INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600
ANATOMICAL LOCALIZATION, SYNAPTIC RELATIONSHIPS, AND
PHYSIOLOGICAL EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE IN THE
MOUSE INFERIOR OLIVARY COMPLEX

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Kathleen V. Gregg

The Ohio State University
1997

Dissertation Committee:                  Approved by
G.A. Bishop, Adviser
J.S. King
R. Nordlander

Neuroscience Graduate Studies Program
ABSTRACT

The inferior olivary complex is the sole source of climbing fiber afferents to the cerebellum. As such, any change in the firing rate of olivary neurons will alter Purkinje cell activity and ultimately, cerebellar output. Neuromodulators are a class of neuromodulators which are known to produce long lasting changes in firing rate in addition to altering neuronal responsiveness to neurotransmitters. The purpose of this dissertation is to investigate the localization, synaptic relationships and physiological effects of the neuromodulator calcitonin gene-related peptide (CGRP) in the adult C57BL/6J mouse. To determine the localization of CGRP, immunohistochemistry was carried out with the indirect PAP antibody method and processed with glucose oxidase. Camera lucida drawings of sections representing four levels of the inferior olive illustrate that CGRP is densely distributed throughout all nuclei of the olive. To study the synaptic relationships of CGRP, pre-embedding immunohistochemistry was carried out and sections were analyzed with the electron microscope. Sections representing all levels of the olive reveal CGRP-IR in dendritic shafts and dendritic spines. Both populations receive synaptic inputs from non-CGRP labeled axon terminals. CGRP-immunoreactive axons have also been identified; double labeling studies in the mouse indicate that these CGRP-containing afferents arise from
the locus coeruleus and the nucleus subparafascicularis (Peltier et al, 1996). Preliminary extracellular recordings indicate that CGRP inhibits both spontaneous firing of olivary neurons and the excitatory effects elicited by specific amino acids.

Therefore, CGRP appears to play two roles in olivary physiology. The locus coeruleus or nucleus subparafascicularis may use CGRP to modulate the responsiveness of olivary neurons in regards to information about the physiological state of an organism. CGRP produced by olivary neurons and released from dendrites and dendritic spines may modulate responsiveness in an autocrine/paracrine fashion. In summary, CGRP is the first peptide present in both afferent terminals and olivary dendrites. This suggests complex interactions between olivary neurons and their afferents sources.
DEDICATED

To my parents,

for your constant love and support

iv
ACKNOWLEDGEMENTS

I would like to thank my adviser, Dr. Georgia Bishop for her amazing patience and guidance over the last four years. She has been an inspiration as a scientist as well as a teacher and I have enjoyