CRF stimulate locomotor activity in both intact and hypophysectomized animals.

The effects of icv injections of CRF on mammalian locomotor activity depend upon the dose administered and the testing environment. For example, an icv injection of CRF dose-dependently increases locomotion in rats tested in photocell cages (Koob and Bloom, 1985; Sutton et al., 1982), while rats tested in novel open field environments exhibit a biphasic response to CRF injection: low doses of the
peptide stimulate the behavior (Sutton et al., 1982; Veldhuis and De Wied, 1984) whereas high doses inhibit it (Sutton et al., 1982).

Opioid substances also influence mammalian locomotion. In rodents, administration of the opioid receptor antagonist, naloxone, either has no effect (Abercrombie et al., 1988), or decreases (Amir et al., 1979; Arnsten and Segal, 1979; Ukai and Kameyama 1985) spontaneous locomotion, suggesting that endogenous opioids sometimes act to stimulate spontaneous locomotor activity. Although many opioid substances can stimulate spontaneous locomotor activity, as reviewed in Olson, et al., 1987, the synthetic opioid k-receptor agonists bremazocine (Römer et al., 1977), tifluadom (Römer et al., 1982), U-50,488 (Clark and Pasternak, 1988; Vonvoigtlander et al., 1983) consistently suppress this behavior in rodents (Castellano and Pavone, 1985; Castellano et al., 1984; Jackson and Cooper, 1986; Vonvoigtlander et al., 1983). Likewise, dynorphin, an endogenous opioid peptide which binds the k-receptor (Chavkin et al., 1982), suppresses locomotion in rodents (Ukai and Kameyama, 1985; Zwiers et al., 1981). These observations are consistent with the proposal that activation of the opioid k-receptor decreases spontaneous locomotor activity (Mansour et al., 1988).

The effects of µ-receptor agonists on spontaneous locomotor activity in mammals are more complex. The opioid µ-receptor agonists morphine (Martin et al., 1976), DAGO (Handa et al., 1981) and FK 33,825 (Römer et al., 1977) given at lower doses stimulate spontaneous locomotor activity in the rat (Babbini and Davis, 1972; Locke and Holtzman, 1986). These same substances, however, given at higher
doses to rats (Babbini and Davis, 1972; Locke and Holtzman, 1986) or morphine at all doses given to DBA/2J mice (Gwynn and Domino, 1984) cause an initial suppression of spontaneous locomotion followed by a period of stimulation.

In mammals, CRF and the opioid system may interact to regulate locomotion. Naloxone treatment in rats either has no effect (Koob et al., 1984) or attenuates (Saunders and Thornhill, 1986) the stimulation of locomotion caused by CRF administration.

It is not known whether the apparent functional interaction between CRF and opioids in the control of locomotor activity in rodents is a widespread phenomenon among vertebrates. This study examines the possibility that such an interaction exists in an amphibian. Agonists for the $k$- and $\mu$- receptors were chosen for study based on their effects on spontaneous locomotion in vertebrates and on binding studies which indicate that opioid receptors in amphibians are predominantly of the $\mu$ and benzomorphan preferring or $k/\sigma$ types (Ruegg et al., 1981; Simon et al., 1982). In this study an amphibian ($T.\ granulosa$) was tested using an open-field testing procedure to investigate the effects on locomotion of CRF and of opioids specific for the $\mu$- and $k$-receptor types administered either separately or in combination.

**GENERAL MATERIALS AND METHODS**

Adult male $T.\ granulosa$, averaging 15 g in body weight, were collected locally (Benton County, Oregon) from freshwater ponds and were maintained in the laboratory for approximately 48 h in two circular holding tanks (85 cm in diameter), each containing 100 l of dechlorinated tap water. These tanks were in a room with
controlled photoperiod (14 h light/10 h dark; lights on at 06.30 h) and an ambient temperature of 19°C. Newts were not handled or disturbed until immediately prior to treatment. Experiments were initiated between 11.00 and 15.30 h.

Synthetic ovine CRF was supplied by Drs. J. Rivier and W. Vale (Salk Institute for Biological Studies, San Diego, CA); naloxone hydrochloride was provided by Endo Pharmaceuticals (Glenolden, NY); (±) bremazocine hydrochloride was provided by Dr. D. Römer (Sandoz; Basel, Switzerland); and morphine sulphate was purchased from Mallinckrodt Chemical Works (St. Louis, MO). Drugs were dissolved in amphibian Ringer’s solution (saline). Control injections consisted of the appropriate volume of saline alone: 2 µl for intracerebroventricular (icv) or 100 µl for intraperitoneal (ip) injections. Intracerebroventricular injections were given through a 0.5 mm hole at the junction of the parietal and frontal bones in the cranial midline using a microsyringe with a tip diameter of 50 µm as described in Moore and Miller, 1983. Solutions (2 µl) were infused over a period of 5 seconds.

Depending on the experiment, newts received one or two injections and, unless otherwise stated, were tested starting 30 minutes following the final injection. During the periods between the two injections in Experiment V and from final injection to testing in all experiments, newts were isolated in temporary circular holding tanks (25 cm in diameter) containing 5 l dechlorinated tap water.

To measure locomotor activity newts were placed individually in a water-filled circular runway with an inside diameter of 70 cm and an outside diameter of 85 cm. Each test arena contained 30 l dechlorinated tap water and was marked with radial
lines to define 16 equal sectors. Starting 4 minutes after placement, locomotor activity was quantified by counting the total number of lines crossed during 3 consecutive minutes. Locomotion consisted of a combination of walking and swimming.

Kruskal-Wallis one-way analysis of variance was used to test for statistical differences among treatment groups within each experiment (n = 15 unless otherwise stated). When appropriate, comparisons between two experimental groups were made using the Mann-Whitney U-test (Siegel, 1956). Two-tailed probabilities $P \leq 0.05$ were considered statistically significant.

**SPECIFIC METHODS AND RESULTS**

*Experiment I. Dose-response study: icv injections of CRF*

To determine if CRF would elicit a dose-dependent behavioral response, locomotor activity was measured 30 minutes after an icv injection. The experiment was conducted in two parts. In Part I newts received 0, 25, 50 or 150 ng CRF; newts in Part II received 0, 6.25, 12.5, or 25 ng CRF. Data obtained from identical treatments (0 and 25 ng) in Parts I and II were combined (n = 30) for statistical analyses since corresponding groups were not different.

Centrally administered CRF stimulated locomotor activity in a dose-dependent pattern (Fig.II.1A). Significant differences were observed between the treatment groups ($T = 41.27; P \leq 0.0001$). Comparison of CRF-injected newts with control newts revealed that doses ranging from 12.5 to 150 ng increased locomotor activity.
The dose of 25 ng, equivalent to about 1.7 μg/kg body weight, resulted in a maximum response and was used in subsequent experiments.

**FIGURE II.1.** Locomotor activity of male rough-skinned newts, defined as the number of crossings in an open field arena during 3 consecutive minutes of observation per animal. Results are presented as median values (±) interquartile ranges. (A) Locomotor activity 30 minutes after icv injection of various doses of CRF (n = 15 except groups which received 0 or 25 ng CRF for which n = 30). (B) Time course of the effect of CRF on locomotor activity. Animals received an icv injection of a control solution (Saline) or of 25 ng CRF (n = 15). *: P ≤ 0.05, 2-tailed probability for comparison of control and corresponding CRF-treated groups (Mann-Whitney U-test).
Experiment II. Time-course study: icv injection of CRF

To determine the temporal relationship between an icv injection and the stimulation of locomotor activity, newts were injected with saline or 25 ng CRF and then were tested 5, 30, 180, and 360 minutes after the injection. Between tests, newts were returned to the temporary holding tanks.

An icv injection of CRF resulted in significant increases in locomotor activity compared to a saline injection. This effect was apparent starting 30 minutes and lasting for at least 360 minutes after injection (Fig.II.1B). Subsequent experiments measured locomotor activity starting 30 minutes after injection of CRF.

TABLE II.1. Locomotor activity in response to an ip injection of 0 or 200 µg naloxone

Newts were tested either 20 minutes or 50 minutes following treatment. Values represent median locomotor activity and interquartile range for each group. No statistically significant differences between the treatment groups were observed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median locomotor activity (crossings/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 min</td>
</tr>
<tr>
<td>No injection</td>
<td>2 (0–10)</td>
</tr>
<tr>
<td>Control injection</td>
<td>2 (0–8)</td>
</tr>
<tr>
<td>200 µg naloxone</td>
<td>2 (0–6)</td>
</tr>
</tbody>
</table>

Experiment III. Injection of naloxone

To investigate a possible involvement of opioids in amphibian spontaneous locomotor activity, newts were injected ip with 200 µg naloxone. Two control groups were included: one group (handled controls) consisted of newts that were handled but
not injected; the other group (saline-injected controls) consisted of newts that were handled and injected ip with amphibian Ringer's solution. Half of the newts in each treatment group were tested for locomotor activity at 20 minutes after the injection; the remaining newts were tested for locomotor activity at 50 minutes after the injection.

Results are presented in Table I. No statistical differences were observed between any of the 3 groups tested 20 minutes (T = .51; P > 0.75) or 50 minutes (T = 4.20; P > 0.10) after treatment.

Experiment IV. Sequential injections of naloxone and CRF: short interinjection interval

To determine if the stimulatory effects of CRF on locomotion are modulated by the opioid system, sequential injections of naloxone and CRF were administered to newts. The first injection was ip and consisted of 0, 40, or 200 μg naloxone. The second injection was icv and consisted of saline or 25 ng CRF. The interval between the injections was approximately 1 minute; testing was conducted 30 minutes after the second injection.

Results of the experiment are presented in Fig.II.2A. There were statistical differences among the four experimental groups (T = 22.17; P ≤ 0.0001). The three groups of newts which received an injection of CRF each moved more than the control group (Sal/Sal). Neither injection of saline nor injection of naloxone affected the CRF-induced stimulation of locomotor activity.
FIGURE II.2. Locomotor activity in response to an icv injection of CRF (25 ng) preceded by an ip injection of a control solution (Sal) or naloxone (Nal; 200 μg in B). The approximate time interval between injections was either 1 minute (A; n = 15) or 20 minutes (B; n = 15). See Fig.II.1 for additional comments. Different superscripts represent significant differences between treatment groups, 2-tailed probability ≤ 0.05 (Mann-Whitney U-test).

Experiment V. Sequential injections of naloxone and CRF: 20 minute interinjection interval

To insure that naloxone would reach its binding sites before CRF was administered, the interval between the injections was increased. As in Experiment IV, newts were given sequential injections of naloxone and CRF. In this experiment, however, the interval between injections was 20 minutes rather than 1 minute.

Results are illustrated in Fig.II.2B. There were significant differences between the three experimental groups (T = 17.05; P ≤ 0.0002). Newts injected with control
solution, then CRF (Sal/CRF), moved more than newts that did not receive CRF (Sal/Sal; P ≤ 0.05). Newts injected with 200 μg naloxone, then CRF, moved more than both control newts (Sal/Sal; P ≤ 0.0001) and newts treated with control solution and CRF (Sal/CRF; P ≤ 0.05). Therefore, the stimulatory effects of CRF were enhanced by prior treatment with naloxone.

**Experiment VI. Sequential injections of bremazocine and CRF**

To test if an exogenously administered opiate k-receptor agonist could alter the behavioral response to CRF, newts received 2 injections: the first injection was ip and consisted of saline or 10 μg bremazocine; the second injection was icv and consisted of saline or 25 ng CRF. A short interinjection interval (approximately 1 minute) was used to avoid possible complications resulting from the release of endogenous opioids associated with initial injection and/or handling of the animals. In addition, because in the previous experiment the effects of naloxone were observed 50 minutes after its injection, newts in this experiment were tested 50 minutes after treatment with bremazocine.

Results of the experiment are presented in Fig.II.3A. There was a significant treatment effect (T = 34.00; P ≤ 0.0001). Treatment with bremazocine followed by an icv injection of control solution decreased locomotor activity. An icv injection of CRF increased locomotor activity whether the injection was preceded by a control injection or by an injection of 10 μg bremazocine.
FIGURE II.3. Locomotor activity in response to icv injections of a control solution or CRF (25 ng) preceded by an ip injection of a control solution (Sal) or bremazocine (Bre; 10 µg) in A; or preceded by a control solution (Sal) or morphine (Mor; 10 µg) in B. The time interval between injections was approximately 1 minute. See Fig.II.1 for additional comments. Different superscripts represent significant differences between treatment groups, 2-tailed probability ≤ 0.05 (Mann-Whitney U-test).

Experiment VII. Sequential injections of morphine and CRF

To determine whether an exogenously administered opiate µ-receptor agonist could alter the behavioral response to CRF, Experiment VI was replicated using morphine (10 µg) instead of bremazocine. The experiment was conducted in two parts. Part I (n=15) and Part II (n=15) were identical but performed on separate days.

Results of the experiment are presented in Fig.II.3B. There was a significant treatment effect (T = 41.49; P ≤ 0.0001). Morphine had no effect on spontaneous
locomotor activity but reduced the behavioral stimulation induced by CRF (P < 0.05).

Experiment VIII. Sequential injections of morphine/naloxone and CRF

To determine if the reduction of CRF-induced locomotion caused by morphine was naloxone-reversible and therefore due to activation of an opiate receptor, three treatment groups were formed based on the nature of the initial injection. The initial injection consisted of either (1) a control solution, (2) morphine (10 µg) alone, or (3) a solution of morphine (10 µg) and naloxone (200 µg). In each case the initial injection was followed by an icv injection of 25 ng CRF. The experiment was conducted in two parts. Part I (n=15) and Part II (n=15) were identical but performed on subsequent days.

![Graph showing locomotor activity in response to icv injections of CRF preceded by ip injections of control solution (Sal), morphine (Mor; 10 µg) or a solution of morphine (Mor; 10 µg) and naloxone (Mor-Nal; 200 µg). The time interval between injections was approximately 1 minute. See Fig.II.1 for additional comments. Different superscripts represent differences between treatment groups, 2-tailed probability ≤ 0.05 (Mann-Whitney U-test).](attachment:image.png)
Results are presented in Fig.II.4. There was a significant treatment effect ($T = 6.92; P \leq 0.05$). As in the previous experiment, prior treatment with morphine reduced the behavioral stimulation induced by CRF ($P \leq 0.05$). This effect of morphine was not evident in the presence of the specific opiate receptor antagonist naloxone, suggesting that the suppressive effect of morphine was mediated by an opiate receptor.

**DISCUSSION**

Results of this study indicate that in rough-skinned newts, an icv injection of CRF stimulates locomotor activity in a dose and time-related pattern. Injection of doses ranging from 12.5 to 150 ng CRF increased locomotion in newts tested 30 minutes after treatment and administration of a dose of 25 ng of the peptide stimulated locomotor activity for up to 6 h. These results are consistent with previous results (Moore et al., 1984) and further characterize the stimulatory effects of CRF on locomotion in *T. granulosa*. They are also consistent with the effects of the peptide in rats tested in photocell cages (Sutton et al., 1982).

Our investigation of the interaction between CRF and the opioid system in the regulation of locomotion is the first to be performed on any nonmammalian vertebrate. The opioid receptor antagonist, naloxone, when given immediately before CRF, did not affect CRF-induced stimulation of locomotor activity in newts. This result would suggest that the stimulatory effects of CRF in the newt are not modulated by the opioid system. However, when naloxone was given 20 minutes before CRF,
naloxone enhanced the stimulatory effects of CRF on locomotor activity. This observation suggests that handling and/or injecting the newts with a control solution 20 minutes before injecting CRF stimulated the release of one or more endogenous opioids which then reduced CRF's potential to stimulate locomotion.

Indeed, opioids are released in response to a multitude of physical stressors in mammals, as reviewed in Olson, et al. (1986) and in Amir et al. (1980), and at least one study has demonstrated opioid involvement in stress-induced behavioral alterations in an amphibian (Pezalla and Dicig, 1984). Under the experimental conditions of this study, naloxone by itself did not affect spontaneous locomotor activity. The endogenous opioid substances which were apparently released in response to handling or injection may not have been present in sufficient amounts to affect spontaneous locomotion, yet they may have been present in sufficient amounts to affect CRF-induced stimulation of locomotion. Treatment with naloxone before treatment with CRF would under these circumstances negate the inhibitory effects of the endogenous opioids, allowing the full expression of CRF-induced stimulation of locomotor activity.

An interaction between CRF and naloxone in the regulation of locomotion has been demonstrated in rats (Saunders and Thornhill, 1986). In this species, naloxone given 10 minutes before an icv injection of CRF attenuates the stimulatory effect of the peptide (Saunders and Thornhill, 1986). This attenuation is consistent with other studies indicating that in rodents, naloxone administration can decrease locomotion (Amir et al., 1979; Arnsten and Segal, 1979; Ukai and Kameyama, 1985). In
contrast, naloxone treatment under some conditions increases locomotion in newts
(Deviche et al., 1989); this observation is consistent with the fact that in newts,
naloxone injection enhances the stimulatory influence of CRF on locomotion. Thus,
while in mammals opioids appear to enhance the effects of CRF (Saunders and
Thornhill, 1986), the opposite may be true in amphibians.

To study the mechanism involved in the interaction between CRF and the
opioid system in the newt, we examined the effects produced by the administration of
the opioid k-receptor agonist, bremazocine (Römer et al., 1980). Bremazocine
injection, when followed immediately by an icv injection of saline, decreased
spontaneous locomotor activity. Bremazocine did not, however, affect the stimulation
of locomotor activity induced by CRF. These results suggest that although opioid
k-receptors play a role in the regulation of amphibian locomotion, they do not
modulate the influence of CRF on this behavior.

A counterpart to the mammalian µ opioid receptor has been characterized in
urodele amphibians (Audigier et al., 1980). Based on experiments performed in the
present study, using morphine, activation of µ-type receptors may reduce the potential
for CRF to stimulate locomotor activity in the newt. This finding is interesting in
light of evidence which suggests that in mammals CRF-induced behavioral activation
may involve stimulation of norepinephrine-containing cells of the locus coeruleus
(Valentino et al., 1983; Velley et al., 1988), a brain nucleus in which both the opioid
k- and µ-receptor subtypes have been located (reviewed in Mansour et al., 1988).
Activation of the \( \mu \) receptor hyperpolarizes locus coeruleus cells (Pepper and Henderson, 1980) and decreases single cell activity (Abercrombie et al., 1988; Korf et al., 1974; North et al., 1987), while application of either \( k \)-type (McFadzean et al., 1987) or \( \delta \)-type (North et al., 1987) receptor agonists has no effect on single cell activity. Only opioid agonists selective for the \( \mu \)-receptor appear to be able to affect locus coeruleus cells directly and this effect is inhibitory.

The nature of the neurohormonal regulation of these norepinephrine-containing cells in the mammal corresponds to the neurohormonal regulation of locomotor behavior in this amphibian.

ACKNOWLEDGEMENTS

We thank Drs. J. Rivier, W. Vale, D. Römer, and Endo Pharmaceuticals for their generous gifts of materials used in these studies. We also thank Miles Orchinik and Cathy Propper for valuable criticism of the manuscript and Dr. Gary DeLander for assistance in experimental design. This research was supported in part by an NICHD grant, RO1 HD13508.
CHAPTER III: CORTICOTROPIN-RELEASING FACTOR (CRF) ANTAGONIST SUPPRESSES STRESS-INDUCED LOCOMOTOR ACTIVITY IN AN AMPHIBIAN

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ABSTRACT

Intracerebroventricular (icv) injections of corticotropin-releasing factor (CRF; 25 ng) given to male rough-skinned newts (*Taricha granulosa*) stimulated locomotor activity tested in a circular arena starting 35 min after the injection. The CRF receptor antagonist, α-helical CRF$_{9,41}$ (ahCRF; 250 or 500 ng), injected icv concurrently with CRF, blocked CRF-induced locomotor activity. In contrast, icv injection of ahCRF had no effect on spontaneous locomotor activity. Other studies examined the effect of ahCRF on the elevated locomotor activity that was observed when the animals were stressed (handled or placed in warm water). The CRF antagonist dose-dependently attenuated the response to either handling or warm stress tested 2 h after drug treatment. We also examined the effect of the $\alpha_2$-adrenergic agonist, clonidine, on spontaneous and CRF-induced locomotor activity. Clonidine injected icv dose-dependently suppressed spontaneous locomotor activity but not CRF-induced locomotor activity. These studies support the hypothesis that endogenous CRF is involved in mediating stress-induced locomotor activity and indicate that the effects of CRF on locomotor activity are independent of activation of the $\alpha_2$-adrenergic system.
INTRODUCTION

Since the identification and characterization of corticotropin-releasing factor (CRF; Vale et al., 1981), CRF-like immunoreactivity has been identified in representatives of each major vertebrate group (reviewed by Peter, 1986). Further, the neuroanatomical distribution of CRF-immunoreactive cell bodies and fibers appears to be very similar among vertebrates, including the distribution of CRF-containing neurons in extrahypothalamic areas (Peter, 1986). This widespread distribution of CRF in the central nervous system suggests that CRF, or CRF-like substances, may have additional functions besides that of controlling the hypothalamic-pituitary-adrenal axis.

There is evidence that in mammals CRF can stimulate the release of adrenocorticotropic hormone (ACTH) and $\beta$-endorphin from the anterior pituitary (Vale et al., 1981). In addition, the administration of CRF elicits electrophysiological (reviewed by Siggins et al., 1985), neurochemical (Dunn and Berridge, 1987; Kalivas et al., 1987), behavioral (Britton et al., 1982; Morley and Levine, 1982; Sirinathsinghji, 1987; Sirinathsinghji et al., 1983; Sutton et al., 1982; Veldhuis and De Wied, 1984), and autonomic (Brown et al., 1982) responses. These diverse effects of CRF have led to the hypothesis that CRF may initiate and coordinate a generalized response to stressful stimuli (Gold and Chrousos, 1985; Koob and Bloom, 1985).

In contrast to the many studies with mammals, few studies have investigated non-hypophysiotropic functions for CRF in nonmammalian vertebrates. However, in
the urodele amphibian, *T. granulosa*, the administration of CRF suppresses male sexual behavior and stimulates male locomotor activity (Moore and Miller, 1984; Moore et al., 1984; Lowry et al., 1990). The effects of CRF on locomotor activity in this species are dose-related and time dependent (Lowry et al., 1990), and appear to be centrally mediated since icv injections of CRF stimulate locomotor activity in hypophysectomized newts (Moore et al., 1984).

In mammals the CRF-receptor antagonist, α-helical CRF_9-41 (ahCRF; Rivier et al., 1984) suppresses stress-induced neuroendocrine (Rivier et al., 1986), autonomic (Brown et al., 1985) and behavioral (Berridge and Dunn 1987; Kalin et al., 1988; Krahn et al., 1986; Tazi et al., 1987a; Winslow et al., 1989) effects. These studies suggest a role for an endogenous CRF in regulating neuroendocrine, autonomic and behavioral responses to stressful stimuli in mammals. To examine whether an endogenous CRF-like substance has a role in regulating the behavioral response to stressful stimuli in a nonmammalian vertebrate, we investigated the effect of ahCRF on stress-induced locomotor activity in *T. granulosa*.

Several studies, electrophysiological (Valentino and Foote, 1988), neurochemical (Dunn and Berridge, 1987), and behavioral (Imaki et al., 1987; Cole and Koob, 1988; Ehlers et al., 1987; Butler et al., 1990), suggest that behavioral effects of CRF in mammals are mediated at least in part by an activation of norepinephrine-containing cells. The neurochemical mechanism by which CRF elicits behavioral effects in nonmammalian vertebrates is unknown. However, since the neuromodulatory control of CRF-induced locomotor activity in male newts is similar
to the neuromodulatory control of norepinephrine-containing cells in mammals; that is, both are suppressed by \( \mu \)-opioid receptor agonists (Abercrombie et al., 1988; Lowry et al., 1990; North and Williams, 1984) and unaffected by \( k \)-opioid receptor agonists (Lowry et al., 1990; McFadzean et al., 1987), we hypothesized that CRF induces locomotor activity in male newts by an activation of norepinephrine-containing cells. Since the single cell activity of norepinephrine-containing cells in mammals is suppressed by \( \alpha_2 \)-adrenergic agonists as well as \( \mu \)-opioid receptor agonists (Aghajanian and Wang, 1986), we investigated the effect of an \( \alpha_2 \)-adrenergic agonist on CRF-induced locomotor activity in male newts.

**GENERAL MATERIALS AND METHODS**

Adult male newts (*T. granulosa*; approximately 15 g in body weight) were collected from local freshwater ponds and transported to two large water-filled holding tanks. These tanks (85 cm in diameter with approximately 100 l dechlorinated tap water and an ambient temperature of 15 °C) and tanks used for testing were in a room with a controlled photoperiod (12 h light/12 h dark; lights on at 07:00 h).

Testing for locomotor activity was done in one of two types of circular testing tanks. These tanks contained either a small circular arena (inside diameter 10 cm; outside diameter 25 cm) or a large circular arena (inside diameter 70 cm; outside diameter 85 cm). Both arenas contained dechlorinated tap water (15 °C) to a depth of approximately 15 cm. Small arenas were marked with 8 equally-spaced radial lines, large arenas were marked with 16 radial lines. Small arenas were used in
experiments requiring testing of unhandled animals. Locomotor activity consisting of integrated swimming and walking movements was recorded as the number of lines crossed during a 3 min test period.

Experimental conditions were varied based on objectives of individual experiments. In experiments I and VII, experimental conditions were selected to optimize the behavioral response to exogenous CRF. These conditions, including the interval between treatment and placement in a testing arena (30 min), and the interval between placement and testing (5 min), were established in previous work (Lowry et al., 1990). In experiments II-V, experimental conditions were selected to optimize the behavioral effects of endogenous CRF. Two treatments were given in these experiments, an initial drug treatment followed by a stress treatment designed to cause the release of endogenous CRF. Since previous results (Lowry et al., 1990) suggest that as a result of endogenous opioid release, endogenous CRF would have no behavioral effect if released within 30 minutes of drug treatment, the interval between drug treatment and testing was increased to 2 hours. In addition since we were interested in the effects of both drug treatment and stress treatment in these experiments, animals were tested immediately following stress treatment.

Synthetic ovine CRF was supplied by Drs. J. Rivier and W. Vale (Salk Institute for Biological Studies, San Diego, CA); ahCRF and clonidine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). In experiments using ahCRF, drugs were dissolved in amphibian Ringer’s with 0.001 M HCl. In other experiments drugs were dissolved in amphibian Ringer’s alone. In each case solutions
were prepared the day of the experiment. For treatment, newts were selected randomly from large holding tanks and given intracerebroventricular (icv) injections through a 0.5 mm hole in the cranial midline at the junction of the parietal and frontal bones as described by Moore and Miller (1983). Solutions (2 μl) were injected over a 5 s period using a microsyringe with a tip diameter of approximately 50 μm.

Two-factor experiments with completely randomized designs (Experiments I, II, VII) were analyzed with a nonparametric two-factor analysis of variance (Scheirer, Ray, and Hare, 1984). Two-factor experiments with repeated measures on one factor (Experiments III, IV, V), were analyzed using the Friedman test for two or more observations per experimental unit (Marascuilo and McSweeney, 1977). Differences within levels of the blocking factors were determined using Kruskal-Wallis one-way analysis of variance (Siegel, 1956; Experiment III) or nonparametric monotonic trend analysis (Ferguson, 1965; Experiments IV, V). Experiment VI was analyzed with a Kruskal-Wallis one-way analysis of variance. When appropriate, nonparametric multiple comparisons were made using Dunn’s Test (Dunn, 1964). Two-tailed probabilities ≤ 0.05 were considered statistically significant.

**SPECIFIC METHODS AND RESULTS**

*Experiment I. CRF-antagonist: Effects on spontaneous and CRF-induced locomotor activity*

To test whether ahCRF would affect spontaneous locomotor activity, newts received a single icv injection of saline or one of two doses of the CRF antagonist (250 or 500 ng; n = 15). Treated newts were isolated for 30 min in temporary
holding tanks (25 cm in diameter) containing 5 l dechlorinated tap water, then placed in large testing arenas and tested 5 min after placement.

**FIGURE III.1.** Corticotropin-releasing factor (CRF; 25ng) stimulated locomotor activity when injected icv by itself (*P ≤ 0.001; Dunn’s Test) but had no effect when injected icv concurrently with ahCRF (CRF-A; 250 or 500 ng). Newts were tested in water-filled arenas 35 min after treatment (n = 15). Locomotor activity was quantified as the number of crossings during a 3 min test period and is expressed as the median value (+) interquartile range. +P ≤ 0.01, ++P ≤ 0.001, two-tailed probabilities for comparison of CRF-injected control and corresponding ahCRF-treated groups (Dunn’s Test).

To test whether ahCRF would affect CRF-induced locomotor activity, newts received either a single icv injection of CRF (25 ng) or a single icv injection of a combination of CRF (25 ng) and one of two doses of ahCRF (250 or 500 ng; n = 15). Newts were then handled and tested as described in the above paragraph. There was a significant interaction between CRF and the CRF antagonist (df = 2, H = 12.56, P ≤ 0.001). As shown in Fig.III.1, ahCRF decreased locomotor activity but only in CRF-injected animals.
Experiments II-III. CRF-antagonist: Effects on spontaneous and handling-induced locomotor activity

Experiment II. To test whether handling would cause an increase in locomotor activity and, pending an increase, to determine whether ahCRF would suppress handling-induced locomotor activity, icv injections of either saline or 250 ng ahCRF (n = 26) were given to newts. Following treatment, newts were placed individually in small testing arenas.

Two h after drug treatment newts from half of each treatment group were subjected to mild handling-stress, the remaining newts were left undisturbed. Handling consisted of grasping the newt lightly behind the forelimbs with the index and middle fingers and placing it in the palm of the opposite hand; light pressure was applied by the thumb on the neck of the newt during a 30 s period. This procedure was intended to restrain rather than to inflict pain by squeezing the newt. Time-matched pairs of handled and unhandled newts were tested simultaneously, immediately upon replacement of the handled newt in the small testing arena. Results are presented in Fig.III.2A. There was a significant effect of handling ($df = 1$, $H = 13.73$, $P \leq 0.001$). Subsequent comparison of unhandled and handled newts indicated that handling significantly increased locomotor activity in saline-injected newts ($z = 3.49$, $P \leq 0.01$) but not ahCRF-injected newts ($z = 1.82$, $P \geq 0.10$).

Experiment III. To determine whether the effect of ahCRF on handling-induced locomotor activity was dose-related, newts received an icv injection of 0, 250 or 500 ng ahCRF (n = 15). These groups were tested before and after handling, 2 h after
FIGURE III.2. Locomotor activity of male rough-skinned newts. Data shown are median values and interquartile ranges. See Fig.III.1 for description of locomotor activity. A: locomotor activity of unhandled and handled (30 s) newts 2 h after treatment with a control solution or ahCRF (250 ng; n = 13). *P ≤ 0.05, two-tailed probability for comparison of unhandled and corresponding handled groups (Dunn's Test). B: locomotor activity (2 h after drug treatment with 0, 250 or 500 ng ahCRF; n =15) of newts before and after a 30 s period of handling. Handling caused a significant increase in locomotor activity (df =1, $X^2 = 20.70$, P ≤ 0.001, Friedman Test for multiple observations per experimental unit). *P ≤ 0.05, two-tailed probability for comparison of saline-injected controls and corresponding ahCRF-treated groups (Dunn's Test).
drug treatment. Results are presented in Fig.III.2B. As in Experiment II, the handling procedure caused a significant increase in locomotor activity \((df = 1, X^2 = 20.70, P \leq 0.001)\). Analysis of the effects of drug treatment indicated that ahCRF affected locomotor activity in handled animals \((df = 2, H = 8.68, P \leq 0.05)\) but not in unhandled animals \((df = 2, H = 2.49, P \geq 0.25)\). Among the handled animals, only a dose of 250 ng ahCRF significantly decreased locomotor activity when compared with control animals \((z = 2.31, P \leq 0.05)\).

Experiments IV and V. CRF-antagonist: Effects on warm stress-induced locomotor activity

Experiment IV. To determine whether ahCRF would elicit a dose-dependent change in locomotor activity induced by an alternative form of stress, placement in a higher ambient temperature, newts received an icv injection of 0, 25, 125, or 250 ng ahCRF \((n = 15)\). Newts were then placed individually in small testing arenas \((15 \, ^\circ C)\) and tested 2 h after drug treatment. Each animal was tested twice, once at 15 \, ^\circ C and again after being transferred to a second small testing arena maintained at 28 \, ^\circ C. Animals were transferred by grasping the tip of the tail, a handling technique which by itself does not affect locomotor activity (data not shown). Results are presented in Table 1. Exposure to warm water significantly increased locomotor activity \((df = 1, X^2 = 36.30, P \leq 0.001)\). Although ahCRF had no effect on locomotor activity of unstressed animals, there was a significant nonparametric monotonic trend for higher doses of ahCRF to reduce warm stress-induced locomotor activity \((z = 1.96; P \leq 0.05)\).
TABLE III.1. Locomotor activity in response to ahCRF treatment in warm-stressed newts. Locomotor activity 2 h after drug treatment (0, 25, 125 or 250 ng ahCRF) either before (15 °C) or during (28 °C, Experiment IV; 30 °C, Experiment V) warm stress (n = 15). See Fig.III.1 for a description of locomotor activity. Data shown are median values and interquartile ranges (line crossings/3 min). Handling increased locomotor activity in both experiments, (Expt. IV, df = 1, $X^2 = 35.3$, $P < 0.001$; Expt. V, df = 1, $X^2 = 85.2$, $P < 0.001$). In addition, in both experiments there was a significant monotonic trend for higher doses of ahCRF to suppress stress-induced increases in locomotor activity (Expt. IV, $z = 1.96$, $P < 0.05$; Expt. V, $z = 2.14$, $P < 0.05$).

<table>
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<th>Increase</th>
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</thead>
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<td></td>
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<td>28°C</td>
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<tr>
<td>Experiment IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
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<td>24 (9-34)</td>
</tr>
<tr>
<td>25</td>
<td>6 (0-15)</td>
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</tr>
<tr>
<td>125</td>
<td>0 (0-4)</td>
<td>14 (8-40)</td>
</tr>
<tr>
<td>250</td>
<td>0 (0-9)</td>
<td>11 (1-31)</td>
</tr>
<tr>
<td></td>
<td>15°C</td>
<td>30°C</td>
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<tr>
<td>Experiment V</td>
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<td>250</td>
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</tbody>
</table>

Experiment V. To determine whether the ability of ahCRF to reduce warm stress-induced locomotor activity is dependent on the intensity of the warm stress, Experiment IV was replicated using a warm stress of 30 °C rather than 28 °C (n = 15). Results are presented in Table 1. Although increases in locomotor activity in response to warm stress were greater in this experiment, (df = 1, $X^2 = 85.2$, $P < 0.001$), there was again a significant nonparametric monotonic trend for ahCRF to reduce warm stress-induced locomotor activity ($z = 2.14$; $P < 0.05$). In other similar experiments (data not shown), higher doses of ahCRF (0.5, 1 and 5 μg) did
not significantly reduce the locomotor activity of unstressed animals or warm stress-induced locomotor activity.

**Experiment VI. Dose response study: icv injection of clonidine**

To determine whether the $\alpha_2$-adrenergic agonist, clonidine, would dose-dependently affect spontaneous locomotor activity, newts were given either an icv injection of saline (n=28) or 0.01, 0.1, 1, or 10 $\mu$g clonidine (n=15). Newts were isolated in temporary holding tanks for 30 min then placed in large testing arenas and tested 5 min after placement. Results are presented in Table III.2. There was a significant treatment effect ($df = 4, H = 20.44, P \leq 0.001$). A dose of 1 $\mu$g clonidine significantly suppressed spontaneous locomotor activity ($z = 3.37; P \leq 0.05$).

**TABLE III.2.** Locomotor activity in response to icv injections of clonidine. Locomotor activity tested 35 min after icv injection of saline (n = 28) or 0.01, 0.1, 1 or 10 ng clonidine (n = 15). See Fig.III.1 for a description of locomotor activity. Data shown are mean responses ± SEM (line crossings/3 min). *P ≤ 0.05, two-tailed probability for the comparisons between the control group and treatment groups (Dunn’s Test).

<table>
<thead>
<tr>
<th>Clonidine ((\mu)g)</th>
<th>Locomotor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.25 (1.26)</td>
</tr>
<tr>
<td>0.01</td>
<td>4.86 (1.67)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.80 (0.31)</td>
</tr>
<tr>
<td>1</td>
<td>0.20 (0.20)*</td>
</tr>
<tr>
<td>10</td>
<td>0.33 (0.13)</td>
</tr>
</tbody>
</table>
Experiment VII. Injection of clonidine and CRF

To determine whether clonidine would affect CRF-induced locomotor activity, newts were given icv injections of either saline, clonidine alone (10 or 100 ng), CRF alone (25 ng), or clonidine and CRF in combination (n = 26). Newts were handled and tested as in experiment VI. Results are presented in Fig.III.3. There was no interaction between clonidine and CRF ($df = 2, X^2 = 1.90, P \geq 0.30$), however, they had independent effects on locomotor activity (clonidine, $df = 2, X^2 = 6.06, P \leq 0.05$; CRF, $df = 1, X^2 = 49.20, P < 0.001$). CRF stimulated locomotor activity ($z = 3.15, P < 0.01$) while the higher dose of clonidine suppressed it ($z = 2.61; P \leq 0.05$).

![Graph showing locomotor activity](image)

FIGURE III.3. Locomotor activity of male rough-skinned newts tested 35 min after drug treatment with control solution, clonidine (10 or 100 ng), CRF (25 ng) or CRF and clonidine together (n = 26). Data shown are median values and interquartile ranges. See Fig.III.1 for a description of locomotor activity. *$P \leq 0.001$, two-tailed probability for comparison of CRF injected and saline-injected control groups, +$P \leq 0.05$, two-tailed probability for comparisons of clonidine-injected groups with corresponding control groups (Dunn's Test).
DISCUSSION

This study provides the first evidence that stress-induced increases in nonmammalian locomotor activity are mediated in part by CRF. Results from this study also indicate that the stimulatory effects of CRF and the sedative effects of clonidine are independent.

As described previously in mammals (Sutton et al., 1982; Veldhuis and De Wied, 1984) and the urodele amphibian, T. granulosa (Moore et al., 1984; Lowry et al., 1990), icv injections of CRF stimulate locomotor activity. In this study using male newts the CRF receptor antagonist (ahCRF), injected icv at doses that did not affect spontaneous locomotor activity, suppressed CRF-induced locomotor activity. Similar effects of ahCRF have been demonstrated in male rats (Britton et al., 1986b). Because studies with ahCRF have shown that it competes with CRF for binding at CRF receptors (Rivier et al., 1984), the effectiveness of this antagonist in these behavioral studies suggests that CRF receptors in mammals and amphibians have similar specificity.

Although CRF has profound effects on locomotor activity in mammals, only one study has demonstrated a suppression of stress-induced locomotor activity by ahCRF (Winslow et al, 1989). In the present study we examined the effects of ahCRF on locomotor activity induced by two different environmental stimuli, handling and exposure to warm ambient temperature. In each case, ahCRF significantly reduced stress-induced locomotor activity. This suggests that a CRF-like substance was released in response to environmental stress and contributed to the
observed increase in locomotor activity. This hypothesis is consistent with studies of mammals that indicate that following exposure to stress, CRF is released in specific brain areas associated with the central nervous system response to stress (Chappell et al., 1986).

The effects of ahCRF on stress-induced locomotor activity were dose related. It is interesting, however, that doses higher than 250 ng did not significantly reduce either handling stress-induced locomotor activity (0.5 μg, Experiment III) or warm stress-induced locomotor activity (0.5, 1 and 5 μg, data not shown). These observations are consistent with studies in mammals in which high doses of ahCRF are less effective (Berridge and Dunn, 1987) or ineffective (Kalin et al., 1988) in reversing stress-induced behaviors. One possible explanation for these observations is that ahCRF may have partial agonist activity (Winslow et al., 1989; Kalin et al., 1988).

Exposure to warm temperatures stimulates ectotherms to search for cooler locations (Reynolds and Casterlin, 1979). The involvement of CRF in this response had not been studied. Although ahCRF significantly reduced warm stress-induced locomotor activity in the newt, it is clear that ahCRF did not completely suppress this response. This may be due to an increased thermoregulatory response or to an increased nociceptive response during exposure to warm temperatures. Both thermoregulatory and nociceptive responses have been shown to be highly dependent on the activation of endogenous opioid systems in vertebrate and invertebrate species (Kavaliers and Hirst, 1986; Kavaliers, 1989), and many opioid substances can
stimulate locomotor activity (see Olson et al., 1987). Regardless, at least one component of the warm stress-induced increase in locomotor activity appears to be mediated by an endogenous CRF-like substance. The handling and warm stress experiments together suggest that a CRF-like substance may be a component of the behavioral response to stressful environmental stimuli in general.

One hypothesis for the mechanism of CRF-induced behavioral arousal in mammals involves activation of norepinephrine-containing cells (see introduction). These cells can be suppressed by activation of presynaptic $\alpha_2$-adrenergic receptors (Cedarbaum and Aghajanian, 1977) which can result in an inhibition of the release of norepinephrine from nerve terminals (reviewed by Langer, 1981). Although the $\alpha_2$-adrenergic system has been implicated in CRF-induced locomotor activity in rats by studies using adrenergic receptor antagonists (Imaki et al., 1987), no studies to date have examined the effect of $\alpha_2$-adrenergic agonists on CRF-induced locomotor activity. In our study we found that the selective $\alpha_2$-adrenergic agonist, clonidine, suppressed spontaneous but not CRF-induced locomotor activity. This suggests that CRF may stimulate locomotor activity independent of $\alpha_2$-adrenergic systems in T. granulosa, consistent with studies in rodents in which treatment with a selective noradrenergic neurotoxin, N-(2-cloroethyl)-N-ethyl-2-bromobenzyl amine hydrochloride (DSP-4), failed to alter CRF-induced increases in emotionality in an open field test (Bakke, Bogsnes and Murison, 1990) or CRF-induced decreases in exploratory behavior (Berridge and Dunn, 1989).
Results from experiments in this study suggest that the function of CRF as a regulator of stress-induced behavior has been conserved during vertebrate evolution. The neuroendocrine mechanism by which CRF induces these changes in *T. granulosa* remains unresolved.

ACKNOWLEDGMENTS

We thank Drs. J. Rivier and W. Vale for kindly providing CRF used in this study. We also thank Christie A. Lowry for reviewing early drafts of the manuscript. This research was supported in part by an NSF Grant No. BNS 8909173 awarded to F.L. Moore.
CHAPTER IV: CORTICOTROPIN-RELEASING FACTOR ENHANCES LOCOMOTION AND MEDULLARY NEURONAL FIRING IN AN AMPHIBIAN

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ABSTRACT

Corticotropin-releasing factor (CRF) administration has been shown to act centrally to enhance locomotion in rats and amphibians. The present study used an amphibian, the roughskin newt (*Taricha granulosa*) to characterize changes in medullary neuronal activity associated with CRF-induced walking and swimming in animals chronically implanted with fine-wire microelectrodes. Neuronal activity was recorded from the raphé and adjacent reticular region of the rostral medulla. Under baseline conditions most of the recorded neurons showed low to moderate amounts of neuronal activity during periods of immobility and pronounced increases in firing that were time-locked with episodes of walking. These neurons sometimes showed further increases in discharge during swimming. Injections of CRF but not saline into the lateral ventricle produced a rapidly-appearing increase in walking and pronounced changes (mostly increases) in firing rates of the medullary neurons. CRF produced diverse changes in patterns of firing in different neurons, but for these neurons as a group, the effects of CRF showed a close temporal association with the onset and expression of the peptide’s effect on locomotion. In neurons that were active exclusively during movement prior to CRF treatment, the post-CRF increase in firing was evident during episodes of walking; in other neurons that also were spontaneously active during immobility prior to CRF infusion, post-CRF activity changes were evident during immobility as well as during episodes of locomotion. Thus, a principal effect of CRF was to potentiate the level of locomotion-related medullary neuronal firing. Due to the route of administration CRF may have acted on multiple
central nervous system sites to enhance locomotion, but the results are consistent with neurophysiological effects involving medullary locomotion-regulating neurons.

INTRODUCTION

Corticotropin-releasing factor (CRF) has a wide range of functions associated with the initiation and coordination of neuroendocrine, autonomic, and behavioral responses to stressful stimuli (reviewed by Dunn and Berridge, 1990). A CRF-induced increase in locomotor activity has been studied in rats (Sutton et al., 1982; Veldhuis and De Wied, 1984; Diamant and De Wied, 1991; Saunders and Thornhill, 1986; Sherman and Kalin, 1987) and the urodele amphibian, Taricha granulosa (Moore et al., 1984; Lowry et al., 1990, 1991, 1993; Lowry and Moore, 1991). In rats and T. granulosa, CRF-induced increases in locomotor activity are dose-related, time-dependent (Sutton et al., 1982; Lowry et al., 1990), and persist in hypophysectomized animals (Eaves et al., 1985; Moore et al., 1984). In addition, CRF- and stress-induced locomotor activity are suppressed by the intracerebroventricular administration of α-helical CRF, a competitive CRF antagonist (Rivier et al., 1984; Britton et al., 1986b; Winslow et al., 1989; Lowry and Moore, 1991). These studies and others (Britton et al., 1985, 1986a) provide evidence that CRF can act at sites within the central nervous system to stimulate locomotion in diverse vertebrate species.

Studies using extracellular recording, lesion, as well as chemical and electrical excitation approaches have described multiple neuroanatomical sites involved in the initiation and expression of locomotion in vertebrates. These sites include the
preoptic basal forebrain, lateral hypothalamus, dorsomedial hypothalamus, the perifornical area, the zona incerta, the ventral tegmental area, the central gray, the dorsal and median raphe, and the area around the pedunculopontine nucleus (for references, see Sinnamon, 1990). Several of these sites share a common feature in that they have excitatory projections to the medial reticulospinal system (Orlovskii, 1970). The convergence of excitatory inputs regulating the initiation and expression of locomotion to the medial reticulospinal system, the involvement of raphe neuronal systems in the regulation of locomotion (Sinnamon, 1984; Jacobs and Fornal, 1993; Viana Di Prisco et al., 1994), and the identification of raphe structures as potential sites of action for CRF (Sharkey et al., 1989; Corley et al., 1990; Singh et al., 1992) led us to select the medial reticular and raphe regions as recording sites for an initial investigation of the neurophysiological correlates of CRF-induced locomotion.

Based on these previous studies our working hypothesis was that CRF stimulates locomotion through direct or indirect actions on rostral medullary locomotor control systems; this led to the prediction that CRF action would be reflected in locomotion-related firing of neurons in or near the raphe region. Thus, in the present study we investigated the effects of CRF on locomotion and behaviorally-correlated single unit activity of rostromedial medullary neurons. These results have been published in part in abstract form (Lowry et al., 1994).
METHODS

Subjects and surgical preparation

Adult male roughskin newts (T. granulosa) were collected in Benton County, Oregon, and held in the laboratory for several months prior to use under conditions described previously (Rose et al., 1993). Implant surgery was conducted with the newts anesthetized in 0.1% MS-222. A cannula guide consisting of a short length of polyethylene tubing plugged with a stainless steel insert was positioned in contact with the surface of the forebrain over a lateral ventricle. Recording electrodes consisted of twisted pairs of 50 μm Diamel-insulated nichrome wires with impedances of approximately 500 kOhms at 1000 Hz. Either one or two pairs of microwires were implanted on the rostral medullary surface at the midline or slightly lateral to the midline. The implant assembly was embedded in dental cement that was anchored by a single 0-90 stainless-steel screw placed in the skull. The microwire electrodes were glued together and extended from the implant for approximately 6 cm before terminating in an Amphenol micro-miniature connector strip.

Recording procedure

Following surgery animals were placed in the recording apparatus while still anesthetized. Brain activity was monitored to determine when animals had fully recovered from the anesthetic. Data collection was begun approximately 30 min to 1 h later. Recording was conducted with the newts in a well-water-filled rectangular plastic pan (6x34x42 cm). Activity from the microwires was led to a preamplifier
through Microdot Mininoise coaxial cables and amplified with a Grass P511 preamplifier with filters set at 100-1 kHz due to the relatively low frequency characteristics of single neuron spikes in this species. During recording a manually-activated pressure transducer was used to signal the onset and offset of walking or swimming and a continuous voice narrative was recorded to describe procedures and the animals' behavior. Data storage, off-line analysis and activity quantification procedures were similar to those previously explained (Rose, 1992), but a brief description follows. Each recording site typically yielded activity from several individual neurons, each of which was identified from tape recordings by means of a Bak dual-window, time-amplitude window discriminator. The properties of single neuron activity and effects of CRF infusions were analyzed from chart records of standard pulses that were electronically integrated (330 msec time constant) and were displayed on the polygraph together with the transducer signal indicating the presence or absence of walking or swimming.

In each experiment, a sample of activity was recorded from several minutes to one hour prior to infusion of CRF (50 ng in 1 µl amphibian Ringer’s solution) or vehicle into the lateral ventricle. In some experiments, the 30 ga. infusion needle was inserted and the solution immediately injected. In other experiments, the infusion needle was inserted, left in place for varying lengths of time and the infusion was accomplished remotely with a microsyringe without further handling of the newt. Leakage of the solutions from the injection needle was prevented by bracketing the volume of solution between small air bubbles. In separate experiments a cannula was
inserted without an infusion or a 1 μl volume of vehicle was injected 20 min prior to CRF as control procedures. At the end of each recording experiment, newts were deeply anesthetized in MS-222, and the brains removed. Cannula placements and recording electrode sites were histologically located in frozen sections stained with neutral red.

Statistics

Statistical analysis of behavior was performed from samples of behavior classified as immobility, walking, or swimming at 10 sec intervals beginning 5 min prior to a treatment and extending to 20 min afterward, yielding 150 time samples per animal (except for one newt where only 144 samples were available). The Change-Point Test for a Sequence of Binomial Variables (Siegel and Castellan, 1988) was used to determine if the amount of locomotion (walking or swimming) changed after CRF infusion or control procedures.

Changes in unit activity were evaluated by comparing the firing rate of each neuron (quantified from polygraph records of a raster-stepper driven by the spike discriminator) during comparable behavioral states before and after CRF or control treatments. In order for a unit’s activity to have been considered altered, second-by-second firing rates for a post-treatment sample of firing from the integrator records had to be uniformly higher or lower than an activity sample of comparable duration recorded just prior to saline or CRF infusion or just prior to cannula placement in the case where there was no infusion. For corroborative analysis, several units were analyzed using the Change-Point Test for Continuous Variables (Siegel and Castellan,
1988). For this test, the behavior-correlated firing rates were calculated for each 10 sec interval of the entire sampling period. The criterion for a sample of unit activity to be used in an analysis was that the corresponding episode of behavior had to be at least 2 sec in duration. The baseline for comparison with post-treatment firing rates was the mean firing rate for a given behavioral state during the 5 min period prior to a treatment. Conclusions about the behavioral and neurophysiological effects of treatment are based on trials where at least 20 min of post-treatment data were available for analysis.

RESULTS

Locations of recorded neurons

The results are based on recordings from 33 single units from a total of 9 sites in the rostral medulla (n=9 male T. granulosa). Locations of recording sites are illustrated in Fig.IV.1. Of these units, 28 were recorded in experiments where a single infusion was given (CRF, n=19; saline, n=9). Within these groups six units were recorded following remote infusion of CRF; five units were recorded following remote infusion of saline. The remaining units in these groups were recorded following a brief period during which the infusion needle was placed in the cannula guide and CRF or saline was infused while the animal was restrained. Two units were recorded in an experiment where remote infusion of saline was followed, 20 min later, by a remote infusion of CRF. Three units were recorded in an experiment where a cannula was placed with no infusion as an additional control procedure.
Unit firing patterns dependent on behavioral state

The principal distinguishing feature of these neurons under baseline conditions was the degree to which their activity was movement-dependent. The majority of the 33 units (73%) were tonically active to some degree in the absence of movement. The remaining units only fired occasionally, or did not fire at all, during immobility. Sixty-seven percent of the neurons in this study showed changes in firing that were associated with the transition to walking in the aquatic testing environment. These neurons showed an onset of firing (or for the tonically-active cells increased firing)
FIGURE IV.2. CRF-enhanced firing in a neuron with movement-related firing. A. Each tracing from top to bottom shows a 58 sec chart record of activity recorded from this neuron. The vertical deflections on the chart are integrated spike discriminator pulses showing the rate of single neuron firing (vertical calibration = 10 spikes/sec). The horizontal lines beneath the integrator records signify episodes of movement, as indicated. Before CRF infusion, this medullary neuron fired small bursts of spikes exclusively during movement. Records at 11 and 22 min after CRF, when locomotion was enhanced, show elevated firing during walking. B. Histograms illustrating changes in behavior and firing rate of this neuron over a 72 min period. Placement of the cannula was performed during the 2 min, 15 sec period immediately prior to CRF infusion (onset of CRF infusion indicated by arrowhead, 30 sec infusion period). The effect of CRF infusion on behavior and unit activity lasted approximately 40 min. C. Digitized spike waveform from the neuron (45 μV amplitude; 2.5 msec spike duration).
FIGURE IV.2

A

Pre CRF

Walking

CRF + 2 min

Head Movement
Backward Movement
Walking
Walking

CRF + 11 min

Walking
Walking

CRF + 22 min

Walking

CRF + 42 min

Walking

B

C

Swimming/Dr. Walking/10s Spikes/10s

Time (min)
during walking. This walking-related firing consisted of either a relatively steady or a randomly fluctuating rate of overall activity that was not temporally correlated with the stepping pattern. Twenty-seven percent of the neurons that showed increases in firing that were associated with the onset of walking displayed a further increase in activity during swimming (see units CRF8-2, CRF8-4, Fig.IV.4). There was no apparent pattern of movement-related unit activity based on the location of the recording electrode; the patterns recorded from electrodes in more lateral positions within the medulla were similar to those recorded from electrodes in midline positions.

Although in most cases the intensity of unit activity was dependent on the behavioral state, this firing showed no specificity for the types of movement patterns that were executed. For example, units that increased firing rates during periods of walking also increased firing rates during orientation movements of the head, shifts in body position, single limb movements and backward locomotion (see Fig.IV.2). In addition, we did not observe any cases of phasic firing (firing that was phase-locked with rhythmic locomotor activities). The chief distinction between firing associated with one type of movement or another was that the more pronounced and sustained movements (walking, swimming) were associated with the highest level of firing.

Sensory responsiveness of rostral medullary neurons

Although sensory responses were not systematically assessed, it was clear that the medullary neurons tended to fire in response to tactile stimuli applied to the face, feet or cloaca, as seen in immobilized newts (Rose et al., 1993). More generalized
FIGURE IV.3. Diverse patterns of unit activity change in five simultaneously-recorded rostromedial medullary neurons during CRF-induced locomotor activation. There was a significant change in movement-related firing by each of these neurons as well as a significant increase in walking by this newt following CRF infusion. The top five histograms show a 35 min sample of firing rate for each neuron. The bottom two histograms show sequential 10 sec samples of walking and swimming, respectively. The histograms of neuronal activity also illustrate transiently increased firing by units 1, 2 and 5 during a 60 sec period of handling (onset indicated by arrow) associated with placement of the infusion needle in the guide cannula 6.5 min after the beginning of each record. The onset of a 30 sec remote CRF infusion (without handling of the newt) is shown by the vertical line through all histograms. CRF infusion caused an increased locomotion-related firing rate (Unit 1) that mirrored the time course of the CRF effect on walking. Units 2 and 5 showed increased locomotion-related firing rates after the CRF infusion that were evident quickly and reached a maximum within 5 min as the behavioral effect of CRF became more evident, and then gradually showed decreased locomotion-related firing rates throughout the remainder of the recording period. Units 3 and 4 displayed no notable changes in firing rates during handling or immediately after CRF infusion, but their firing rates slowly decreased as the CRF-induced locomotion became more pronounced; these neurons eventually ceased firing altogether. Note the difference in ranges of the vertical axes indicating different dynamic ranges of firing in these units. Unit 1 was moderately active while Units 2 and 4 fired at low rates during periods of walking and immobility. Unit 3 displayed bursts of activity at the onset of movement and was only active during movement or sensory stimulation. Unit 5 fired primarily during walking but also fired at a low rate during periods of immobility. Scale: 1 msec, 20 µV (Units 2-5), 10 µV (Unit 1).
FIGURE IV.3

Unit 1

Unit 2

Unit 3

Unit 4

Unit 5

Swimming (sec)

Walking (sec)

Time (min)

Spikes/10 sec
stimulation associated with manual restraint of the newt during cannula insertion was monitored for 26 units. The majority of these neurons (69%) increased their firing rates substantially during the period of handling, even if the newt was not moving. Typically, unit activity returned to baseline levels immediately following the release of the animal, although the activity of some units appeared to be suppressed for several minutes following handling or other forms of sensory stimulation.

Effects of CRF administration on behavioral and unit activity

Corticotropin-releasing factor infusion increased the incidence of walking to a highly significant degree in five out of six CRF-injected newts [CRF2, P < 0.001; CRF3, P < 0.001; CRF4, P = 0.05, CRF5, Fig.IV.5, P < 0.001; CRF6, Fig.IV.3, P < 0.005; CRF7 did not respond, P > 0.05]. Visual inspection of behavioral records in the statistically significant cases indicated that an increase in walking behavior was clearly evident within 2-3 min of CRF infusion. Only one animal (CRF2) showed increased swimming, a behavior that was much less frequent than walking either before or after CRF administration; the effect on swimming behavior in this animal was delayed approximately 20 min and was much less pronounced than the effect on walking behavior. In the five newts showing increased wal activity of the 14 units studied following application of these control procedures (see Fig.IV.2).

Changes in unit activity during periods of immobility following CRF infusion

Examination of integrator records of firing rates for units with firing during periods of immobility (i.e. "spontaneous discharge") revealed that three neurons
FIGURE IV.4. Behavioral and single-unit activity before and after infusion of saline into the lateral ventricle, showing an absence of effect on neuronal firing or behavior. Episodes of walking and swimming are shown in the two bottom histograms. Insertion of the infusion needle into the cannula preceded the illustrated period of recording by 30 sec. Activity of four different neurons that were recorded simultaneously from a single site (Units 1-4) are illustrated in the top four histograms. The spike waveform for each of these units is shown at the right of these histograms. These neurons showed no significant changes in firing rates after saline infusion (indicated by the vertical line through all histograms). All these neurons were moderately active during periods of immobility and to varying degrees, more active during periods of locomotion. Units 2 and 4 displayed highest levels of firing during episodes of swimming. Calibration: 1 msec, 10 μV.
FIGURE IV.4

Unit 1

Unit 2

Unit 3

Unit 4

Salamander movements

Time (min)

Spikes/10 sec

Walking (sec)

Swimming (sec)
FIGURE IV.5. CRF-induced increases in firing rate in a medullary neuron that were independent of behavioral state. The top tracing in each 58 sec record shows integrated neuronal activity (vertical calibration = 10 spikes/sec), with horizontal lines beneath each integrator record signifying walking or swimming. Firing rates are illustrated for a brief period prior to CRF infusion (A) and at five successive times afterward (B-E). As was typical for newts in this study, bouts of locomotion were often characterized by an initial period of walking followed immediately by a transition to a period of swimming (A,B,D,E). Bouts of swimming were usually followed by periods of immobility (A,B,E). Prior to CRF infusion, this neuron was active during periods of immobility as well as locomotion, showing no reliable association between firing rate and movement. CRF infusion resulted in a significant increase in walking and concurrently elevated neuronal firing rate during immobility as well as locomotion, with a maximum effect at approximately 25-30 min.
FIGURE IV.5

A

Pre CRF

Walking

Swimming

B

CRF + 9 min

Walking

Swimming

C

CRF + 23 min

Walking

Swimming

D

CRF + 31 min

Walking

Swimming

E

CRF + 51 min

Walking

Swimming
showed increased tonic firing and three showed decreased tonic firing after CRF infusion. Latencies for changes in firing rates during immobility were determined for five neurons with the Change-Point Test for Continuous Variables. Three of these units showed increased discharge and two showed decreased discharge following CRF infusion with latencies ranging from 3 min, 10 sec to 13 min, 10 sec. The majority of these neurons showed changes in less than 4.5 min. For all 14 neurons recorded during control procedures (all of which showed some degree of spontaneous activity), quantified values of firing rates during periods of immobility were essentially identical before and after control procedures.

**DISCUSSION**

*Behavior-related properties of medullary neurons*

A conspicuous feature of these medullary neurons was the close association between their firing and episodes of movement. Although most cells exhibited some degree of firing during immobility, most showed a pronounced increase in activity that was associated with changes in movement intensity and duration but not specifically to movement type, a pattern similar to the movement-related firing of ventromedial midbrain neurons in hamsters (Rose, 1985). Thus, in this urodele amphibian as in cyclostomes (Grillner et al., 1988), teleost fish (Grillner et al., 1988; Mos and Roberts, 1994), and mammals that have been studied (Ross and Sinnamon, 1984; Millhorn, 1986), these medullary neurons show properties consistent with the modulatory control of central pattern generators, including the central pattern
generator for locomotion. Since this study is the first to record neuronal activity during behavior in a species showing both quadrupedal locomotion as well as swimming, which involves waves of trunk and tail flexion as in fish, it is noteworthy that the same neurons were active during both forms of movement.

**CRF effects on neuronal activity and behavior**

This study provides the first description of single neuron activity correlates of CRF-induced behavior in a nonmammalian vertebrate. In newts that displayed a pronounced increase in walking in response to CRF administration, the great majority of the recorded rostromedial medullary neurons displayed pronounced changes, usually increases, in movement-related firing. In contrast, in animals that failed to show a behavioral response to CRF or to control treatments, none of the recorded single units displayed altered neuronal activity. This close association between neural and behavioral effects of CRF was also seen in the timing of the peptide’s effects; behavioral and neurophysiological effects were comparably rapid, beginning in some cases within 3-4 min of CRF infusion.

The time course of CRF effects on neuronal activity was more complex than that on behavior. Neurons exhibited increases -- or in a few cases decreases -- in activity that spanned the time course of the CRF effect on behavior, but also showed changes involving initial increases in activity followed by decreases. Thus, while the overall neuronal effect of CRF corresponded with the behavioral effect, the peptide’s effect on individual neurons differed in form, probably due to sampling of heterogeneous neuron types with this recording technique. Of particular importance,
the diversity in the neurophysiological effects of CRF shows that these changes were not simply a reflection of the intensity of sensory feedback from more pronounced locomotion.

Our results showing predominantly increased neuronal activity following CRF infusion are similar to results from previous neurophysiological studies that investigated the effects of CRF on diverse brain regions of the rat central nervous system, including the preoptic region, hypothalamus, locus coeruleus, solitary tract complex, hippocampus, and cortex (Eberly et al., 1983; Siggins et al., 1985; Siggins, 1990; Valentino, 1990). The latency of the fastest electrophysiological effects observed in the present study (as early as 3-4 min) was similar to latencies of electrophysiological effects of direct administration of CRF to neural tissue in other vertebrates (Valentino, 1990). The presence of behavior-related neuronal activity properties allowed for detection of more diverse patterns of CRF action than would be possible with preparations that use brain slices or anesthetized animals. For example, some units which under baseline conditions displayed enhanced activity during episodes of walking displayed progressively less activity during CRF-induced locomotion and ultimately ceased activity altogether, illustrating that not all locomotion-related neuronal activity is enhanced by CRF administration (CRF6-3, CRF6-4, Fig.IV.3). Since CRF may alter -- and in some cases suppress -- a variety of sensory, integrative, and motor processes involved in the regulation of complex physiological and behavioral responses (reviewed by Dunn and Berridge, 1990), it is not surprising that we observed diverse neuronal responses in the present study.
In the present study one of six animals did not respond behaviorally or neurophysiologically to CRF administration (CRF7). This failure to respond could reflect a mechanical error in the administration of the peptide, or alternatively, the physiological condition of this animal may have altered the behavioral and neurophysiological responses to CRF. For example, the nonresponsive animal was handled for cannula placement approximately 30 min prior to CRF administration; in all other cases in the present study CRF was administered within four min of cannula placement. We have shown previously that exposure to a stressful stimulus 20 min but not 1 min prior to administration of CRF inhibits CRF-induced locomotion in a naloxone- reversible manner (Lowry et al., 1990). Thus, it is possible that during the initial handling associated with placement of the cannula in the nonresponsive animal the release of endogenous opiates may have suppressed the subsequent behavioral and neurophysiological responses to CRF administration.

**CRF actions in the central nervous system**

The present study focused on the raphe and paramedian region of the rostral medulla due to evidence for locomotion-controlling neurons in these regions. One characteristic of the raphe region that appears to be evolutionarily conserved across all vertebrate classes is the presence of a complex of serotonergic neuronal cell bodies. This complex of neuronal cell bodies provides a widespread serotonergic innervation of the central nervous system through ascending projections within the medial forebrain bundle (Steinbusch, 1981, 1984; Steinbusch and Nieuwenhuys, 1983; Parent 1981). Although most of the recording sites in this study were optimally positioned
to record from serotonergic raphé neurons (Yoshida et al., 1983; Ueda et al., 1984; Fasolo et al., 1986; Corio et al., 1992), we did not detect neurons firing with the highly regular, relatively slow patterns characteristic of mammalian serotonergic raphé neurons (Jacobs and Fornal, 1991).

Although it remains unresolved whether or not CRF administration altered neuronal activity of raphé serotonergic neurons, there is independent evidence that CRF-induced locomotion in *T. granulosa* is associated with a widespread activation of serotonergic neuronal systems. Intracerebroventricular injections of CRF dose-dependently enhance locomotion and alter serotonin or 5-hydroxyindoleacetic acid (5-HIAA; a serotonin metabolite) concentrations in the ventral forebrain and dorsomedial hypothalamus, as measured by microdissection and high performance liquid chromatography with electrochemical detection techniques (Lowry et al., 1991). In related studies, the specific serotonin reuptake inhibitor, fluoxetine (Fuller and Wong, 1977), enhanced locomotor activity in *T. granulosa* treated with a behaviorally subthreshold dose of CRF (but not in saline-treated animals); this effect was associated with a widespread depletion of serotonin throughout the central nervous system (Lowry et al., 1993). In rats, CRF infused into the lateral ventricles dose-dependently increases the activity of tryptophan hydroxylase, the rate-limiting enzyme for serotonin synthesis (Corley et al., 1990; Singh et al., 1992). These studies suggest that the central actions of CRF involve the activation of serotonergic neuronal systems.
There is evidence that serotonin facilitates locomotor activity in vertebrates by actions at caudal brainstem as well as spinal levels (Harris-Warrick and Cohen, 1985; Grillner et al., 1987, Cazalets et al., 1992). Of particular importance to this study is the evidence that serotonin potentiates firing of reticulospinal neurons in response to excitatory input (Viana Di Prisco et al., 1992). Activation of reticulospinal neurons is one means of initiating locomotion (Ross and Sinnamon, 1984; Grillner et al., 1988) and in the thoroughly-studied lamprey model, serotonin-containing neurons may synapse directly on locomotion-triggering reticulospinal neurons (Viana Di Prisco et al., 1994). In the present study with *T. granulosa* the increases in the maximal discharge rate of locomotion-related medullary reticular neurons are consistent with prior studies of serotonin effects on motor output (Grillner et al., 1987; Cazalets et al., 1992; Viana Di Prisco et al., 1992, 1994; Schotland and Grillner, 1993; Harris-Warrick and Cohen, 1985; Jacobs and Fornal, 1993). Further neurophysiological studies that directly manipulate serotonin action coupled with CRF administration will be required to establish the neurochemical processes underlying the neural effects of CRF.

Multiple neuroanatomical sites may be involved in the expression of CRF-induced locomotion. In *T. granulosa* CRF-induced locomotor activity is associated with site-specific changes in concentrations of neurotransmitters (or their metabolites) in the basal forebrain and the dorsomedial infundibular hypothalamus (Lowry et al., 1991; 1993). These sites, as well as the dorsal and median raphe nuclei and the medial reticulospinal system are important locations for the initiation and expression
of locomotion in vertebrates (Sandner et al., 1979; Di Scala et al., 1984; Ross and Sinnamon, 1984; Sinnamon, 1984; Brudzynski and Mogenson, 1985; Sinnamon, 1987; Swerdlow and Koob, 1987; Lammers et al., 1988; Marciello and Sinnamon, 1990). Multiple neuroanatomical levels of CRF regulation of locomotor activity would allow for greater refinement in the neurobehavioral control effected by this peptide.

**Summary and conclusions**

Infusions of CRF (but not saline) into the lateral ventricle stimulated a rapidly-appearing increase in walking and pronounced changes (mostly increases) in firing of rostromedial medullary neurons. In general, the neuronal effects of CRF showed a close temporal association with the onset and expression of the peptide’s effect on locomotion. In neurons that were active exclusively during movement prior to CRF treatment, the post-CRF increase in firing was evident during episodes of walking. In other neurons that also were spontaneously active during immobility prior to CRF infusion, post-CRF activity changes were evident during immobility as well as during episodes of locomotion. The results of the present study together with previous investigations of *T. granulosa* suggest that CRF acts centrally at multiple neuroanatomical sites to enhance the action of brainstem reticular and spinal locomotor-controlling regions, thereby producing more frequent and sustained episodes of locomotion.
ACKNOWLEDGEMENTS

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CHAPTER V: CATECHOLAMINES AND INDOLEAMINES IN THE CENTRAL NERVOUS SYSTEM OF A URODELE AMPHIBIAN: A MICROSURGERY STUDY WITH EMPHASIS ON THE DISTRIBUTION OF EPINEPHRINE

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ABSTRACT

Individual brain nuclei and regions of the central nervous system of adult male roughskin newts (*Taricha granulosa*) were microdissected and the concentrations of norepinephrine, epinephrine, 3,4-dihydroxyphenylacetic acid, dopamine, 5-hydroxyindoleacetic acid, and serotonin were determined using high performance liquid chromatography (HPLC) with electrochemical detection. The pattern of distribution of these catecholamines and indoleamines revealed many similarities between this urodele and other vertebrates. The highest concentrations of biogenic amines were observed in brainstem, hypothalamic, and basal forebrain structures; the lowest concentrations were observed in the internal granule layer of the olfactory bulb and pallial structures of the telencephalon. High concentrations of catecholamines and indoleamines were found in hypothalamic periventricular regions that are known to include cerebrospinal fluid-contacting, monoamine-containing neuronal cell bodies. The rostral diencephalon, which included the preoptic recess organ, had high concentrations of the primary catecholamines, norepinephrine and dopamine, and extremely high concentrations of the secondary catecholamine epinephrine. The dorsomedial infundibular hypothalamic region, which included the paraventricular organ, had high concentrations of dopamine and serotonin. The lateral infundibular hypothalamic region, which included the nucleus infundibularis dorsalis, had high concentrations of each of the biogenic amines. The results revealed unique patterns of distribution for each of the catecholamines and indoleamines studied, and provided evidence that regions of the hypothalamus that include cerebrospinal fluid-contacting,
monoamine-containing neuronal cell bodies are focal regions for the metabolism of multiple biogenic amines.

INTRODUCTION

Catecholamine and indoleamine systems have been visualized using the original Falck-Hillarp formaldehyde histofluorescence technique (Falck et al., 1962; Falck and Owman, 1965), or variations of this technique (De la Torre and Surgeon, 1976; Lorén et al., 1980; Schöler and Armstrong, 1982) in the central nervous system of several amphibians including *Xenopus laevis* (Goos and van Halewijn, 1968; Terlou and Ploemacher, 1973), *Rana esculenta* (Braak, 1970), *Rana temporaria* (Bartels, 1971; Parent, 1973; Prasada Rao and Hartwig, 1974), *Bufo poweri* (Chacko et al., 1974), *Rana pipiens* (Parent, 1975), *Ambystoma mexicanum* (Sims, 1977), *Necturus maculosus* (Dubé and Parent, 1982), and *Triturus alpestris* (Corio and Doerr-Schott, 1988; see also Lamas et al., 1988). Immunohistochemical techniques have been used to describe the distribution of catecholamine-synthesizing enzymes (Hökfelt et al., 1973a) in *Rana catesbeiana* (Yoshida et al., 1983; Carr et al., 1991), *Triturus cristatus carnifex* (Franzoni et al, 1986), *Rana ridibunda* and *Pleurodeles waltlii* (Smeets and González, 1990; González and Smeets, 1991, 1994; González et al., 1994a), *T. alpestris* (Corio et al., 1992), *X. laevis* (González and Smeets, 1993, 1994; González et al., 1993a, 1994a), *Siren lacertina* (González and Smeets, 1994), and *Typhlonectes compressicauda* (González et al., 1994b). Immunohistochemistry has also been used to describe the distribution of specific amines, dopamine, norepinephrine, and serotonin (5-hydroxytryptamine, 5-HT; Steinbusch et al., 1978;

The distribution of biogenic amines in these amphibians reveals many similarities to a generalized vertebrate pattern (reviewed by Parent, 1979; Parent, 1981; Parent, 1984; Parent et al., 1984; Parent, 1986; González and Smeets, 1994). Principle components of this pattern are ascending fiber systems that extend from monoaminergic cell bodies in brainstem nuclei. These components are evident in anuran and urodele amphibians with an ascending serotonergic system arising from columns of neurons situated along the raphe and mesencephalic reticular formation (Yoshida et al., 1983; Ueda et al., 1984a; Fasolo et al., 1986; Corio et al., 1992), an ascending dopaminergic system (presumed homologue of the mesostriatal and mesolimbocortical systems of mammals) arising from the ventromedial tegmentum of the rostral midbrain, rostral or dorsomedial to the root of the oculomotor nerve (Yoshida et al., 1983; Franzoni et al., 1986; Smeets and González, 1990; Carr et al., 1991; González and Smeets, 1991, 1994; González et al., 1993a,b), as well as ascending noradrenergic and adrenergic systems arising from lateral regions of the medulla oblongata (Franzoni et al., 1986; Yoshida et al., 1983; González et al., 1993b; González and Smeets, 1994). In addition, recent evidence suggests that anuran and urodele amphibians also may have an ascending noradrenergic system
homologous to the isthmocortical (locus coeruleus) system of other vertebrates (Dubé et al., 1990, González and Smeets, 1993, 1994). In general, these ascending monoaminergic systems appear to be more extensive and complex in birds and mammals than in fishes, amphibians, and reptiles (Parent et al., 1984).

Another aspect of the vertebrate pattern of monoamine distribution is the presence of monoaminergic systems, characterized immunohistochemically by the presence of monoamine-synthesizing enzymes and the monoamines themselves, that arise from neurons in the hypothalamus (for amphibians see Yoshida et al., 1983; Franzoni et al., 1986; González and Smeets, 1991, 1994; and González et al., 1993a; for a review of monoaminergic neuronal systems in the hypothalamus of mammals, see Everitt et al., 1992).

Studies of monoamine-containing systems in the hypothalamus reveal some apparent differences between nonmammalian and mammalian vertebrates. Associated with the hypothalamic monoaminergic systems in nonmammalian vertebrates, but not mammalian vertebrates, are bipolar monoamine-containing neurons with apical processes that contact the cerebrospinal fluid (CSF) (reviewed by Parent et al., 1984). In amphibians, immunohistochemical studies found immunoreactivities to dopamine (Smeets and González, 1990; González and Smeets, 1991, 1994; Corio et al., 1992; González et al., 1993a), norepinephrine (González and Smeets, 1993, 1994), and 5-HT (Sano et al., 1983; Shimizu et al., 1983; Yoshida et al., 1983; Ueda et al., 1984a; Fasolo et al., 1986; Corio et al., 1992) in these hypothalamic CSF-contacting neurons.
Conspicuously absent from the patterns of distribution of aminergic systems described above is a description of the distribution of epinephrine in the vertebrate central nervous system. Although immunohistochemistry has been used to detect epinephrine in the adrenal medulla (Verhofstad et al., 1980, 1983), this technique has not been used successfully to visualize epinephrine in the central nervous system (Hökfelt et al., 1980; Steinbusch and Tilders, 1987). The lack of success in immunohistochemical studies, as well as the relatively low sensitivity of the Falck-Hillarp formaldehyde histofluorescence technique for the detection of epinephrine (Falck et al., 1962), has limited our understanding of the regional distribution of epinephrine in amphibians. Information available on the regional distribution of epinephrine in urodele amphibians is limited to high performance liquid chromatography with electrochemical detection (HPLC-ED) studies which measured concentrations of epinephrine in major subdivisions of the brain (olfactory bulb, telencephalon, hypothalamus, and midbrain or brainstem) in *Cryptobranchus alleganiensis*, *Ambystoma tigrinum*, and *N. maculosus* (Cooney et al., 1985; Hamilton et al., 1987; Norris et al., 1992).

As indirect evidence for epinephrine-containing neuronal structures, immunohistochemical studies have used antibodies raised against the epinephrine-forming enzyme, phenylethanolamine N-methyltransferase (PNMT) to visualize PNMT-immunoreactive neuronal structures. Unfortunately, although immunohistochemical studies have proven successful for visualizing the distribution of PNMT-immunoreactive neuronal systems in mammals (Hökfelt et al., 1974, 1984a;
Howe et al., 1980; Ross et al., 1984; Foster et al., 1985; Ruggiero et al., 1985, 1986, 1992; Kitahama et al., 1985, 1986, 1988; Tillet, 1988; Iwamoto et al., 1989; Komori et al., 1990; Dormer et al., 1993), this approach has had limited success in nonmammalian vertebrates. Of the studies that have described the distribution of PNMT-immunoreactive structures in nonmammalian vertebrates (Yoshida et al., 1983; Steeves et al., 1987; Sas et al., 1990; Smeets and Jonker, 1990; Smeets and Steinbusch, 1990; González and Smeets, 1994), only the studies by Yoshida and colleagues of *R. catesbeiana* (1983) and by González and Smeets of *P. waltl*i (1994) describe the distribution of PNMT-immunoreactive structures in amphibians. These studies, however, focus on the distribution of medullary PNMT-immunoreactive neuronal cell bodies; the distribution of PNMT-immunoreactive fibers throughout the amphibian central nervous system has not been described. Furthermore, PNMT immunoreactivity and PNMT activity are not reliable indicators of the regional distribution of epinephrine in vertebrate brain (Fuller and Hemrick-Luecke, 1983; Sved, 1989).

Microdissection techniques combined with HPLC-ED lack the neuroanatomical resolution of histofluorescence and immunohistochemical techniques but complement them by providing valuable information in the form of independent, quantitative estimates of neurotransmitters or metabolites in specific brain areas (Brownstein and Palkovits, 1984).

The present study used microdissection and HPLC-ED procedures to determine the neuroanatomical distribution of specific catecholamines, indoleamines, and
selected metabolites in the brain of the male roughskin newt (*T. granulosa*), providing for the first time in any amphibian detailed quantitative information on the relative concentrations of monoamines in specific brain areas. This information complements previous histochemical and biochemical studies of the central nervous system in amphibians and is valuable for comparisons with studies which have used similar techniques to examine other vertebrate species. Our main objective was to determine, using quantitative measures, which brain areas are characterized by relatively high concentrations (and, conversely, which brain areas are characterized by relatively low concentrations) of specific biogenic amines. We focus attention on the regional distribution of epinephrine and the aminergic content of regions of the hypothalamus that include cerebrospinal fluid-contacting, monoamine-containing (CSF-MA) neuronal structures. These data have been presented in part in preliminary form (Lowry et al., 1991).

**MATERIALS AND METHODS**

The original research reported herein was performed in accordance with the U.S. Public Health Service’s *Guide to the Care and Use of Laboratory Animals*.

*Preparation of tissue for microdissection*

Adult male roughskin newts (*Taricha granulosa*) were collected in February, 1990 (Benton County, Oregon). For preparation of tissues animals were killed by rapid decapitation and each animal was rapidly dissected (1-2 min) to remove the intact brain and rostral spinal cord. These were then embedded in Tissue-Tek®
O.C.T. Compound, frozen on dry ice, and stored in airtight microcentrifuge tubes at -80 °C until sectioning. Serial 300 µm sections of frozen brain were cut at -12 °C in a cryostat. Sections were placed on gelatin-coated glass slides and collectively thaw-mounted by briefly warming the slide. Sections were refrozen within approximately 30 sec. Slides were sealed in slide boxes and stored at -80 °C.

**Preparation of microdissection atlas**

The microdissection atlas (Fig.V.1), was prepared using adult male *T. granulosa* (n=4) that were treated as above, with the following modifications. Intact heads were fixed for 24 h in modified Zamboni fixative (4% paraformaldehyde, 3% sucrose and 7.5% picric acid in 0.1 M sodium phosphate buffer (SPB), pH 7.4). After 24 h the intact brain and spinal cord was removed, washed 2x12 h in SPB, and stored in SPB containing 30% sucrose and 0.1% sodium azide. Tissue was sectioned (20 µm) and sections were thaw-mounted on gelatin-coated glass slides, immersed in SPB containing 4% paraformaldehyde and 1.5% sucrose for 5 min, and then stained using the Lillie modification of the Weigert method (Lillie, 1965). The atlas illustrated in Fig.V.1b is a series of camera lucida drawings prepared from sections from a single animal. Brain regions were identified using descriptions of the urodele amphibian telencephalon (Herrick, 1927; Northcutt and Kicliter, 1980), diencephalon and midbrain (Herrick, 1917), and hindbrain (Herrick, 1930), as well as the comprehensive study by Herrick (1948). The glyoxylic acid-induced histofluorescence technique (De la Torre and Surgeon, 1976; De la Torre, 1980) was used to verify the precise locations in *T. granulosa* of the preoptic recess organ (PRO;
Vigh-Teichmann et al., 1969), paraventricular organ (PVO; Vigh-Teichmann and Vigh, 1983; nucleus organi paraventricularis, Vigh et al., 1967; periventricular organ, Smeets and González, 1990), and nucleus infundibularis dorsalis (NID; Terlou and Ploemacher, 1973). The dissection of the PVO included the rostral region of this complex, the organon vasculosum hypothalami (Hanke, 1976).

Microdissection of discrete brain regions

Microdissection of individual brain regions (April, 1990; n=8 animals) was accomplished using the methods described for the microdissection of mammalian brain tissues (Palkovits, 1973; Palkovits and Brownstein, 1982) as previously applied in T. granulosa (Zoeller and Moore, 1986). Slides representing an entire brain were placed on a cold stage (Thermoelectric Cold Plate, TCP-2, Thermoelectrics Unlimited, Inc.) and maintained at -10 °C. Using a stereomicroscope (25X magnification), brain regions were identified using the ventricular systems and white matter and gray matter as landmarks. Tissue punches (300 μm i.d.) were expelled into 60 μl of acetate buffer (pH 5) containing 0.5 x 10⁻⁷ M internal standard, α-methyldopamine, and then stored at -80 °C until analyzed for monoamine content. If a brain region was damaged during preparation of the tissue for microdissection and landmarks could not be recognized, the region was not sampled.

Monoamine assay

Norepinephrine (NE), epinephrine (E), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (3,4-dihydroxyphenylethylamine; DA), 5-hydroxyindoleacetic
FIGURE V.1. Camera lucida drawings of brain tissues of an adult male *T. granulosa* illustrating diagrammatically the relationships between the intact brain and individual 300 μm sections which were prepared for microdissection. *Figure V.1A.* Camera lucida drawings illustrating dorsal *(above)* and lateral *(below)* views. The brain was illustrated while the meninges were intact and therefore melanocytes are visible as large irregularly-shaped dots on the diencephalic and brainstem surfaces. The reticular structure in the center of the caudal telencephalic lobes is the choroid plexus. The numbered scale indicates the section numbers that correspond to 300 μm transverse sections. *Figure V.1B.* Camera lucida drawings of transverse sections from the serially sectioned (20 μm) brain illustrated in *Figure V.1A* following removal of the meninges and the choroid plexus. The sections illustrated are approximately 300 μm apart. The dotted lines represent (except in the lower regions of sections 21 and 22 where they represent connective tissue associated with the pituitary) borders between gray matter and white matter which provide excellent landmarks for microdissection of tissues. Numerical labels indicate the section number corresponding to *Figure V.1A* followed by the section number in the series of 20 μm sections used for illustration. The location of microdissected punches (300 μm i.d.) is indicated on the right hand side of each section. Microdissection of all areas was bilateral except for the anterior preoptic area, rostral ventral hypothalamus, and the interpeduncular nucleus. Stippling within the microdissected regions represents relative concentrations of epinephrine in these regions *(light stippling, 10 pg/μg protein; medium stippling, 10-20 pg/μg protein; and solid, >20 pg/μg protein).* Scale bar = 1 mm.
FIGURE V.1B (Continued)
acid (5-HIAA), and serotonin (5-hydroxytryptamine; 5-HT) were analyzed using HPLC-ED. The method of analysis was based on a previously described procedure (Renner and Luine, 1984; Renner and Luine, 1986) with several modifications. The tissue samples were thawed and centrifuged at 15,000 x g for 2 min. The supernatant was treated with 2 µl of 0.02% ascorbate oxidase (Boehringer Mannheim) to minimize ascorbic acid contributions to the solvent front (McKay et al., 1984) and directly injected into a Waters chromatographic system (Waters Associates, Inc.). Chromatographic separation was accomplished using a C-18, 4 µm radial compression cartridge and a mobile phase consisting of (wt/vol), 0.84% sodium acetate, 1.2% citric acid, 0.015% octanesulfonic acid sodium salt (Eastman Kodak), 0.02% disodium EDTA, in (vol/vol) 15% methanol in water. Electrochemical detection was provided by a laboratory built potentiostat and a glassy carbon electrode (Bioanalytical Systems) set at +0.7 V with respect to an Ag/AgCl reference electrode. The tissue pellet was dissolved in 0.3 N NaOH and analyzed for protein content according to the method of Bradford (1976).

Calculations

The pg/cm peak heights of known concentrations of the standards were determined from the mean peak heights of 3 chromatograms for each respective standard. The α-methyldopamine internal standard was injected 3 times to determine the peak height for 100 % sample recovery. Amine concentrations were calculated from the standard values and corrected for % recovery and injection volume using a Waters 730 Data Module. The amine concentrations were divided by µg protein to
yield pg amine/µg protein. Outlier values identified using the Grubbs method were not included in reported values (two-tailed probability, $P \leq 0.05$; Grubbs and Beck, 1972).

RESULTS

Table I summarizes the concentrations of monoamines and monoamine metabolites in microdissected regions of the central nervous system of male $T.$ granulosa. Concentrations of norepinephrine were highest in microdissected areas in the preoptic-hypothalamic region, including the microdissected regions that contained the PRO and the NID, and in the brainstem. Norepinephrine concentrations were moderately high in the ventral striatum, amygdala pars medialis and amygdala pars lateralis of the basal forebrain, thalamus and habenula of the diencephalon, and the microdissected region that included the PVO. The lowest concentrations of norepinephrine were found in the internal granule layer of the olfactory bulb and pallial structures of the telencephalon.

Concentrations of epinephrine were highest in the microdissected regions that contained the PRO and the NID. Relatively high epinephrine concentrations were observed in the remaining areas of the diencephalon, amygdala complex, and brainstem. Epinephrine concentrations were lower in the remaining telencephalon, including the dorsal striatum, ventral striatum, and septal structures.

Table II shows the percentage of epinephrine (relative to the total amount of epinephrine and norepinephrine) in the major subdivisions of the brain. In general, norepinephrine concentrations exceeded epinephrine concentrations in the
telencephalon, but norepinephrine concentrations were exceeded by epinephrine concentrations in most diencephalic regions.

Concentrations of dopamine were highest in areas in the basal forebrain (striatum, septal structures, and amygdala complex), and in the preoptic-hypothalamic region (ventral hypothalamus and the microdissected regions that contained the PRO, the PVO, and the NID). All other regions measured had relatively low concentrations of dopamine.

The highest concentrations of 5-HT were observed in the raphe region of the brainstem (interpeduncular nucleus), and the microdissected regions that contained the PVO and the NID. High 5-HT concentrations also were observed in the striatum, the microdissected region that included the PRO, the tectum, and the ventral tegmentum. Moderately high 5-HT concentrations were observed in microdissected areas throughout the remaining basal forebrain, diencephalon, and brainstem. The lowest levels of 5-HT, as with the catecholamines, were found in the internal granule layer of the olfactory bulb and pallial structures of the telencephalon. In general, 5-HT concentrations were higher than catecholamine concentrations.

Concentrations of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were highest in microdissected tissues of the basal forebrain, diencephalon and hindbrain structures. The metabolite to monoamine ratio, [DOPAC]/[DA], which has often been interpreted as an index of utilization or turnover of dopamine, was lowest in areas with the highest concentrations of dopamine (Fig.V.2a). The lowest
TABLE V.1. Monoamine Levels and Monoamine Metabolite Levels* in Microdissected Brain Regions of *T. granulosa.* *Mean values ± S.E.M. expressed as pg/µg protein; bold-faced and italicized text are used to facilitate identification of the regions with the highest and lowest concentrations, respectively, for each of the amine neurotransmitters. Abbreviations: DA, dopamine, 3,4-dihydroxyphenylethylamine; DOPAC, 3,4-dihydroxyphenylacetic acid; E, epinephrine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin, 5-hydroxytryptamine; NE, norepinephrine.
<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>NE</th>
<th>E</th>
<th>DOPAC</th>
<th>DA</th>
<th>DOPAC/DA</th>
<th>5-HIAA</th>
<th>5-HT</th>
<th>5-HIAA/5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telencephalon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal Granule Layer, Medial</td>
<td>5.26 ± 0.91</td>
<td>1.47 ± 0.15</td>
<td>1.73 ± 0.10</td>
<td>3.36 ± 0.46</td>
<td>0.563 ± 0.056</td>
<td>4.12 ± 0.43</td>
<td>22.75 ± 1.98</td>
<td>0.191 ± 0.011</td>
</tr>
<tr>
<td>Internal Granule Layer, Lateral</td>
<td>3.18 ± 0.40</td>
<td>0.99 ± 0.17</td>
<td>1.20 ± 0.08</td>
<td>2.01 ± 0.13</td>
<td>0.627 ± 0.069</td>
<td>2.25 ± 0.26</td>
<td>9.10 ± 0.78</td>
<td>0.250 ± 0.019</td>
</tr>
<tr>
<td>Medial Pallium</td>
<td>4.19 ± 0.41</td>
<td>2.04 ± 0.10</td>
<td>2.11 ± 0.20</td>
<td>4.09 ± 0.55</td>
<td>0.450 ± 0.055</td>
<td>4.86 ± 0.38</td>
<td>22.64 ± 2.50</td>
<td>0.199 ± 0.016</td>
</tr>
<tr>
<td>Dorsal Pallium</td>
<td>3.52 ± 0.27</td>
<td>1.53 ± 0.12</td>
<td>1.81 ± 0.23</td>
<td>3.12 ± 0.26</td>
<td>0.576 ± 0.045</td>
<td>2.67 ± 0.29</td>
<td>12.05 ± 1.83</td>
<td>0.240 ± 0.031</td>
</tr>
<tr>
<td>Lateral Pallium</td>
<td>4.49 ± 0.42</td>
<td>2.18 ± 0.35</td>
<td>2.86 ± 0.33</td>
<td>5.44 ± 0.40</td>
<td>0.553 ± 0.093</td>
<td>3.60 ± 0.26</td>
<td>17.30 ± 3.17</td>
<td>0.256 ± 0.040</td>
</tr>
<tr>
<td>Dorsal Striatum</td>
<td>9.88 ± 1.70</td>
<td>3.62 ± 0.83</td>
<td>5.46 ± 1.84</td>
<td>14.55 ± 1.48</td>
<td>0.274 ± 0.074</td>
<td>5.44 ± 0.32</td>
<td>72.81 ± 8.38</td>
<td>0.083 ± 0.011</td>
</tr>
<tr>
<td>Ventral Striatum</td>
<td>13.08 ± 2.67</td>
<td>6.80 ± 1.02</td>
<td>3.41 ± 0.39</td>
<td>20.29 ± 1.72</td>
<td>0.177 ± 0.020</td>
<td>4.99 ± 0.37</td>
<td>68.36 ± 3.96</td>
<td>0.078 ± 0.008</td>
</tr>
<tr>
<td>Septum</td>
<td>7.79 ± 0.97</td>
<td>5.09 ± 1.07</td>
<td>4.22 ± 0.60</td>
<td>13.30 ± 1.42</td>
<td>0.332 ± 0.052</td>
<td>8.74 ± 1.09</td>
<td>53.84 ± 3.73</td>
<td>0.139 ± 0.012</td>
</tr>
<tr>
<td>Amygdala Pars Medialis</td>
<td>19.14 ± 4.14</td>
<td>22.83 ± 4.31</td>
<td>5.73 ± 1.44</td>
<td>17.04 ± 1.66</td>
<td>0.452 ± 0.083</td>
<td>10.28 ± 1.69</td>
<td>45.17 ± 4.89</td>
<td>0.208 ± 0.024</td>
</tr>
<tr>
<td>Amygdala Pars Lateralis</td>
<td>21.32 ± 3.52</td>
<td>14.62 ± 3.74</td>
<td>4.82 ± 0.76</td>
<td>12.14 ± 1.96</td>
<td>0.412 ± 0.060</td>
<td>8.10 ± 0.61</td>
<td>52.04 ± 6.41</td>
<td>0.145 ± 0.012</td>
</tr>
<tr>
<td>Diencéphalon</td>
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<td></td>
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<tr>
<td>Preoptic Recess Organ</td>
<td>37.44 ± 5.81</td>
<td>73.28 ± 10.93</td>
<td>9.03 ± 1.78</td>
<td>25.03 ± 3.15</td>
<td>0.339 ± 0.052</td>
<td>8.04 ± 0.95</td>
<td>59.82 ± 11.59</td>
<td>0.178 ± 0.019</td>
</tr>
<tr>
<td>Thalamus</td>
<td>16.93 ± 2.34</td>
<td>22.66 ± 3.07</td>
<td>4.53 ± 1.06</td>
<td>8.54 ± 1.59</td>
<td>0.525 ± 0.099</td>
<td>6.76 ± 0.76</td>
<td>52.17 ± 3.72</td>
<td>0.128 ± 0.016</td>
</tr>
<tr>
<td>Habenula</td>
<td>20.28 ± 5.75</td>
<td>12.94 ± 2.56</td>
<td>7.57 ± 2.06</td>
<td>5.72 ± 0.71</td>
<td>1.241 ± 0.209</td>
<td>9.96 ± 2.29</td>
<td>48.59 ± 12.88</td>
<td>0.184 ± 0.059</td>
</tr>
<tr>
<td>Ventral Hypothalamus</td>
<td>8.33 ± 1.33</td>
<td>13.30 ± 2.10</td>
<td>3.82 ± 0.93</td>
<td>11.92 ± 1.06</td>
<td>0.212 ± 0.025</td>
<td>5.56 ± 0.97</td>
<td>53.64 ± 6.90</td>
<td>0.114 ± 0.022</td>
</tr>
<tr>
<td>Nucleus Infundibularis Dorsalis</td>
<td>24.94 ± 2.91</td>
<td>39.77 ± 5.32</td>
<td>6.31 ± 0.78</td>
<td>29.26 ± 1.66</td>
<td>0.317 ± 0.090</td>
<td>12.62 ± 2.21</td>
<td>103.51 ± 16.52</td>
<td>0.130 ± 0.021</td>
</tr>
<tr>
<td>Paraventricular Organ</td>
<td>14.64 ± 1.78</td>
<td>20.20 ± 2.30</td>
<td>3.49 ± 0.63</td>
<td>28.30 ± 1.53</td>
<td>0.184 ± 0.043</td>
<td>5.05 ± 0.37</td>
<td>80.63 ± 8.30</td>
<td>0.065 ± 0.011</td>
</tr>
<tr>
<td>Region</td>
<td>Value 1 ± SD</td>
<td>Value 2 ± SD</td>
<td>Value 3 ± SD</td>
<td>Value 4 ± SD</td>
<td>Value 5 ± SD</td>
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<tr>
<td><strong>Midbrain</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tectum</td>
<td>6.69 ± 0.70</td>
<td>9.68 ± 1.27</td>
<td>1.67 ± 0.17</td>
<td>5.92 ± 0.26</td>
<td>0.330 ± 0.041</td>
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</tr>
<tr>
<td>Dorsal Tegmentum Mesencephali</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.43 ± 2.20</td>
<td></td>
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</tr>
<tr>
<td>Isthmic Tegmentum</td>
<td>9.06 ± 1.15</td>
<td>7.85 ± 0.75</td>
<td>3.59 ± 0.74</td>
<td>8.75 ± 0.33</td>
<td>0.418 ± 0.105</td>
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<td></td>
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<tr>
<td><strong>Hindbrain</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interpeduncular Nucleus (Raphe)</td>
<td>13.73 ± 2.26</td>
<td>11.96 ± 0.63</td>
<td>3.46 ± 0.64</td>
<td>6.33 ± 0.99</td>
<td>0.501 ± 0.101</td>
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</tr>
<tr>
<td>Ventral Tegmentum</td>
<td>24.77 ± 4.12</td>
<td>15.88 ± 0.95</td>
<td>4.16 ± 0.51</td>
<td>7.51 ± 0.74</td>
<td>0.553 ± 0.061</td>
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</tr>
<tr>
<td>Dorsal Tegmentum</td>
<td>15.41 ± 3.45</td>
<td>16.03 ± 2.88</td>
<td>5.39 ± 1.24</td>
<td>8.43 ± 0.88</td>
<td>0.528 ± 0.109</td>
<td></td>
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</tr>
</tbody>
</table>
TABLE V.2. Percent epinephrine relative to norepinephrine in major brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Epinephrine (% of Total)*</th>
<th># Microdissected Regions (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory bulb</td>
<td>22.79 ± 0.95</td>
<td>2</td>
</tr>
<tr>
<td>(Internal granule layer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pallium</td>
<td>31.91 ± 0.80</td>
<td>3</td>
</tr>
<tr>
<td>Subpallium</td>
<td>39.13 ± 0.45</td>
<td>5</td>
</tr>
<tr>
<td>Diencephalon</td>
<td>57.22 ± 3.88</td>
<td>6</td>
</tr>
<tr>
<td>Midbrain and Hindbrain</td>
<td>48.43 ± 3.29</td>
<td>5</td>
</tr>
</tbody>
</table>

*Values for percent methylated amine (concentration of epinephrine x 100/total concentration of epinephrine and norepinephrine) represent mean values ± S.E.M. using values from Table V.1.

[DOPAC]/[DA] ratios occurred in the ventral striatum and the microdissected region that included the PVO. Concentrations of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) were high in microdissected tissues of the septal structures, amygdala complex, diencephalon, dorsal tegmentum mesencephali, and ventral tegmentum. As with dopamine the metabolite to monoamine ratio, [5-HIAA]/[5-HT], was lowest in microdissected areas with the highest concentrations of 5-HT (Fig.V.2b). The lowest (5-HIAA)/(5-HT) ratios occurred in the raphe region and the microdissected region that included the PVO.
DISCUSSION

The present microdissection with HPLC-ED study reveals the neuroanatomical distribution of catecholamines and indoleamines in the central nervous system of an amphibian, *T. granulosa*. These data complement previous histofluorescence and immunohistochemical studies (reviewed by Parent et al., 1984; Parent, 1986; González and Smeets, 1994) by providing quantitative measures of biochemically identified monoamines and their metabolites in specific brain areas. The overall pattern in this amphibian resembles that of other vertebrates, including the most fundamental characteristic of monoaminergic systems, that is, a widespread distribution throughout the central nervous system (Parent, 1981, 1984; Parent et al., 1984). These monoaminergic systems are not necessarily diffuse and nonspecific but, instead, can be highly organized topographically (see Lindvall and Björklund, 1983; Cunningham and Sawchenko, 1988).

The technique used in the present study has a high level of sensitivity. We observed measurable levels of monoamines in all regions of the central nervous system studied.

**Regional distribution of norepinephrine**

Early biochemical studies using quantitative fluorometric techniques were unable to measure norepinephrine in amphibian brain tissues (*R. pipiens*, *Hyla cinerea*, *Bufo americanus*, *B. marinus*, *A. tigrinum*, and *N. maculosus*; Bogdanski et al., 1963). In contrast, in recent studies which used HPLC-ED techniques in urodele...
FIGURE V.2. The ratios of metabolites to monoamines in the brain of adult male *T. granulosa* were higher in regions of the brain with low concentrations of the parent amines. Mean values for each brain region were included. Coefficients of correlation: (DOPAC)/(DA) vs (DA), -0.7309, *P* ≤ 0.0005; (5-HIAA)/(5-HT) vs (5-HT), -0.7729, *P* ≤ 0.0001.
amphibians (*A. tigrinum*, Hamilton et al., 1987; Norris et al., 1992; *Ambystoma maculata*, *Amphiuma means*, *C. alleganiensis*, *N. maculosus*, *Plethodeon glutinosus*, and *S. lacertina* Cooney et al., 1985) norepinephrine was detected in whole brain homogenates or in each major region of the central nervous system analyzed (olfactory bulb, telencephalon, hypothalamus, and midbrain or brainstem). Our microdissection and HPLC-ED study expands these findings by quantifying norepinephrine in specific brain areas. These observations, together with immunohistochemical studies of *P. waltlii* and *X. laevis* (González and Smeets, 1993, 1994), suggest that noradrenergic systems are more extensive in some amphibians than previously recognized.

Early histofluorescence studies failed to detect catecholamine (norepinephrine) neuronal somata within the isthmic tegmentum of amphibians (Braak, 1970; Bartels, 1971; Parent, 1973; Terlou and Ploemacher, 1973; Chacko et al., 1974; Parent, 1975; Sims, 1977), or the presence of an isthmotelencephalic catecholaminergic pathway (Parent, 1975; Dubé and Parent, 1982). However, immunohistochemistry provided evidence for noradrenergic neuronal somata within the isthmic tegmentum of *X. laevis*, *R. ridibunda*, and *P. waltlii* (González and Smeets, 1991, 1993, 1994). These observations, together with previous descriptions of ascending projections from the isthmic tegmentum to the midbrain tectum and telencephalon (Wilczynski and Northcutt, 1977; Finkenstädt et al., 1983; Dubé et al., 1990; Lázár and Kozicz, 1990) led González and Smeets to label the noradrenergic cell mass in the isthmic tegmentum as the locus coeruleus. In the present study, the moderately high
norepinephrine concentrations in the thalamus, amygdala pars medialis and amygdala pars lateralis are consistent with biochemical and immunohistochemical observations in mammals in which the thalamic and amygdaloid structures receive moderately high norepinephrine innervation originating in the locus coeruleus (Kobayashi et al., 1974; Fallon et al., 1978; Lindvall and Björklund, 1978; Moore and Bloom, 1979); these observations are consistent with the hypothesis that amphibians have a norepinephrine cell body region with homology to the locus coeruleus of other vertebrates (González and Smeets, 1993; 1994).

**Regional distribution of epinephrine**

Our understanding of the regional distribution of epinephrine in the amphibian central nervous system is incomplete (see Introduction). However, in all studies that used either quantitative fluorometric techniques (*Bufo arenarum*, Segura and Biscardi, 1967; *R. temporaria*, Juorio, 1973) or HPLC-ED techniques (*R. catesbeiana*, Fuller and Hemrick-Luecke, 1983; *Scaphiopus holbrookii*, *Bufo terrestris*, *N. maculosus*, *C. alleganiensis*, Cooney et al., 1985; *T. granulosa*, present study) the hypothalamus contains higher epinephrine concentrations than the cerebral hemispheres, brainstem, or spinal cord. A similar pattern has been observed in several species of reptiles, birds, and mammals (Fuller and Hemrick-Luecke, 1983).

The distribution of epinephrine described in the present study is similar to the pattern of distribution described in microdissection studies of other vertebrates (Juorio and Vogt, 1967; Koslow and Schlumpf, 1974; Mefford et al., 1978; 1982; Brownstein and Palkovits, 1984), with the highest concentrations in microdissected
areas in the hypothalamus (preoptic-hypothalamic region in amphibians, see definition by Herrick, 1948; discussion by Peter, 1986), thalamus, habenula, and brainstem.

The neuroanatomical distribution of adrenergic neuronal systems can be inferred from immunohistochemical studies of the epinephrine-forming enzyme, PNMT. The PNMT-immunoreactive neurons in the mammalian systems are typically identified as two separate populations within the rostral medulla, the C1 neurons in the reticular formation of the ventrolateral medulla, and the C2 neurons in the dorsomedial medulla, including the periventricular region, medial aspects of the dorsal vagal motor nucleus, and portions of the medial and basal aspects of the nucleus of the solitary tract (Hökfelt et al., 1974, 1984a; Ruggiero et al., 1985; Iwamoto et al., 1989; Ruggiero et al., 1992; Dormer et al., 1993). A third group of PNMT-immunoreactive neurons have been described in the rostral midline raphe of some mammals, Rattus rattus, Sus scrofa, and canines (C3 Group; Howe et al., 1980; Ruggiero et al., 1992; Dormer et al., 1993), but not other mammals (Kitahama et al., 1985, 1986, 1988; Tillet, 1988). The distribution of PNMT-immunoreactive neuronal cell bodies in these mammals is comparable to the distribution of medullary PNMT-immunoreactive neurons in the lizard Gekko gecko, the turtle Pseudemys scripta elegans (Smeets and Jonker, 1990; Smeets and Steinbusch, 1990), and the white pekin duck Anas platyrhyncos (Steeves et al., 1987). There is considerable interspecies variation, however, in the precise location and number of groups of medullary PNMT-immunoreactive neurons (see discussion by Smeets and Jonker, 1990).
Among the Amphibia PNMT-immunoreactive neurons have been described in *R. catesbeiana* and *P. waltlii* (Yoshida et al., 1983; González and Smeets, 1994). The PNMT-immunoreactive neurons in the medulla oblongata of *R. catesbeiana* and *P. waltlii* are located in the nucleus of the solitary tract and the neighboring reticular formation (Yoshida et al., 1983; González and Smeets, 1994). It is likely but not certain that these brainstem neurons are a significant source of epinephrine throughout the amphibian central nervous system. The only reference to ascending PNMT-immunoreactive fibers in amphibians is the presence of a few weakly stained fibers which course through the ventral tegmentum to the basal forebrain in *P. waltlii* (González and Smeets, 1994). In addition, there is some debate regarding whether or not PNMT activity or PNMT-immunoreactive structures are well correlated with adrenergic systems (Fuller and Hemrick-Luecke, 1983; Sved, 1989). Although lesion studies indicate that the majority of hypothalamic epinephrine originates from within the central nervous system (*Rattus rattus*, Palkovits et al., 1980; Mefford et al., 1981), the mechanisms through which hypothalamic tissues in *T. granulosa* and other vertebrates accumulate high concentrations of epinephrine are uncertain.

**Relative concentrations of norepinephrine and epinephrine**

In the present study all diencephalic regions, except for the habenula, had higher epinephrine concentrations than norepinephrine concentrations. Conversely, within the rostral telencephalon including the internal granule layer of the olfactory bulb, pallial structures and the striatum, norepinephrine concentrations were higher than epinephrine concentrations. This pattern is similar to that observed in *N.*
maculosus, where norepinephrine concentrations exceeded epinephrine concentrations in the cerebral hemispheres but not the hypothalamus (Cooney et al., 1985). Based on the regions sampled in this study, the epinephrine concentration was less than 50% of the total (relative to the sum of epinephrine and norepinephrine concentrations) in T. granulosa (Table II).

Studies using quantitative fluorometric techniques on whole brain homogenates (R. pipiens, H. cinerea, B. americanus, Bufo marinus, A. tigrinum, and N. maculosus, Bogdanski et al., 1963) or homogenates of major regions of the central nervous system (B. arenarum, Segura and Biscardi, 1967; R. temporaria, Juorio, 1973) demonstrated that the ratio of epinephrine to norepinephrine is unusually high in amphibians (norepinephrine was not detectable, Bogdanski et al., 1963; or concentrations of epinephrine were approximately 80% of the total; Juorio, 1973; cf. other nonmammalian vertebrates, Juorio and Vogt, 1967; Juorio, 1973). In contrast, in mammals epinephrine concentrations are low compared to norepinephrine concentrations (approximately 5-15% of the total; Vogt, 1954; Gunne, 1962).

Based on the more recent study by Cooney and colleagues (1985) and the present study, there appears to be considerable variation among Amphibia with respect to the relative concentrations of epinephrine and norepinephrine in the central nervous system. In fact, in some urodele amphibians which belong to the suborders Cryptobranchoidea and Salamandroidea (Duellman and Trueb, 1986), norepinephrine can be considered to be the predominant amine relative to epinephrine (ie., T. granulosa, present study; C. alleganiensis, where epinephrine is 38.4% of total; A.
maculata, where epinephrine is 41.7% of total; A. means, where epinephrine is 46.6% of total, Cooney et al., 1985). Thus, although it remains a cogent generalization that in anuran amphibians epinephrine is the predominant amine relative to norepinephrine, this is not a cogent generalization for Amphibia.

The predominance of norepinephrine over epinephrine in the mammalian central nervous system is most extreme in the cerebral cortices (e.g. Mefford et al., 1982). Since cortical structures are a major target of the locus coeruleus (isthmocortical) noradrenergic pathways in vertebrates (i.e. the locus coeruleus gives rise to the norepinephrine innervation of the entire neocortex in the rat; for references see Lindvall and Björklund, 1978), it seems likely that, as suggested previously for the midbrain dopaminergic system (Parent et al., 1984), hypertrophy of the neocortex may account for the extensive development of the locus coeruleus complex in mammals. Conversely, lack of extensive cortical targets in amphibians might account in part for the difficulties encountered (reviewed by Parent, 1986; see discussions by González and Smeets, 1993, 1994) in the identification of noradrenergic isthmotelencephalic pathways in amphibians.

Regional distribution of dopamine

In T. granulosa dopamine concentrations were highest in microdissected areas of the striatum, septum and the amygdala complex in the basal forebrain, as well as in diencephalic regions which contained CSF-MA neuronal cell bodies. Dopamine concentrations were low in microdissected areas of the internal granule layer of the olfactory bulb and pallial structures.
In mammals, the neurons that comprise the majority of the ascending dopaminergic system are located ventrally at the mesodiencephalic junction and give rise to well defined mesostriatal, mesolimbocortical, and mesodiencephalic systems (reviewed by Parent, 1986; see Lindvall and Björklund, 1983). These nuclei -- A8 (the retrorubral nucleus), A9 (the substantia nigra), and A10 (the ventral tegmental area, a medial extension of the substantia nigra) -- extend rostrally toward the diencephalon from the level of the oculomotor nucleus (reviewed by Lindvall and Björklund, 1983). A similar but smaller complex of dopaminergic neurons in the ventromedial portion of the rostral midbrain tegmentum has been described in amphibians using histofluorescence (Parent, 1973; Sims, 1977; Dubé and Parent, 1982; reviewed by Parent, 1986) and immunohistochemical techniques (Yoshida et al., 1983; Franzoni et al., 1986; Smeets and González, 1990; Carr et al., 1991; González and Smeets, 1991, 1994; González et al., 1993a, 1994a). Although not described as such in all studies (i.e. Corio et al., 1992), and certainly not definitive (see discussion by González and Smeets, 1994; González et al., 1994a), dopaminergic neurons in the rostral ventral midbrain tegmentum of amphibians may represent a complex which is homologous to the A8, A9, A10 complex described in mammals.

In rats microdissection and HPLC-ED studies found that the highest concentrations of dopamine are observed in elements of the basal ganglia (olfactory tubercle, nucleus accumbens, caudate-putamen, ventral striatum, anterior amygdala area, and globus pallidus; Versteeg et al., 1976; Brownstein and Palkovits, 1984). In *T. granulosa* we observed high concentrations of dopamine within microdissected
regions of the basal forebrain (the dorsal striatum, ventral striatum, septal structures, and amygdala complex; Northcutt and Kicliter, 1980; nucleus accumbens, unpublished observations). These observations are consistent with the hypothesis that T. granulosa has functional mesostriatal and mesolimbic dopaminergic systems, however, more detailed topographical, hodological (as in, for example, González et al., 1993b), and neurochemical studies (see Fasolo et al., 1990) are necessary before direct comparisons can be made with a high degree of confidence between elements of the basal forebrain of this amphibian and elements of the basal ganglia of mammals or other vertebrates; (in particular, it should be noted that the use of the term ventral striatum in the present study does not imply homology with similarly labeled structures in amniotes). Interestingly, however, there are clear differences between our observations in T. granulosa and those in R. rattus described by Brownstein and Palkovits (1984). Most notably, although the concentrations of dopamine in the basal forebrain of T. granulosa were high relative to most regions of the central nervous system, the highest dopamine concentrations occurred in regions of the hypothalamus that contain CSF-MA neuronal cell bodies.

*Regional distribution of serotonin*

In T. granulosa 5-HT concentrations in the telencephalon were greater than catecholamine concentrations. This observation is consistent with studies of amphibians using quantitative fluorometric (Bogdanski et al., 1963), histofluorescence (Parent, 1975) and HPLC-ED methods (Hamilton et al., 1987; Norris et al., 1992). In fact, in T. granulosa all regions contained appreciable levels of 5-HT, with the
highest concentrations in the areas which, based on previous studies, include 5-HT-containing neurons (raphe region, PVO, NID). The pattern of 5-HT distribution is similar to that observed in other vertebrates (Saavedra, 1977; Gaudin-Chazal et al., 1979; Mefford et al., 1982; Brownstein and Palkovits, 1984) with high concentrations in the raphe region, as well as in regions of the hypothalamus, ventral forebrain, and brainstem. This pattern most likely represents a widespread innervation of the brain by 5-HT neurons in the raphe region of the brainstem, a neuronal complex which has been described as a major source of 5-HT innervation, via ascending projections within the medial forebrain bundle, in virtually all vertebrate species studied (reviewed by Parent et al., 1984).

*Preoptic-Hypothalamic CSF-MA Neuronal Systems*

The highest concentrations of monoamines in the brain of *T. granulosa* (excluding the serotonergic raphe system) were observed in microdissected samples from the preoptic-hypothalamic regions that contain CSF-MA perikarya in amphibians.

*Preoptic Recess Organ*

The microdissected region of the PRO contained the highest concentrations of norepinephrine and epinephrine compared to all other regions in the present study. The concentrations of dopamine in this region were also relatively high, exceeded only by the concentrations measured in the microdissected regions of the PVO and the
NID. In addition, the concentrations of 5-HT were moderately high in the region of the PRO compared to other regions of the central nervous system.

These observations are consistent with the description of CSF-MA neurons in the PRO of several amphibians including *A. mexicanum, Bombinator igneus, Bufo bufo, P. waltlii, R. esculenta*, and *R. ridibunda* (Vigh-Teichmann et al., 1969; see also Braak, 1970; Bartels, 1971; Parent, 1973; Terlou and Ploemacher, 1973; Chacko et al., 1974; Prasada Rao and Hartwig, 1974; Sims, 1977; Kikuyama et al., 1979; Dubé and Parent, 1982; Yoshida et al., 1983; Miyakawa et al., 1984; Ueda et al., 1984a; Franzoni et al., 1986; Corio and Doerr-Schott, 1988; Lamas et al., 1988; Carr et al., 1991; Corio et al., 1992; González and Smeets, 1994). These neurons are distributed around the entire surface of the preoptic recess, from the most rostral portion of the recess caudally to the margin of the neurosecretory preoptic nucleus (Vigh-Teichmann et al., 1969). Several studies have described a rostral extension of this nucleus with monoamine-containing neurons at the posterior and upper surface of the anterior commissure ("PRO-additional cells" of Chacko et al., 1974; see also Vigh-Teichmann et al., 1969; Dubé and Parent, 1982; Lamas et al., 1988).

A monoamine neuronal component of rostral preoptic area CSF-contacting neurons was not demonstrated in initial studies of fishes, reptiles, birds, or mammals (Vigh-Teichmann et al., 1969) using the formaldehyde-induced histofluorescence technique (Falck and Owman, 1965). More recent studies have, however, identified a potential monoaminergic preoptic recess organ in elasmobranch (*Scyliorhinus canicula* L., Wilson and Dodd, 1973), and teleost fishes (*Anguilla vulgaris*, Lefranc et al.,
1969), a few weakly fluorescent CSF-MA neurons in the preoptic recess area of the
turtle, *Chrysemys picta* (Parent and Poirier, 1971; Parent and Poitras, 1974), and
numerous tyrosine hydroxylase-immunoreactive, CSF-contacting neurons in the
preoptic recess area of the reptile, *Caiman crocodilus* (Brauth, 1988).

Based on the results of the present study, the possibility exists that regions
with high concentrations of epinephrine, especially the regions of the anterior preoptic
area and the caudal infundibular hypothalamus, may be important sites for the
synthesis, storage, transport or utilization of epinephrine in *T. granulosa* brain. In
comparison, based on measurements using gas chromatography-mass spectrometry
(Koslow and Schlumpf, 1974), radiometric methods (Van der Gugten et al., 1976), or
HPLC-ED techniques (Gereau et al., 1993), periventricular regions at the anterior
hypothalamic level and the infundibular hypothalamus of the rat contain several-fold
higher concentrations of epinephrine than other regions measured, suggesting these
regions may be important loci for the synthesis, storage, transport or utilization of
epinephrine in a mammalian species as well.

In addition to high concentrations of epinephrine in the microdissected region
that included the PRO, the concentrations of the primary catecholamines were also
relatively high. These observations are consistent with previous studies which have
identified CSF-MA neurons in the preoptic region using anti-tyrosine hydroxylase
antisera in *R. catesbeiana* (Nagatsu et al., 1982; Yoshida et al., 1983; Carr et al.,
1991), *R. ridibunda* and *P. waltlii* (González and Smeets, 1991, 1994), and *T.
alpestris* (Corio et al., 1992). Although these observations and the reserpine-sensitive
properties of CSF-contacting neurons in the preoptic recess organ are consistent with
the presence of catecholamines (Vigh-Teichmann et al., 1969), it was until recently
unclear which catecholamines were represented. Spectrofluorometric analysis in R.
temporaria (Prasada Rao and Hartwig, 1974) suggested that the catecholamine
localized in the cells of the PRO was dopamine, while the ability of repeated
treatments with DL-m-tyrosine to deplete the catecholamine content of these neurons
favored the presence of norepinephrine (Vigh-Teichmann et al., 1969). These initial
characterizations of the PRO are supported by the presence of dopamine-
immunoreactive, cerebrospinal fluid-contacting neurons within the PRO of T. alpestris
(Corio et al., 1992), R. ridibunda, P. waltlii, and X. laevis (González and Smeets,
1991, 1994), as well as norepinephrine-immunoreactive, dopamine-β-hydroxylase-
immunonegative cerebrospinal fluid-contacting neurons within the PRO of P. waltlii
(González and Smeets, 1994). In addition, immunohistochemical studies have
identified 5-HT in CSF-contacting cells in the PRO of R. catesbeiana (Yoshida et al.,
1983; Ueda et al., 1984a). These observations and the quantitative measurements in
the present study suggest that the periventricular anterior preoptic area of amphibians
is an important locus for the metabolism of biogenic amines.

Paraventricular Organ

The region containing the PVO had high concentrations of dopamine and 5-
HT in T. granulosa. Cerebrospinal fluid-contacting neurons in the hypothalamic PVO
contain dopamine, identified using immunohistochemical techniques, but lack
immunoreactivity for tyrosine hydroxylase in representatives of teleost fishes
including *Gasterosteus aculeatus* (Ekström et al., 1990), the electric mormyrid teleost, *Gnathonemus petersii* (Meek et al., 1989), and the electric gymnotiform teleost, *Apteronotus leptorhynchus* (Sas et al., 1990), in urodele and anuran amphibians (Smeets and González, 1990; González and Smeets, 1991, 1994; González et al., 1993a), the lizard *G. gecko* and the turtle, *P. scripta elegans* (Smeets and Steinbusch, 1990), and the bird, *Gallus domesticus* (Smeets and González, 1990). Studies in the anuran amphibian, *X. laevis* (González and Smeets, 1993) and the reptiles, *G. gecko*, and *P. scripta elegans* (Smeets and Steinbusch, 1989, 1990) have determined that these neurons also contain norepinephrine and are immunonegative for dopamine β-hydroxylase (DBH), the enzyme which converts dopamine to norepinephrine in noradrenergic neurons. It is uncertain if dopamine and norepinephrine immunoreactivities are located within the same cell bodies (González and Smeets, 1994).

In addition, histofluorescence studies (Terlou and Ploemacher, 1973; Chacko et al., 1974; Prasada Rao and Hartwig, 1974; Dubé and Parent, 1982; Corio and Doerr-Schott, 1988) and immunohistochemical studies (Sano et al., 1983; Shimizu et al., 1983; Yoshida et al., 1983; Ueda et al., 1984a; Fasolo et al., 1986) indicate the presence of 5-HT within CSF-MA PVO neurons. There does not, however, appear to be a good correspondence between immunoreactivities for 5-HT and tryptophan hydroxylase, the rate-limiting enzyme for biosynthesis of 5-HT, in this region of the hypothalamus. For example, in the chick embryo, numerous CSF-contacting 5-HT-containing neurons are located in the PVO, while the majority of tryptophan
hydroxylase-immunoreactive neurons are located lateral to the PVO. A much smaller number of tryptophan hydroxylase-immunoreactive neurons are located in the PVO, compared to 5-HT-immunoreactive neurons, suggesting that the presence of 5-HT-containing cells in the PVO is probably not due to local intraneuronal synthesis of 5-HT (Gabaldon et al., 1992; J.W. Wallace, personal communication).

Collectively, these studies indicate that CSF-contacting cells of the PVO in amphibians contain dopamine, noradrenaline, and 5-HT, yet do not contain immunoreactivity for enzymes normally required for biosynthesis of monoamines. These observations and the ability of these neurons to accumulate radiolabeled dopamine following intracerebroventricular injection in *Rana nigromaculata* and *R. catesbeiana* (Nakai et al., 1977), has led to the proposal that the CSF-contacting cells of the PVO accumulate catecholamines from the cerebrospinal fluid (*i.e.* dopamine, Nakai et al., 1977; Smeets and González, 1990; and norepinephrine, González and Smeets, 1993). This proposal is consistent with the observation that in reptiles the dopamine synthesis inhibitor α-methyl-p-tyrosine, which significantly reduces dopamine immunoreactivity in the dopaminergic cell bodies of the ventral midbrain tegmentum, has little effect on dopamine immunoreactivity in CSF-MA neurons of the PVO (Smeets et al., 1991).

Although discrete clusters of monoamine-accumulating, CSF-contacting neurons have not been described in mammals, it is clear that specific populations of non-monoaminergic neurons in the preoptic area and hypothalamus of mammals can also accumulate monoamines (Fuxe and Ungerstedt, 1968; Lidbrink et al., 1974;
Chan-Palay, 1977; Kent and Sladek, 1978; Beaudet and Descarries, 1979; Karasawa et al., 1994). It is also clear that monoamine-containing neurons project into the cerebral ventricles of normal animals (Richards et al., 1973; Aghajanian and Gallager, 1975; Chan-Palay, 1976). It is not clear, however, whether the monoamine-accumulating neurons that are located in the preoptic area and hypothalamus of mammals project to the third ventricle. In fact, evidence suggests that 5-HT-containing cerebrospinal fluid-contacting projections in mammals originate in the midbrain median and dorsal raphe nuclei (Aghajanian and Gallager, 1975).

Nonetheless, the conservation of these functional and structural features of monoamine systems, that is, monoamine-accumulating capabilities of non-monoaminergic preoptic area and hypothalamic neurons and CSF-contacting structural elements in all classes of vertebrate organisms suggests that these features may be important components of monoaminergic systems in the central nervous system (see Steinbusch and Niewenhuys, 1983). As suggested by Parent and colleagues (Parent, 1984; Parent et al., 1984) monoamine-containing neurons may provide an important link between the cerebrospinal fluid and neural tissues in all vertebrates.

**Nucleus Infundibularis Dorsalis**

Relatively high concentrations of norepinephrine, epinephrine, dopamine, and 5-HT were observed in the region of the NID (terminology of Terlou and Ploemacher, 1973) of *T. granulosa*. Microspectrofluorometric characteristics of this nucleus in *X. laevis* suggest that it has a similar monoaminergic composition as the PVO, with both dopamine and 5-HT present (Terlou and van Kooten, 1974). In
support of these observations, immunohistochemical studies in amphibians have identified dopamine (Corio et al., 1985; Corio et al., 1992) and 5-HT (Shimizu et al., 1983; Yoshida et al., 1983; Ueda et al., 1984a; Fasolo et al., 1986; Corio et al., 1992) in NID CSF-contacting neurons.

As with other hypothalamic CSF-MA neuronal systems in amphibians, it is uncertain whether or not NID CSF-contacting neurons contain epinephrine. However, the topographical position of NID neurons, in the caudal infundibular hypothalamus of amphibians, is strikingly similar to the distribution of a population of cerebrospinal fluid-contacting tyrosine hydroxylase-immunoreactive, PNMT-immunoreactive neuronal cell bodies adjacent to the caudal infundibular recess of a gymnotiform teleost fish, *A. leptorhynchus* (nucleus tuberis lateralis pars anterior; Sas et al., 1990), and a population of PNMT-immunoreactive neurons in the rat (Foster et al., 1985). The presumed hypothalamic adrenergic system of mammals is characterized by PNMT-immunoreactive neurons within regions of the dorsal and medial hypothalamus, as well as lateral, perifornical, zona incerta (Ross et al., 1984; Ruggiero et al., 1985), and caudal arcuate nuclei (Foster et al., 1985). It was suggested by Sas and colleagues (1990) that cerebrospinal fluid-contacting PNMT-immunoreactive neurons in the caudal infundibular hypothalamus of *A. leptorhynchus* may correspond to the PNMT-immunoreactive neurons in the caudal arcuate nucleus of the rat. These neurons in the rat, however, do not appear to contain other catecholamine-synthesizing enzymes, including tyrosine hydroxylase, aromatic L-amino acid decarboxylase (AADC), and DBH. Since the PNMT-immunoreactive
neurons described in the rat hypothalamus do not contain immunoreactivity for tyrosine hydroxylase, it was suggested that these neurons may accumulate norepinephrine from extracellular sources as a precursor for epinephrine synthesis (Ruggiero et al., 1985), a proposal which is consistent with a hypothalamic source of PNMT activity, independent of the medullary adrenergic cells (Brownstein et al., 1976; Mefford et al., 1981; Saavedra et al., 1983; Masana and Mefford, 1989). Furthermore, the possibility that these PNMT-immunoreactive neurons in the caudal infundibular hypothalamus of the rat may synthesize, store, transport, or utilize epinephrine is consistent with the observation that the arcuate nucleus is among the regions of the rat central nervous system with the highest tissue concentrations of epinephrine (Brownstein and Palkovits, 1984). In this context, further studies of parvocellular monoamine-accumulating neuronal systems in the caudal arcuate nucleus and ventral premammillary nucleus of the rat (see Fig.V.3), as well as potentially homologous neuronal systems associated with the mammillary recess in the caudal infundibular hypothalamus of nonmammalian vertebrates, promise to be rewarding in our effort to understand hypothalamic monoamine-metabolizing systems.

The observations outlined above and others (see discussion below) suggest that metabolism of multiple biogenic amines in the infundibular hypothalamus may be an evolutionarily conserved feature of vertebrate brain.
Do hypothalamic monoamine-containing neurons contribute to the pattern of monoamine distribution in extrahypothalamic regions?

Evidence suggests that hypothalamic monoamine-containing neuronal systems in anuran and urodele amphibians contribute to the innervation of the median eminence, intermediate pituitary (Terlou and Ploemacher, 1973; Prasada Rao and Hartwig, 1974; Aronsson and Enemar, 1981; Dubé and Parent, 1982; Tuinhof et al., 1993; see also Artero et al., 1994; Tuinhof et al., 1994) and the larval pars distalis (Aronsson and Enemar, 1981). Although in the urodele amphibian, *T. cristatus*, retrograde labeling studies suggest that the region containing the PVO neurons provides a major projection to the striatum (Dubé et al., 1990), the full extent to which these neurons contribute to the pattern of monoamine distribution in hypothalamic and extrahypothalamic areas of the amphibian central nervous system is uncertain. Likewise, with the exception of the well-characterized diencephalospinal projection of mammalian (*R. rattus*) A11 dopaminergic neurons (Skagerberg and Lindvall, 1985), the extent to which the hypothalamic dopaminergic (tyrosine hydroxylase-immunoreactive, DBH-immunonegative) neuronal systems innervate extrahypothalamic regions is uncertain in amphibians (Yoshida et al., 1983; González and Smeets, 1994) and in mammals (reviewed by Everitt et al., 1992).

The infundibular hypothalamus as a focal region for metabolism of biogenic amines

Our observation that the highest concentrations of norepinephrine, epinephrine, dopamine, and 5-HT (excluding the serotonergic raphe system) occur in periventricular regions of the diencephalon with CSF-MA neurons indicates that these
regions are important loci for metabolism of multiple biogenic amines. Based on previous studies in mammalian and nonmammalian vertebrates, such a complex of amine-metabolizing structures within the infundibular hypothalamus may be an evolutionarily conserved trait of the vertebrate brain (see Fig.V.3).

Of particular interest are the striking similarities between amine-metabolizing structures in the nonmammalian PVO and the mammalian dorsomedial hypothalamic nucleus (DMN). These structures share a similar topographical position in the periventricular region of the dorsal infundibular hypothalamus, well-removed caudally from the hypothalamic neurosecretory nuclei (Vigh et al., 1967). In addition, these regions are each characterized by discrete populations of monoamine-containing or monoamine-accumulating neuronal cell bodies (for references see previous discussion of PVO).

The presence of 5-HT-accumulating neurons in the DMN of the rat is supported by studies in which 5-HT is visualized immunohistochemically following systemic pretreatment with a monoamine oxidase inhibitor, either pargyline or nialamide, and the precursors of 5-HT synthesis, either L-tryptophan (Frankfurt et al., 1981; Steinbusch et al., 1982; Frankfurt and Azmitia, 1983; Steinbusch and Nieuwenhuys, 1983; Sakamoto et al., 1984; Steinbusch, 1984; Arezki et al., 1985; Ugrumov et al., 1988, 1989) or 5-hydroxytryptophan (5-HTP; Sakamoto et al., 1984; Staines et al., 1986; Ugrumov et al., 1989; also in the cat, Denoyer et al., 1989; Sakamoto et al., 1984), or by high dosages of monoamine oxidase inhibitor alone (Ueda et al., 1984b; Ugrumov et al., 1989). Alternatively, following treatment of
rats with the 5-HT neurotoxin, 5,7-dihydroxytryptamine, which is resistant to metabolism by monoamine oxidase, these neuronal cell bodies were visualized using a monoclonal antibody to 5-HT which cross-reacts with the 5,7-dihydroxytryptamine antigen (Howe et al., 1982). The presence of 5-HT-accumulating neurons in the rat DMN is further supported by studies in which serotonin binding protein -- a protein that is believed to be an intrinsic and a specific component of serotonergic neurons -- is visualized immunohistochemically within DMN neuronal cell bodies in the absence of the pharmacological treatments necessary for the visualization of the same neurons using anti-5-HT antisera (Kirchgessner et al., 1988).

Details about the mechanisms through which DMN neurons accumulate 5-HT remain unclear, however, the ability of pretreatment with the selective 5-HT uptake inhibitor, fluoxetine, to prevent the visualization of 5-HT immunoreactivity following treatment with pargyline and L-tryptophan suggests that the 5-HT immunostaining is a result of specific uptake of 5-HTP or 5-HT from extracellular fluids rather than intraneuronal synthesis from the precursor L-tryptophan (Ugrumov et al., 1988, 1989).

Congruent with the ability of neuronal cell bodies in the mammalian DMN to accumulate 5-HT following administration of the 5-HT precursors, L-tryptophan or 5-HTP, as well as to exhibit catecholamine histofluorescence or dopamine immunoreactivity following administration of the precursor of dopamine synthesis, L-3,4-dihydroxyphenylalanine (L-DOPA; Lidbrink et al., 1974; Karasawa et al., 1994), neuronal cell bodies of the adult X. laevis PVO accumulate both radiolabeled 5-HTP
and L-DOPA (Peute and van Oordt, 1974), indicating that neuronal cell populations within the dorsomedial hypothalamus of these representatives of Mammalia and Amphibia may have similar functional characteristics.

There are similarities in the distribution of 5-HT-synthesizing enzymes associated with the mammalian DMN and nonmammalian PVO. There are laterally displaced tryptophan hydroxylase-immunoreactive neurons within the rostral DMN (Weissmann et al., 1987), an observation which is consistent with the ability of hypothalamic cell cultures from 16-day-old rat fetuses to synthesize 5-HT from tryptophan (Becquet et al., 1990), and the observation that surgical isolation of the medial basal hypothalamus in adult rats does not eliminate either tryptophan hydroxylase or 5-HT content of the DMN (Brownstein et al., 1976). This arrangement in the rat with presumed 5-HT-accumulating neurons located medially within the DMN and tryptophan hydroxylase-immunoreactive neurons located laterally within the DMN is very similar to the arrangement in the PVO of the chick embryo (see previous discussion of PVO).

There are similarities in the distribution of catecholamine-synthesizing enzymes associated with the mammalian DMN and nonmammalian PVO. The CSF-MA neurons of the nonmammalian PVO are immunonegative for catecholamine-synthesizing enzymes including tyrosine hydroxylase and DBH, but are clearly associated with laterally displaced tyrosine hydroxylase-immunoreactive neurons (PVO-accompanying cells in amphibians; Smeets and González, 1990; González and Smeets, 1991, 1993, 1994; González et al., 1993a; presumed homologue of the
FIGURE V.3. Schematic representation of multiple biogenic amine-metabolizing systems in the mammalian (above) and urodele amphibian (below) infundibular hypothalami based on histochemical studies.

**Mammalian hypothalamus.** Filled circles, dopaminergic cell group of the arcuate nucleus (A12) (Dahlström and Fuxe, 1964) and the dorsal hypothalamic dopaminergic cell group (A13) (Fuxe et al., 1969); open circles, serotonin binding protein-immunoreactive (Kirchgessner et al., 1988), 5-HT-accumulating cells within the DMN (Fuxe and Ungerstedt, 1968; Chan-Palay, 1977; Beaudet and Descarries, 1979; Frankfurt et al., 1981; Howe et al., 1982; Steinbusch et al., 1982; Frankfurt and Azmitia, 1983; Steinbusch and Nieuwenhuys, 1983; Sakamoto et al., 1984; Steinbusch, 1984; Ueda et al., 1984b; Staines et al., 1986; Ugrumov et al., 1986, 1988, 1989; Denoyer et al., 1989). These cells are associated with catecholamine-accumulating cells (Lidbrink et al., 1974; Karasawa et al., 1994). These 5-HT-and/or catecholamine-accumulating neurons in the DMN are associated with and may correspond to tyrosine hydroxylase-immunonegative, aromatic L-amino acid decarboxylase- (AADC)-immunoreactive neurons in the periventricular part of the DMN (Group D12) described by Jaeger and colleagues (1984); filled circles with ventricular contact, bipolar subependymal catecholamine-containing cells (Sladek, 1978; Knigge et al., 1980); asterisks, phenylethanolamine N-methyltransferase (PNMT)-immunoreactive perikarya within the lateral and perifornical nuclei, zona incerta, and dorsal and medial hypothalamus (Ruggiero et al., 1985; PNMT-immunoreactive perikarya in the caudal arcuate nucleus are not illustrated, Foster et al., 1985). Within the infundibular hypothalamus, caudal to the level illustrated, are parvocellular 5-HT-accumulating neurons which do not follow classical neuroanatomical boundaries but are, instead, scattered around the mammillary recess in the dorsocaudal extreme of the arcuate nucleus, extending into the region around the premammillary nuclei (Chan-Palay, 1977; Maeda et al., 1984; Sakamoto et al., 1984; tuberal and ventral subdivisions of the tuberomammillary nucleus according to Staines et al., 1986), which may correspond to parvocellular AADC-immunoreactive neurons (Group D9) described in this region by Jaeger and colleagues (1984). These 5-HT-accumulating neurons are adjacent to a group of medium-sized catecholamine-accumulating neurons within the ventral premammillary nucleus, extending dorsally into the dorsal premammillary nucleus (Odake, 1967; Björklund et al., 1968; Fuxe and Ungerstedt, 1968, Loizou, 1971; Hökfelt and Fuxe, 1972; Lidbrink et al., 1974), which may correspond to medium-sized AADC-immunoreactive neurons (Group D8) described in this region by Jaeger and colleagues (1984; see also Karasawa et al., 1994). Also at a level caudal to that illustrated, norepinephrine-containing cell bodies are intermingled with dopamine-containing cell bodies in the caudal dopaminergic cell group (A11; Dahlström and Fuxe, 1964; Björklund and Nobin, 1973). Topography of neuroanatomical structures is based on the atlas by Paxinos and Watson (1986).

**Urodele amphibian hypothalamus.** Filled circles, tyrosine hydroxylase-immunoreactive cells commonly referred to as the PVO-accompanying cells which do not appear to contact the CSF, and tyrosine hydroxylase-immunoreactive cells frequently of the CSF-contacting type (Franzoni et al., 1986; González and Smeets,
1991; Corio et al., 1992; González and Smeets, 1994), associated with the NID (a structure which is in turn closely associated with the primordial mammillary region, Herrick, 1948; or more specifically with the primordial tuberomammillary nucleus, Airaksinen and Panula, 1990); open circles with ventricular contacts, bipolar 5-HT-containing CSF-contacting cells associated with the PVO and NID (Dubé and Parent, 1982; Fasolo et al., 1986; Corio and Doerr-Schott, 1988; Corio et al., 1992); filled circles with ventricular contacts, bipolar catecholamine-containing CSF-contacting cells within the PVO and NID (Sims, 1977; Dubé and Parent, 1982; Corio and Doerr-Schott, 1988; Lamas et al., 1988; González and Smeets, 1991, 1994; Corio et al., 1992).
mammalian A13 group in the caudal medial hypothalamus of *C. crocodilus*, Brauth, 
1988). Likewise, the monoamine-accumulating perikarya within the DMN of the rat 
are associated with laterally displaced tyrosine hydroxylase-immunoreactive neurons 
(A13, A13l; A14l; Hökfelt et al., 1984a,b).

Also described in the DMN of the rat, within the periventricular aspect of the 
nucleus, are a population of AADC-immunoreactive neurons which are not 
immunoreactive for tyrosine hydroxylase (D12 Group; Jaeger et al., 1984). These 
cells may correspond to the catecholamine- or 5-HT-accumulating cells in the same 
region (see discussion by Hökfelt et al., 1984a). This is an attractive hypothesis since 
a single or multiple AADC enzyme(s) catalyze the conversion of L-DOPA to 
dopamine and 5-HTP to 5-HT in mammalian tissues (Hökfelt et al., 1973b; Sourkes, 
1987). In support of this hypothesis studies of cultured embryonic mouse 
hypothalamus indicate that a population of cells which is tyrosine hydroxylase- and 
tryptophan hydroxylase-immunonegative has capabilities for 5-HTP-uptake and 
decarboxylation as well as ³⁵H-5-HT accumulation (De Vitry et al., 1986). The 
presence of AADC-immunoreactive neuronal cell bodies in the mammalian DMN is 
consistent with the arrangement in the nonmammalian PVO. The CSF-MA neurons 
of the PVO of the bullfrog, *R. catesbeiana*, and the posterior PVO of a teleost fish, 
*C. auratus*, are immunonegative for tyrosine hydroxylase (Carr et al., 1991; Beltramo 
et al., 1994), but contain immunoreactivities for AADC (Kani et al., 1987; Beltramo 
et al., 1994).
In accordance with a role for these hypothalamic structures in the metabolism of biogenic amines, both are characterized by the presence of monoamine oxidase (MAO), an enzyme with a wide substrate selectivity which converts catecholamines to their corresponding aldehydes, or 5-HT to the metabolite 5-HIAA (reviewed by Berry et al., 1994). In MAO enzyme histochemical preparations, the intraventricular projections of PVO neurons in *X. laevis* show a strong MAO activity, whereas the neuronal cell bodies show a more moderate MAO activity (Terlou and Stroband, 1973). This is paralleled by the description of small MAO-B-positive neuronal cell bodies within the DMN in studies of the cat using a sensitive enzyme histochemical method (Kitahama et al., 1989).

Elegant studies have described the projections of the PVO in the dipnoan fish (*Protopterus dolloi*; von Bartheld and Meyer, 1990), and the DMN of the rat (ter Horst and Luiten, 1986). These studies of diverse species of vertebrates indicate that there are impressive similarities in intrahypothalamic, ascending and descending projections of the PVO and the DMN. In these studies intrahypothalamic projections were described for the PVO and DMN to the median eminence, lateral regions of the infundibular hypothalamus, as well as ventral and lateral aspects of the anterior hypothalamic-preoptic area. Ascending projections were evidenced by terminal labeling in the habenular nuclei and adjacent thalamic structures, as well as in elements of the basal forebrain, including septal structures. Descending pathways were observed extending caudally along the ventral brainstem, innervating multiple mesencephalic, isthmic, and rhombencephalic regions. These included the ventral
mesencephalic tegmentum, the isthmic region (locus coeruleus and subcoeruleus regions in the rat), the cerebellum, multiple raphe structures at mesencephalic and lower brainstem levels, and portions of the reticular formation throughout the rhombencephalon. Thus, the generalized projections of the PVO in *P. dolloi*, a dipnoan fish, and the DMN in the rat are remarkably similar. Equally thorough descriptions of the projections of the PVO in representatives of intermediate classes of vertebrates are necessary to adequately evaluate the extent to which the nonmammalian PVO and the mammalian DMN innervate homologous regions of the central nervous system.

Based on connectivity as well as topographical, immunohistochemical, biochemical, and functional characteristics of the nonmammalian PVO and the mammalian DMN, we propose that these structures may be homologous, and that they represent important components of an evolutionarily conserved complex of amine-metabolizing structures in the infundibular hypothalamus.

**Metabolites**

The regions of the brain with the highest concentrations of monoamines, (DA) and (5-HT), had the lowest ratios of metabolites, [DOPAC] and [5-HIAA], to monoamines (Fig.V.2). This pattern has been described previously in mammals (Gaudin-Chazal et al., 1979; Mefford et al., 1982). The lowest [DOPAC]/[DA] ratios and the lowest [5-HIAA]/[5-HT] ratios were in regions of the brain with monoamine-containing neuronal cell bodies, for example in the raphe region and the region of the PVO for the serotonergic system. It is likely that these regions had low
ratios of metabolites to monoamines as a result of the relatively high synthesis, storage and transport properties of these neurons, compared to the levels of synaptic interactions.

The ratios of metabolites to monoamines in this species were approximately an order of magnitude lower than those described in studies of mammals (cf. Gaudin-Chazal et al., 1979; Mefford et al., 1982). This suggests that some aspects of synthesis, storage, transport, and utilization of monoamine neurotransmitters in this amphibian are different than in mammalian systems. Although few studies have made direct comparisons of these processes in mammalian and amphibian systems, several studies suggest that there are fundamental differences. For example, injections of either reserpine, a catecholamine depleter, or α-methyl-p-tyrosine, an effective catecholamine synthesis inhibitor in mammals, have little or no effect on total brain concentrations of monoamines in the anuran amphibian *R. pipiens* using biochemical (Baumgarten, 1972) or histofluorescence techniques (Parent, 1975). These observations are supported by the inability of α-methyl-p-tyrosine (1.2, 9, or 18 mg; intraperitoneally), in animals killed two and four h after treatment, to alter concentrations of catecholamines in several microdissected brain regions of *T. granulosa* (*unpublished observations*). As previously suggested (Baumgarten, 1972; Parent, 1975) it may be that due to the slower metabolic rates of ectotherms, turnover rates of monoaminergic systems are much slower and turnover cannot be quantified when using sampling protocols developed for mammalian systems. Consequently,
chronic injections of pharmacological agents may be more efficient for manipulations of monoamine neuronal systems in some amphibians.

Conclusions

Quantitative measurements of norepinephrine, epinephrine, dopamine, and 5-HT in the central nervous system of *T. granulosa* revealed many similarities between this urodele and other vertebrates, although the sources of these neurotransmitters (single or multiple sources in hypothalamic or brainstem regions) have not been determined. In addition, the highest concentrations of monoamines, with the exception of 5-HT in the raphe region, were observed in the regions of the hypothalamus that contain the CSF-contacting cells of the PRO, PVO, and NID. Of particular interest is the observation that epinephrine concentrations were high in these regions relative to other regions of the central nervous system, raising the possibility that these regions may be important sites for the synthesis, storage, transport, or utilization of epinephrine. In addition, regions which include CSF-MA neuronal systems may be focal regions for metabolism of multiple biogenic amines. As such, they may have important neuroendocrine, physiological or behavioral functions. An understanding of the characteristics of these conserved systems in diverse vertebrate classes may help distinguish functional aspects of hypothalamic aminergic systems from technical artifacts, and lead to a more thorough understanding of catecholamine and indoleamine systems in the vertebrate central nervous system.
ACKNOWLEDGMENTS

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ABBREVIATIONS

AADC, aromatic L-amino acid decarboxylase; CSF-MA, cerebrospinal fluid-contacting, monoamine-containing; DBH, dopamine β-hydroxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; HPLC-ED, high performance liquid chromatography with electrochemical detection; 5-HT, serotonin, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; L-DOPA, L-3,4-dihydroxyphenylalanine; PNMT, phenylethanolamine N-methyltransferase

ABBREVIATIONS USED IN FIGURES

AM amygdala
APL amygdala pars lateralis
APM amygdala pars medialis
APOA anterior preoptic area (microdissected region that included the PRO)
Arc arcuate hypothalamic nucleus
DA dorsal hypothalamic area
DMN dorsomedial hypothalamic nucleus
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<th>Abbreviation</th>
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<tr>
<td>DP</td>
<td>dorsal pallium</td>
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<tr>
<td>DT</td>
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<td>EPL</td>
<td>external plexiform layer</td>
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<td>GL</td>
<td>glomerular layer</td>
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<td>ic</td>
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<td>interpeduncular nucleus</td>
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<td>LH</td>
<td>lateral hypothalamic area</td>
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<td>mammillothalamic tract</td>
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<td>NID</td>
<td>nucleus infundibularis dorsalis</td>
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<td>Pe</td>
<td>periventricular hypothalamic nucleus</td>
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<td>PMV</td>
<td>premammillary nucleus, ventral</td>
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<td>PVO</td>
<td>paraventricular organ</td>
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<td>Abbreviation</td>
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<td>PRO</td>
<td>preoptic recess organ</td>
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<td>TEG</td>
<td>tegmentum (dorsal and ventral regions)</td>
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<tr>
<td>TI</td>
<td>isthmic tegmentum</td>
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<td>TM</td>
<td>dorsal tegmentum mesencephali</td>
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<td>V</td>
<td>3rd ventricle</td>
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<td>VH</td>
<td>ventral hypothalamus</td>
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<td>VMH</td>
<td>ventromedial hypothalamic nucleus</td>
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<tr>
<td>VT</td>
<td>ventral thalamus</td>
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<td>ZI</td>
<td>zona incerta</td>
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CHAPTER VI: ELEVATION OF SEROTONIN, DOPAMINE, AND SELECTED METABOLITE CONCENTRATIONS IN THE DORSOMEDIAL HYPOTHALAMUS FOLLOWING CORTICOTROPIN-RELEASING FACTOR OR CORTICOSTERONE ADMINISTRATION

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ABSTRACT

The male roughskin newt (*Taricha granulosa*) was used to investigate -- using microdissection and high performance liquid chromatography with electrochemical detection techniques -- the effects of corticotropin-releasing factor (CRF) or corticosterone administration on tissue concentrations of norepinephrine, epinephrine, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin, and 5-hydroxyindoleacetic acid (5-HIAA). In experiments I and II we injected control solutions or CRF (25 or 50 ng), measured locomotor activity 34 min after treatment, then measured monoamine and monoamine metabolite levels in microdissected brain areas. Compared to control animals CRF-injected animals were hyperactive and had altered levels of monoamines or monoamine metabolites in two of 12 brain areas, the ventral striatum and the dorsomedial hypothalamus. In the ventral striatum concentrations of the serotonin metabolite 5-HIAA were lower in CRF-treated animals than in control animals. In the dorsomedial hypothalamus, a region that contains dopamine- and serotonin-accumulating neuronal cell bodies, dopamine and serotonin concentrations were higher in CRF-treated animals than in control animals. In experiment III male newts were treated as follows: 1) no injection, no handling, 2) saline injection, or 3) 10 µg corticosterone (20 min). Monoamine and monoamine metabolite concentrations were similar in the unhandled and saline-injected controls. In contrast, corticosterone-injected newts had elevated concentrations of serotonin, 5-HIAA, and the dopamine metabolite DOPAC in the dorsomedial hypothalamus (but not in seven other brain areas studied). Together these observations suggest that CRF
and corticosterone act at the level of the dorsomedial hypothalamus to regulate serotonergic and dopaminergic systems.

**INTRODUCTION**

In addition to the well-established hypophysiotropic role for corticotropin-releasing factor (CRF; Vale et al., 1981; Rivier et al., 1990), there is evidence that this 41-amino acid neuropeptide acts within the central nervous system as a neurotransmitter or neuromodulator. For example, there is evidence that (1) CRF-immunoreactive neuronal cell bodies and fibers, receptor proteins, and mRNA are widely distributed throughout the central nervous system (Swanson et al., 1983; De Souza et al., 1984, 1985; Merchenthaler, 1984; Skofitsch et al., 1985; Wong et al., 1994), (2) CRF is localized within nerve terminals (Cain et al., 1991), (3) depolarization of neuronal cell bodies containing CRF results in calcium-dependent CRF release (Smith et al., 1986; Clarke et al., 1987; Widmaier et al., 1989; Hillhouse and Reichlin, 1990; Hu et al., 1992; Cratty and Birkle, 1994; Gabr et al., 1994), and (4) iontophoretic or intracerebroventricular (i.c.v.) application of CRF alters the spontaneous firing rates of extrahypothalamic neurons in rats (Valentino et al., 1983; Siggins et al., 1985; Siggins, 1990; see also Valentino and Wehby, 1988; Valentino et al., 1991) and the urodele amphibian *Taricha granulosa* (Lowry et al., 1994).

In addition to the evidence that CRF may act as a neurotransmitter or neuromodulator, recent studies suggest that the glucocorticoid steroid hormone corticosterone can rapidly modulate neuronal excitability within the central nervous system.
system. For example, iontophoretically applied corticosterone or cortisol rapidly (within 1-10 sec) alters spontaneous and glutamate-induced increases in firing of electrophysiologically identified units in the hypothalamic paraventricular nucleus (Saphier and Feldman, 1988) as well as of single units within the medial tuberal hypothalamus of the rat (Mandelbrod et al., 1974). Rapid electrophysiological effects of corticosterone also have been described in studies of *T. granulosa* (Rose et al., 1993). These rapid electrophysiological effects of corticosterone in the rat and in *T. granulosa* may be mediated by specific binding sites for glucocorticoids in neuronal membranes (Towle and Sze, 1983; Orchinik et al., 1991; 1992; Sze and Towle, 1993; Orchinik and McEwen, 1995).

Together these studies indicate that CRF and corticosterone can rapidly alter neuronal activity in diverse vertebrate species. Among the many central actions of CRF and corticosterone is the modulation of monoaminergic metabolism. Microdialysis as well as studies using microdissection with high performance liquid chromatography and electrochemical detection (HPLC-ED) techniques indicate that both CRF and corticosterone can alter elements of the synthesis, storage, transport, or utilization of catecholamines and indoleamines in vertebrates (Dunn and Berridge, 1987; Kalivas et al., 1987; Losada, 1988; Barrett et al., 1989; Matsuzaki et al., 1989; Shimizu and Bray, 1989; Emoto et al., 1993; Lavicky and Dunn, 1993; Lowry et al., 1993; Lee et al., 1994). Consistent with these observations CRF can stimulate the activities of tyrosine hydroxylase (Olianas and Onali, 1989) and tryptophan
hydroxylase (Corley et al., 1990; Singh et al., 1992), the rate-limiting enzymes for synthesis of dopamine and serotonin (5-hydroxytryptamine, 5-HT), respectively.

Although in *T. granulosa* CRF and corticosterone can act within minutes to alter behavior (Moore and Miller, 1984; Moore et al., 1984, 1995; Boyd and Moore, 1990; Lowry et al., 1990, 1991, 1993, 1994; Orchinik et al., 1991), it is currently unknown where in the central nervous system these behaviorally active neurochemicals act. It is also unclear which neurotransmitter systems may be involved in mediating the effects of CRF and corticosterone on behavior. As an initial step in addressing these questions we have investigated, using *T. granulosa*, the effects of i.c.v. CRF and intraperitoneal corticosterone administration on tissue concentrations of norepinephrine, epinephrine, DOPAC, dopamine, 5-HIAA, and 5-HT in microdissected brain regions. Portions of these data have been presented in preliminary form (Lowry et al., 1991; Moore et al., 1995).

**MATERIALS AND METHODS**

Adult male roughskin newts (*Taricha granulosa*) were collected locally from freshwater ponds on separate occasions for each of three experiments. Animals were maintained in the laboratory for approximately 48 h in holding tanks containing dechlorinated tap water. These tanks were in a room with controlled photoperiod (14 h light:10 h dark; lights on at 0630 h) and an ambient temperature of 19°C (Experiments I and II), or in a room with natural photoperiod and an ambient temperature of 23°C (Experiment III). Newts were not handled or disturbed until immediately prior to treatment.
In Experiments I and II animals received i.c.v. injections of either amphibian Ringer's solution or vehicle containing synthetic ovine corticotropin-releasing factor (CRF; generous gift from Drs. Wylie Vale and Jean Rivier, Salk Institute for Biological Studies, San Diego, CA; Experiment I, 25 ng; Experiment II, 50 ng; n=10). Intracerebroventricular injections were given through a 0.5 mm hole at the junction of the parietal and frontal bones in the cranial midline using a microsyringe with a tip diameter of 50 μm as described in Moore and Miller (1983). Solutions (2 μl) were infused over a period of 5 seconds. Following i.c.v. injection newts were isolated in temporary circular holding tanks, (25 cm in diameter) containing 5 L of dechlorinated tap water, until behavioral testing. For behavioral testing newts were placed individually in a dechlorinated water-filled (10 cm depth) circular runway with an inside diameter of 70 cm and an outside diameter of 85 cm; each testing arena was marked with radial lines to define 16 equal sectors. Newts were placed in the testing arena 30 minutes after treatment. Starting 4 minutes after placement, locomotor activity was quantified by counting the total number of lines crossed during 3 consecutive minutes. Locomotion consisted of a combination of walking and swimming movements. The Mann-Whitney U-test was used to test for statistical differences in locomotor activity between treatment groups (Siegel, 1956). Two-tailed probabilities P ≤ 0.05 were considered statistically significant.

In Experiment III male newts were treated as follows: 1) no injection, no handling, 2) saline injection, or 3) 10 μg corticosterone (Sigma Chemical Company; intraperitoneal injection, 0.1 ml volume; n=12). Following treatment, newts were
isolated in temporary circular holding tanks as described above, but were not tested for locomotor activity; previous studies in _T. granulosa_ indicate that intraperitoneal injection of corticosterone does not alter locomotor activity (Moore, Roberts, and Bevers, 1984).

Immediately after behavioral testing (Experiments I and II), or 20 min after treatment (Experiment III), newts were killed by rapid decapitation. Each animal was rapidly dissected (1-2 min) to remove the intact brain and rostral spinal cord. These were then embedded in Tissue-Tek® O.C.T. Compound, frozen on dry ice, and stored in airtight microcentrifuge tubes at -80 °C until sectioning. Serial 300 μm sections of frozen brain were cut at -12 °C in a cryostat. Sections were placed on gelatin-coated glass slides and collectively thaw-mounted by briefly warming the slide. Sections were refrozen within approximately 30 sec. Slides were sealed in slide boxes and stored at -80 °C.

Microdissection of individual brain regions was accomplished using the methods described for the microdissection of mammalian brain tissues (Palkovits, 1973; Palkovits and Brownstein, 1982) as previously applied in _T. granulosa_ (Zoeller and Moore, 1986; Lowry et al., 1995). Slides representing an entire brain were placed on a cold stage (Thermoelectric Cold Plate, TCP-2, Thermoelectrics Unlimited, Inc.) and maintained at -10 °C. Tissue punches (300 μm i.d.) were expelled into 60 μl of acetate buffer (pH 5) containing internal standard, 0.5 x 10^{-7} \text{M} α-methylldopamine (Experiment I and II), or 3.2 x 10^{-8} \text{M} 3,4-dihydroxybenzylamine hydrobromide (Experiment III), and then stored at -80 °C until analyzed for
monoamine content. A microdissection atlas described previously for *T. granulosa* (Lowry et al., 1995) was used. Brain regions were identified based on descriptions of the urodele amphibian telencephalon (Herrick, 1927; Northcutt and Kicliter, 1980), diencephalon and midbrain (Herrick, 1917), and hindbrain (Herrick, 1930), as well as the comprehensive study by Herrick (1948).

Tissue concentrations of norepinephrine, epinephrine, DOPAC, dopamine, 5-HIAA, and 5-HT were analyzed using HPLC-ED. The method of analysis was based on a previously described procedure (Renner and Luine, 1984; Renner and Luine, 1986) with several modifications (Lowry et al., 1995). The tissue samples were thawed and centrifuged at 15,000 x g for 2 min. The supernatant was treated with 2 µl of 0.02% ascorbate oxidase (Boehringer Mannheim) to minimize ascorbic acid contributions to the solvent front (McKay et al., 1984) and directly injected into a Waters chromatographic system (Waters Associates, Inc.). Chromatographic separation was accomplished using a C-18, 4 µm radial compression cartridge and a mobile phase consisting of (wt/vol), 0.84% sodium acetate, 1.2% citric acid, 0.015% octanesulfonic acid sodium salt (Eastman Kodak), 0.02% disodium EDTA, in (vol/vol) 15% methanol in water. Electrochemical detection was provided by a laboratory built potentiostat and a glassy carbon electrode (Bioanalytical Systems) set at +0.7 V with respect to an Ag/AgCl reference electrode. The tissue pellet was dissolved in 0.3 N NaOH and analyzed for protein content according to the method of Bradford (1976).
The pg/cm peak heights of known concentrations of the standards were determined from the mean peak heights of 3 chromatograms for each respective standard. The internal standard was injected 3 times to determine the peak height for 100 % sample recovery. Amine concentrations were calculated from the standard values and corrected for % recovery and injection volume using a Waters 730 Data Module. The amine concentrations were divided by µg protein to yield pg amine/µg protein. Outlier values identified using the Grubbs method were not included in reported values (two-tailed probability, P \leq 0.05; Grubbs and Beck, 1972). In Experiments I and II, Student’s t-test was used to test for differences between treatment groups in tissue concentrations of monoamines, monoamine metabolites, or ratios of monoamines to their respective metabolites. In Experiment III, differences among treatment groups were analysed using one-way analysis of variance; multiple comparisons to the same control (saline versus unhandled, uninjected; saline versus corticosterone) were made when appropriate using Dunnett’s Test. Two-tailed probabilities P \leq 0.05 were considered statistically significant.

RESULTS

Corticotropin-releasing factor (Figs.VI.1, VI.2) and corticosterone (Fig.VI.3) administration resulted in site-specific changes in tissue concentrations of DOPAC, dopamine, 5-HIAA, and 5-HT. With a single exception the CRF- and corticosterone-induced changes in monoamine and monoamine metabolite concentrations were restricted to the dorsomedial hypothalamus (DMH). In contrast to the differences
observed in the tissue concentrations of DOPAC, dopamine, 5-HIAA, and 5-HT there were no differences in tissue concentrations of either norepinephrine or epinephrine.

**TABLE VI.1.** Locomotor activity in response to an i.c.v. injection of saline or CRF

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mean locomotor activity (crossings/3 min)</th>
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<tbody>
<tr>
<td></td>
<td>Control injection</td>
</tr>
<tr>
<td>Experiment I</td>
<td>9.0 ± 3.3</td>
</tr>
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<td>(25 ng CRF)</td>
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<tr>
<td>Experiment II</td>
<td>10.7 ± 3.2</td>
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<td>(50 ng CRF)</td>
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Values represent mean locomotor activity ± S.E.M (n=10). *Significantly different from saline-injected control, two-tailed probability ≤ 0.05 (Mann-Whitney U-test).

following treatment with either CRF or corticosterone in any brain region (Fig.VI.4).

In Experiments I and II i.c.v. injection of CRF stimulated locomotor activity compared to saline-injected controls (Table VI.1). Concurrently CRF had site-specific effects on tissue concentrations of monoamines or monoamine metabolites that were dependent on the dosage of CRF given. Injection of the lower dose of CRF (25 ng) did not alter 5-HT, 5-HIAA, or DOPAC concentrations in any of the brain regions studied, however this dose resulted in elevated levels of dopamine in the dorsomedial hypothalamus when compared to saline-injected controls (Fig.VI.1).

There was a corresponding decrease in the ratio of DOPAC/DA in this region (*saline*,
FIGURE VI.1. Effects of CRF (25 ng) on monoamine and monoamine metabolite levels in microdissected brain regions measured approximately 35 min following treatment. Effects of i.c.v. injection of saline or CRF (25 ng) on 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in microdissected brain regions of adult male T. granulosa measured approximately 35 min following treatment. Injection of CRF resulted in elevated concentrations of dopamine in the dorsomedial hypothalamus compared to saline-injected controls. Abbreviations: DMH, dorsomedial hypothalamus; DTM, dorsal tegmentum mesencephali; IST, isthmic tegmentum; LH, dorsolateral hypothalamus (region containing the nucleus infundibularis dorsalis); MP, medial pallium; R, raphe region; S, septum; TH, thalamus; TT, trigeminal tegmentum; VS, ventral striatum.
0.29 ± 0.06; CRF, 0.13 ± 0.01; \( t = 3.47, 11 \, df, \, P \leq 0.01 \)). The higher dose of CRF (50 ng) did not alter dopamine or DOPAC concentrations in any of the brain regions studied, however this dose resulted in elevated levels of 5-HT in the dorsomedial hypothalamus and lower levels of 5-HIAA in the ventral striatum (Fig. VI.2). There was a corresponding decrease in the ratio of 5-HIAA/5-HT in the ventral striatum in CRF-injected newts compared to saline-injected controls (saline, 0.13 ± 0.01; CRF, 0.08 ± 0.01; \( t = 3.15, 16 \, df, \, P \leq 0.01 \)).

In Experiment III, there were differences in tissue concentrations of monoamines and monoamine metabolites among treatment groups, but only in the dorsomedial hypothalamus. Specifically, there were differences among the treatment groups in tissue concentrations of 5-HT (\( F_{2,31} = 4.85, \, P \leq 0.05 \)), 5-HIAA (\( F_{2,30} = 7.017, \, P \leq 0.01 \)), dopamine (\( F_{2,31} = 3.47, \, P \leq 0.05 \)), and DOPAC (\( F_{2,31} = 3.34, \, P \leq 0.05 \)) in this region. There was no effect of handling and saline injection on tissue concentrations of any of the monoamines or monoamine metabolites in any of the eight brain regions studied compared to unhandled controls. There were, however, significant increases in the tissue concentrations of 5-HT, 5-HIAA, and DOPAC in the dorsomedial hypothalamus 20 min following corticosterone administration compared to saline-injected controls (Fig. VI.3).
FIGURE VI.2. Effects of CRF (50 ng) on monoamine and monoamine metabolite levels in microdissected brain regions measured approximately 35 min following treatment. Effects of i.c.v. injection of saline or CRF (50 ng) on 5-HT, 5-HIAA, dopamine, and DOPAC concentrations in microdissected brain regions of adult male *T. granulosa* measured approximately 35 min following treatment. Injection of CRF resulted in elevated concentrations of 5-HT in the dorsomedial hypothalamus and lower levels of 5-HIAA in the ventral striatum compared to saline-injected controls. See Fig.VI.1 for abbreviations.
FIGURE VI.3. Effects of corticosterone on monoamine and monoamine metabolite levels in microdissected brain regions measured 20 min following treatment. A. Effects of intraperitoneal injection of saline or corticosterone (10 μg) on 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in microdissected brain regions of adult male T. granulosa measured 20 min following treatment. Although the handling and injection of saline did not alter 5-HT or 5-HIAA concentrations in any of the brain regions compared to unhandled controls, injection of corticosterone resulted in elevated concentrations of both 5-HT and 5-HIAA in the dorsomedial hypothalamus compared to saline-injected controls.

B. Effects of intraperitoneal injection of saline or corticosterone (10 μg) on dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in microdissected brain regions of adult male T. granulosa measured 20 min following treatment. As observed for 5-HT and 5-HIAA, the handling and injection of saline did not alter dopamine or DOPAC concentrations in any of the brain regions compared to unhandled controls. Injection of corticosterone resulted in elevated concentrations of DOPAC in the dorsomedial hypothalamus compared to saline-injected controls. Although a one-way analysis of variance indicated significant differences among treatment groups for the concentration of dopamine in the dorsomedial hypothalamus, the comparison between saline injected controls and corticosterone-injected animals was not significant (P = 0.07). Abbreviations: APM, amygdala pars medialis; DMH, dorsomedial hypothalamus; LH, dorsolateral hypothalamus (region containing the nucleus infundibularis dorsalis); NA, nucleus accumbens; NTS, nucleus of the solitary tract; POA, preoptic area; R, raphe region; VS, ventral striatum.
FIGURE VI.3

[Graph showing neurotransmitter levels in different brain regions under various conditions.]
raises the possibility that corticosterone may have similar effects on serotonin metabolism in the mediobasal hypothalamus of nonmammalian and mammalian vertebrates.

*Neurochemical changes within the dorsomedial hypothalamic nucleus following exposure to stressful stimuli*

The effects of CRF (elevation of dopamine and 5-HT) on monoamine concentrations in the dorsomedial hypothalamus of *T. granulosa* are strikingly similar to the effects of exposure of rats to certain types of stressful stimuli. For example, exposure of rats to electric foot shock results in a three- to four-fold increase in the tissue concentration of 5-HT within the DMN (but not the adjacent lateral hypothalamus) 24 h following exposure to electric foot shock (Shekhar et al., 1994). Since this study used microdissection and HPLC-ED techniques as in the present study, it is uncertain where within the DMN the accumulation of 5-HT is taking place. For example, the elevation of 5-HT may be localized within neuronal perikarya which are intrinsic to the DMN or, alternatively, within 5-HT fibers and terminals arising from serotonergic neurons within the brainstem raphe.

In addition, exposure to a psychological stressor, fear-potentiated startle (but not exposure to elevated plus-maze and social interaction tests) results in increases in dopamine concentrations within the DMN after 24 h (Katner et al., 1994). This change in dopamine concentration was evident even though there was no change in tyrosine hydroxylase activity within the DMN compared to controls. These studies indicate that accumulation of dopamine and 5-HT within the DMN may be important
DISCUSSION

We have identified site-specific effects of behaviorally active doses of CRF and corticosterone administration on the tissue concentrations of specific monoamines and monoamine metabolites in the central nervous system of male *T. granulosa*. These effects were restricted to changes in tissue concentrations of DOPAC, dopamine, 5-HIAA, and 5-HT within the dorsomedial hypothalamus and changes in tissue concentrations of 5-HIAA in the ventral striatum.

The most remarkable observation in the present study is the site-specificity of the effects of i.c.v. CRF and intraperitoneal corticosterone administration on tissue concentrations of monoamines and monoamine metabolites. Depending on the dosage, CRF administration resulted in either an increase in the concentration of dopamine or 5-HT within the dorsomedial hypothalamus. Corticosterone administration resulted in an increase in the concentrations of DOPAC, 5-HIAA, and 5-HT within the dorsomedial hypothalamus compared to vehicle-injected controls. This is intriguing because the dorsomedial hypothalamus in vertebrates contains populations of dopamine- and 5-HT-accumulating neuronal cell bodies. In nonmammalian vertebrates these dopamine- and 5-HT-accumulating neuronal cell bodies are components of a vascularized structure referred to as the paraventricular organ; in mammalian vertebrates these cell bodies are localized within the dorsomedial hypothalamic nucleus (DMN; for references, see Lowry et al., 1995). Based on connectivity as well as topographical, immunohistochemical, biochemical, and functional characteristics of the nonmammalian PVO and the mammalian DMN, it
appears that the monoamine-accumulating neurons in DMN and the neuronal component of the PVO are homologous structures (Lowry et al., 1995).

The increases in dopamine and 5-HT in the dorsomedial hypothalamus following i.c.v. CRF administration may reflect an accumulation of these neurotransmitters by the dopamine- and serotonin-accumulating neuronal cell bodies in this region of the hypothalamus or, alternatively, by serotonergic fibers and terminals which arise from neuronal cell bodies within the brainstem raphe (Frankfurt and Azmitia, 1983). The possibility that stress hormone-induced elevations of 5-HT and dopamine concentrations in the dorsomedial hypothalamus may be localized within neuronal cell bodies intrinsic to the dorsomedial hypothalamus is based on descriptions of populations of dopamine- and 5-HT-accumulating neuronal cell bodies in this region in mammals (Fuxe and Ungerstedt, 1968; Lidbrink et al., 1974; ChanPalay, 1977; Beaudet and Descarries, 1979; Frankfurt et al., 1981; Howe et al., 1982; Steinbusch et al., 1982; Frankfurt and Azmitia, 1983; Steinbusch and Nieuwenhuys, 1983; Sakamoto et al., 1984; Steinbusch, 1984; Ueda et al., 1984; Arezki, 1985; Staines et al., 1986; Ugrumov et al., 1986, 1988, 1989; Denoyer et al., 1989; Karasawa et al., 1994) and amphibians (Peute and van Oordt, 1974; Nakai et al., 1977). Although dopamine-accumulating neuronal cell bodies have been described in the dorsomedial hypothalamus of the rat (Lidbrink et al., 1974), the laboratory shrew, *Suncus murinus* (Karasawa et al., 1994), and the anuran amphibians, *Rana catesbeiana, Rana nigromaculata,* and *Xenopus laevis* (Peute and van Oordt, 1974; Nakai et al., 1977), the characteristics of the 5-HT-accumulating
neuronal cell bodies in this region have been described in more detail. The term "5-HT-accumulating neuronal cell bodies" is used because, although these cells do not normally contain tryptophan hydroxylase (the rate limiting enzyme for de novo synthesis of 5-HT) or 5-HT immunoreactivity, 5-HT immunoreactivity is observed following systemic pretreatment with a monoamine oxidase inhibitor and 5-HT precursors, either L-tryptophan or 5-hydroxytryptophan. Alternatively, 5-HT immunoreactivity can be visualized within these cells following high dosages of monoamine oxidase inhibitor alone (Ueda et al., 1984). These same neurons contain immunoreactivity for serotonin binding protein -- a protein that is believed to be an intrinsic and a specific component of serotonergic neurons -- in normal animals (Kirchgessner et al., 1988; Tamir et al., 1994). Furthermore, systemic treatment of rats with dopaminergic or noradrenergic agonists can result in an accumulation of 5-HT immunoreactivity within DMN neuronal cell bodies (Arezki et al., 1985), suggesting that 5-HT accumulation within these neurons may be regulated in vivo; however, there is currently no physiological or behavioral function ascribed to this unique population of neurons.

Although studies in mammals have not assessed the effects of i.c.v. CRF on tissue concentrations of monoamines within the DMN, intraperitoneal injection of corticosterone results in dramatic increases in tissue concentrations of 5-HT and 5-HIAA in the mediobasal hypothalamus of the rat, a structure which includes the DMN, measured 30 min after treatment (Losada, 1988). This observation, together with the observation in the present study that corticosterone administration results in
an elevation of 5-HT and 5-HIAA in the dorsomedial hypothalamus of *T. granulosa*, raises the possibility that corticosterone may have similar effects on serotonin metabolism in the mediobasal hypothalamus of nonmammalian and mammalian vertebrates.

*Neurochemical changes within the dorsomedial hypothalamic nucleus following exposure to stressful stimuli*

The effects of CRF (elevation of dopamine and 5-HT) on monoamine concentrations in the dorsomedial hypothalamus of *T. granulosa* are strikingly similar to the effects of exposure of rats to certain types of stressful stimuli. For example, exposure of rats to electric foot shock results in a three- to four-fold increase in the tissue concentration of 5-HT within the DMN (but not the adjacent lateral hypothalamus) 24 h following exposure to electric foot shock (Shekhar et al., 1994). Since this study used microdissection and HPLC-ED techniques as in the present study, it is uncertain where within the DMN the accumulation of 5-HT is taking place. For example, the elevation of 5-HT may be localized within neuronal perikarya which are intrinsic to the DMN or, alternatively, within 5-HT fibers and terminals arising from serotonergic neurons within the brainstem raphe.

In addition, exposure to a psychological stressor, fear-potentiated startle (but not exposure to elevated plus-maze and social interaction tests) results in increases in dopamine concentrations within the DMN after 24 h (Katner et al., 1994). This change in dopamine concentration was evident even though there was no change in tyrosine hydroxylase activity within the DMN compared to controls. These studies
indicate that accumulation of dopamine and 5-HT within the DMN may be important
components of the response to certain types of stressful stimuli. Although the
neurochemical responses to electric foot shock and fear potentiated startle in rats
appear similar to the neurochemical responses to CRF and corticosterone in the
present study, it is uncertain at this time whether the effects of electric foot shock or
fear potentiated startle on 5-HT and dopamine levels in the DMN involve the actions
of CRF, corticosterone, or other neuroactive chemicals.

Mechanisms for accumulation of dopamine and serotonin in the dorsomedial
hypothalamus

We are unable to conclude if CRF is acting independently from corticosterone
to elevate tissue concentrations of 5-HT and dopamine in the dorsomedial
hypothalamus. Corticotropin-releasing factor may stimulate the accumulation of
dopamine or serotonin in the dorsomedial hypothalamus through its actions on the
hypothalamic-pituitary-adrenal (HPA) axis and the consequent elevation of plasma
corticosterone levels or, alternatively, through direct or indirect actions within the
central nervous system independent from activation of the HPA axis. For example,
the DMN has reciprocal connections with the parvocellular CRF cell body region of
the paraventricular nucleus (Luiten et al., 1985; ter Horst and Luiten, 1986), contains
CRF receptors (Aguilera et al., 1990), and, together with the ventral premammillary
nucleus, is a region of the hypothalamus which is noted to express CRF-binding
protein (Potter et al., 1992); these observations suggest that the DMN may be an
important site for direct actions of CRF. In addition, studies in rats indicate that
activation of dopaminergic or β-adrenergic receptors can stimulate the accumulation of 5-HT in identified 5-HT-accumulating neuronal cell bodies within the DMN (Arezki et al., 1985). Since CRF can activate dopaminergic and noradrenergic systems (reviewed by Dunn and Berridge, 1990), it is possible that CRF may stimulate the accumulation of 5-HT within intrinsic neuronal cell bodies within the DMN indirectly through activation of dopaminergic or noradrenergic systems.

We are also unable to conclude if corticosterone is acting independently from CRF to elevate tissue concentrations of DOPAC, 5-HIAA, and 5-HT in the dorsomedial hypothalamus. Corticosterone may stimulate the accumulation of DOPAC, 5-HIAA, and 5-HT, in the dorsomedial hypothalamus through the stimulation of select CRF neuronal populations within the central nervous system or, alternatively, through actions within the central nervous system independent of CRF activation. For example, since corticosterone can activate some CRF neuronal populations within the central nervous system (Lee et al., 1994; Makino et al., 1994; Schulkin et al., 1994), corticosterone may stimulate the accumulation of DOPAC, 5-HIAA, or 5-HT in the dorsomedial hypothalamus through its actions on CRF neurons. Alternatively, corticosterone may act directly within the dorsomedial hypothalamus to rapidly stimulate the uptake of 5-HT precursors. Based on studies using mice glucocorticoids may regulate the uptake of tryptophan, the amino acid precursor of 5-HT (Neckers and Sze, 1975; Towle and Sze, 1983). A single injection of hydrocortisone acetate elevated the brain levels of tryptophan by 50% within 1 hour and enhanced the accumulation of 5-HT in whole brain in the presence of a
monoamine oxidase inhibitor (Neckers and Sze, 1975). Treatment did not appear to alter tryptophan hydroxylase or aromatic L-amino acid decarboxylase activity. Injection of hydrocortisone acetate 60 minutes before sacrifice elevated tryptophan but not tyrosine (the amino acid precursor for catecholamines) concentrations in subcellular synaptosomal fractions of brain tissue, indicating that the effect was selective for accumulation of the precursor of 5-HT. Consistent with these studies, in vitro synaptosomal preparations, hydrocortisone acetate or corticosterone stimulated uptake of L-[3H]-tryptophan by the synaptosomes (Neckers and Sze, 1975; Towle and Sze, 1983). In contrast, androgen-, estrogen-, and progesterone-like steroids were without effect. The highest concentration of corticosterone binding sites in the synaptosomal plasma membrane preparation was found in the hypothalamus, compared to binding capacities in the cortex and hippocampus (Towle and Sze, 1983), suggesting that the hypothalamus may be an important site of action for the corticosterone-induced uptake of L-[3H]-tryptophan. These results indicate that glucocorticoids can regulate 5-HT accumulation through direct actions on synaptosomal elements to stimulate uptake of L-tryptophan. A rapid effect of corticosterone on the accumulation of L-tryptophan by neuronal structures within the dorsomedial hypothalamus may account for the corticosterone induced elevation of 5-HT and 5-HIAA observed in T. granulosa in the present study.

The dorsomedial hypothalamus as a substrate for CRF and stress-mediated effects

The observations that both CRF and corticosterone administration result in elevation of monoamines or monoamine metabolites in the dorsomedial hypothalamus
are consistent with studies in mammals which have identified the dorsomedial hypothalamus as an important substrate for the central nervous system response to stress. Studies in rats have demonstrated that the DMN is an important neuroanatomical site for the integration of physiological and behavioral responses to stressful stimuli (Soltis and DiMicco, 1991; Inglefield et al., 1994). These studies in rats are supported by the observations that exposure to a variety of stressors, or to i.c.v. administration of CRF, results in altered c-fos expression (Pezzone et al., 1992; Rivest et al., 1992; Imaki et al., 1993), and that exposure to a variety of stressors also alters neurotransmitter and neuropeptide concentrations within this nucleus (Siegel et al., 1987; McCarthy et al., 1993; Katner et al., 1994; Shekhar et al., 1994). Intracerebroventricular administration of corticotropin-releasing factor, immobilization stress for 60 min, or electric foot-shock stress induces c-fos mRNA expression within the dorsomedial hypothalamus, as well as in regions of the limbic system which receive projections from the DMH, including the lateral septal nucleus, the hippocampus, the anterior corticomedial and medial amygdaloid nuclei, and the paraventricular and supraoptic nuclei of the hypothalamus, within 30-60 min (Pezzone et al., 1992; Imaki et al., 1993). C-fos expression was also enhanced in select diencephalic and brainstem regions innervated by projections from the DMH, including some thalamic nuclei, the habenula, the pontine nucleus and the locus coeruleus.

The dorsomedial hypothalamus has particularly strong projections to the locus coeruleus, as well as projections to the dorsal raphe nucleus, the pontine reticular
formation lateral to the medial raphe nucleus, the median raphe nucleus, the pontine raphe nucleus, the nucleus raphe magnus, raphe obscuris, and raphe pallidus nucleus (ter Horst and Luiten, 1986; Behzadi et al., 1990). This raises the possibility that alterations in neuronal activity within the DMH, mediated by CRF or corticosterone, may affect locus coeruleus noradrenergic and raphe serotonergic function.

The dorsomedial hypothalamus as a neuroanatomical substrate for maintenance of the homeostatic condition in vertebrates

The present study provides no information about the functional consequences of CRF or corticosterone-induced elevation of DOPAC, dopamine, 5-HIAA, or 5-HT within the DMN; however, one of the most well characterized functional roles for the DMH is the regulation of cardiovascular and behavioral responses to stress. These studies indicate that endogenous gamma-aminobutyric acid (GABA) suppresses the activity of a sympatho-excitatory mechanism in the DMH in rats which results in tachycardic and pressor responses (Anderson and DiMicco, 1990; Soltis and DiMicco, 1991a,b; Stoltz-Potter et al., 1994). Thus, microinjection of drugs that impair GABA-mediated synaptic inhibition in the DMH of rats generates cardiovascular and behavioral responses that mimic the response to stress. The cardiovascular responses, marked tachycardia and modest elevation of arterial pressure, following inhibition of GABA function are dependent on activation of local ionotropic and metabotropic excitatory amino-acid receptors (Soltis and DiMicco, 1991b, 1992; DiMicco and Monroe, 1994). Associated with the stress-induced tachycardia responses attributed to the DMH are stress-induced behavioral responses which are
dependent on the DMH (Shekhar et al., 1987, 1993; Inglefield et al., 1994; Shekhar and Katner, 1995). The elevations of dopamine and 5-HT observed following exposure to stressful stimuli as well as CRF or corticosterone administration may have pronounced effects on these autonomic and behavioral processes. Since both CRF and corticosterone alter the monoaminergic content of the dorsomedial hypothalamus in T. granulosa, it is unlikely that the effects on monoaminergic systems are responsible for CRF-induced locomotor activity. However, our observation that both CRF and corticosterone result in an increase in the tissue concentration of 5-HT within the DMN does not allow us to determine if CRF and corticosterone have the same effect on synaptic release of 5-HT in this region; thus synaptic release of 5-HT and consequent behavioral and physiological responses mediated within the dorsomedial hypothalamus may be affected differently by CRF and corticosterone. Regardless, since blockade of GABA_A receptor function in the region of the dorsomedial hypothalamus elicits escape-oriented locomotor activity (Shekhar and DiMicco, 1987), it remains possible that CRF-induced accumulation of dopamine or 5-HT may modulate escape-oriented locomotor activity or other autonomic or behavioral processes.

For example, in a study using 5,7-dihydroxytryptamine (5,7-DHT; a selective neurotoxin for serotonergic systems), hypothalamic lesions but not lesions of the raphe region resulted in facilitation of female sexual behavior (Luine et al., 1983). This suggests that serotonergic neuronal systems within the hypothalamus are critical for regulation of female sexual behavior. This is consistent with lesion studies in the
urodele amphibian which indicate that the monoamine-accumulating neurons of the
dorsomedial hypothalamus are critical for the performance of male sexual behavior
(Dubé et al., 1990). The observation in the present study that CRF and
corticosterone -- which both have suppressive effects on the performance of sexual
behavior in male *T. granulosa* (Moore and Miller, 1984) -- alter the tissue
concentrations of 5-HT in the dorsomedial hypothalamus is consistent with the
hypothesis that intrahypothalamic serotonergic systems regulate sexual behavior, and
raises the possibility that stress-related neurochemicals may regulate sexual behavior
in part through actions within the dorsomedial hypothalamus.

*Associations between CRF-induced locomotor activity and monoaminergic systems in vertebrates*

In the present study i.c.v. CRF administration resulted in increased locomotor
activity compared to saline-injected controls. This demonstrates that the doses of
CRF used were behaviorally active. This behavioral effect of CRF is consistent with
previous studies in rats (Sutton et al., 1982; Veldhuis and De Wied, 1984; Saunders
and Thornhill, 1986; Sherman and Kalin, 1987; Diamant and De Wied, 1991; Lee et
al., 1994) and the urodele amphibian, *Taricha granulosa* (Moore et al., 1984; Lowry
et al., 1990, 1993, 1994; Lowry and Moore, 1991). In rats and *T. granulosa*, CRF-
induced increases in locomotor activity are dose-related, time-dependent (Sutton et al.,
1982; Lowry et al., 1990), and persist in hypophysectomized animals (Moore et al.,
1984; Eaves et al., 1985). In addition, CRF- and stress-induced locomotor activity
are suppressed by the intracerebroventricular administration of α-helical CRF, a
competitive CRF antagonist (Rivier et al., 1984; Britton et al., 1986b; Winslow et al., 1989; Lowry and Moore, 1991). These studies and others (Britton et al., 1985, 1986a) provide evidence that CRF can act at sites within the central nervous system to stimulate locomotion in diverse vertebrate species. The sites of action for CRF-induced locomotor activity remain unclear.

Although the neurochemical mechanisms through which CRF induces locomotion have not been determined, the present study and previous studies (Dunn and Berridge, 1987; Kalivas et al., 1987; Lee et al., 1994; Lowry et al., 1993; Matsuzaki et al., 1989) clearly indicate that CRF-induced locomotor activity is associated with changes in metabolism of multiple monoaminergic systems, including noradrenergic, dopaminergic and serotonergic systems. For example, studies using microdissection with HPLC-ED techniques indicate that i.c.v. CRF-induced locomotor activity is associated with increased DOPAC or DOPAC:DA ratios in the prefrontal cortex, septum, striatum, nucleus accumbens, hypothalamus and brainstem (Dunn and Berridge, 1987; Kalivas et al., 1987; Matsuzaki et al., 1989), as well as increased 3-methoxy-4-hydroxy-phenylglycol (MHPG) or MHPG:norepinephrine ratios in the prefrontal cortex, amygdala, hypothalamus midbrain, locus coeruleus region, and brainstem (Dunn and Berridge, 1987; Emoto et al., 1993). Studies using in vivo microdialysis in freely moving rats indicates that i.c.v. CRF-induced locomotor activity is associated with elevated dialysate concentrations of norepinephrine in the hypothalamus (Lee et al., 1994). Consistent with this observation, i.c.v. administration of CRF elevates dialysate concentrations of norepinephrine, dopamine,
and several of their measurable metabolites in the medial hypothalamus and medial prefrontal cortex of rats (Lavicky and Dunn, 1993). Although dialysate concentrations of serotonin were not determined, concentrations of the metabolite 5-HIAA also were elevated in the medial hypothalamus and medial prefrontal cortex. These studies, together with studies in *T. granulosa* (Lowry et al., 1993), provide evidence that i.c.v. administration of CRF results in a concomittant activation of dopaminergic, noradrenergic, and serotonergic systems.

Several studies have addressed the question of whether or not CRF-induced locomotor activity is dependent on activation of monoaminergic receptor systems. Studies have demonstrated that i.c.v. CRF-induced locomotor activity can be suppressed by $\alpha$-flupenthixol and haloperidol, dopamine receptor antagonists, but only at doses that induced catalepsy (Koob et al., 1984; Imaki et al., 1985). In addition, destruction of dopamine nerve terminals using 6-hydroxydopamine within the nucleus accumbens did not block CRF-induced locomotor activity (Swerdlow and Koob, 1985). Microinjection of CRF directly into the dopamine cell body-containing region of the ventral tegmental area (VTA) of rats also induces increases in locomotor activity, but this effect also is insensitive to the dopaminergic receptor antagonist haloperidol (Kalivas et al., 1987), providing further evidence that CRF-induced locomotor activity is not dependent on activation of dopaminergic receptor systems.

The involvement of noradrenergic receptor systems in CRF-induced locomotor activity is more equivocal. For example, behavioral studies in rats indicate that $\alpha_1$ and $\alpha_2$ adrenergic receptor antagonists (phenolamine, prazosin, and yohimbine) can
suppress CRF-induced locomotor activity measured in a novel environment (Imaki et al., 1987); however the observation that administration of the $\alpha_2$ adrenergic receptor agonist, clonidine -- which can result in an inhibition of norepinephrine release from nerve terminals by acting on $\alpha_2$ receptors within the locus coeruleus (Langer, 1981) --, suppresses spontaneous but not CRF-induced locomotor activity in *T. granulosa* (Lowry et al., 1991) and the observation that microinfusion of CRF directly into the locus coeruleus did not alter locomotor activity (Butler et al., 1990), suggest that CRF-induced locomotor activity is not dependent on activation of the noradrenergic system at the level of the locus coeruleus.

Studies in *T. granulosa* suggest that CRF-induced locomotion is associated with a widespread activation of serotonergic neuronal systems. The selective serotonin reuptake inhibitor fluoxetine (Fuller et al., 1991) enhances locomotor activity in *T. granulosa* treated with a behaviorally subthreshold dose of CRF (but not in saline-treated animals); this effect is associated with a widespread depletion of serotonin throughout the central nervous system measured using HPLD-ED techniques (Lowry et al., 1993). In rats, CRF infused into the lateral ventricles dose-dependently increases the activity of tryptophan hydroxylase, the rate-limiting enzyme for serotonin synthesis (Corley et al., 1990; Singh et al., 1992). Collectively, these studies suggest that the central actions of CRF involve the activation of serotonergic neuronal systems. It remains uncertain if CRF-induced locomotor activity is dependent on the activation of serotonergic receptor systems.
Summary

In summary, the dorsomedial hypothalamus in vertebrates integrates behavioral and autonomic responses to physiological or environmental challenges to the organism. The accumulation of catecholamines and indoleamines within the dorsomedial hypothalamus following exposure to stressful stimuli, i.c.v. administration of CRF, or intraperitoneal administration of corticosterone in vertebrate species may alter homeostatic processes regulated by this structure. The mechanisms by which stress or stress-related neurochemicals alter the accumulation of monoamines in the dorsomedial hypothalamus, as well as the precise neuroendocrine, autonomic and behavioral consequences, are important questions for future investigations.

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CHAPTER VII: DISCUSSION

These studies establish that, in male *T. granulosa*, administration of the neuropeptide CRF into the cerebral ventricle stimulates locomotor activity in a dose- and time-dependent manner. In addition, these studies provide the first evidence that an endogenous CRF may be involved in the adaptive locomotor responses to stressful stimuli; administration of the CRF receptor antagonist α-helical CRF₉₋₄₁ suppressed the adaptive locomotor responses to handling and to exposure to a dangerously warm ambient temperature. These studies as well as the rapid nature of the behavioral responses to CRF administered directly into the lateral ventricle demonstrate that CRF can act to alter locomotor activity in a time-frame consistent with a role for this peptide in the "fight or flight" response. This rapid change in behavior elicited by intraventricular administration of CRF is associated with rapid changes in medullary neuronal activity, suggesting that there is a rapid functional reconfiguration of neuronal activity in response to CRF administration in this amphibian.

Microdissection and high performance liquid chromatography with electrochemical detection techniques were used to first describe the distribution of catecholamines and indoleamines throughout the central nervous system of male *T. granulosa*, then to identify site-specific effects of CRF and corticosterone administration on tissue concentrations of specific monoamines. In the survey of monoaminergic systems in male *T. granulosa* it was determined that the highest concentrations of monoamines, with the single exception of 5-HT concentrations in the serotonergic raphe system, are in regions of the hypothalamus that contain
cerebrospinal fluid-contacting, monoamine-containing neuronal cell bodies. A thorough review of recent literature suggests that these hypothalamic monoamine-containing neuronal systems may be more conserved evolutionarily than originally believed. For example, since the original description of the paraventricular organ (PVO) in nonmammalian vertebrates, it has been believed that there is no homologous structure present in the central nervous system of mammalian vertebrates. Based on connectivity as well as topographical, immunohistochemical, biochemical, and functional characteristics of this region, however, the PVO of nonmammalian vertebrates appears to be homologous to the dorsomedial hypothalamic nucleus (DMN) of mammals.

This is an important observation because in studies of the effects of CRF- and corticosterone-administration on the tissue concentrations of catecholamines and indoleamines, the region containing the PVO consistently responded with elevations in dopamine, DOPAC, 5-HT, and 5-HIAA. These responses are remarkably similar to the neurochemical changes observed within the DMN of rats following exposure to certain types of stressful stimuli. Together, these studies suggest that the dorsomedial hypothalamus of vertebrates may be an important neuroanatomical site for the actions of stress and stress-related neurochemicals on central nervous system function.

The changes in tissue concentrations of monoamines and monoamine metabolites within the dorsomedial hypothalamus following administration of CRF or corticosterone -- or following exposure to stressful stimuli -- may have important neuroendocrine, physiological, or behavioral consequences for the organism.
Although it is impossible at this time to determine what those consequences may be, studies of the DMH of rats demonstrate that excitation of this structure elicits 1) increases in heart rate, respiration, and blood pressure (DiMicco and Abshire, 1987; Anderson and DiMicco, 1990; Soltis and DiMicco, 1991a,b; Soltis and DiMicco, 1992; Shekhar, 1993; DiMicco and Monroe, 1994; Stoltz-Potter et al., 1994), 2) hyperglycemic reactions accompanied by increases in plasma catecholamine levels (Frohman and Bernardis, 1971; Hasegawa et al., 1993), 3) increases in brown fat temperature (Kelly and Bielajew, 1991), 4) hyperthermia (Sellami and Beaurepaire, 1993), and 5) behavioral effects including a suppression of feeding (Takaki et al., 1992), an increase in locomotor activity (Shekhar and DiMicco, 1987), as well as increases in indices of fear and anxiety (Shekhar et al., 1987, 1990; Shekhar, 1993; Inglefield et al., 1994). Since all of these physiological and behavioral effects also have been identified as central actions of CRF (reviewed by Dunn and Berridge, 1990), it is reasonable to propose the hypothesis that CRF may stimulate elements within the dorsomedial hypothalamus and thereby initiate and coordinate this suite of physiological and behavioral responses. Testing the validity of this hypothesis -- as well as determining the cellular mechanisms through which CRF and corticosterone alter tissue concentrations of dopamine, DOPAC, 5-HT, and 5-HIAA within the dorsomedial hypothalamus -- are important areas for future research.

In summary, these studies establish that administration of CRF into the cerebral ventricle of male T. granulosa rapidly elicits behavioral and neurochemical effects that are also observed following exposure of vertebrate organisms to stressful
stimuli. These observations and others described in this thesis provide evidence that CRF actions are important in the adaptive behavioral response to stressful stimuli in this amphibian and, consequently, that this functional role for CRF is an evolutionarily ancient feature of this neuropeptide.
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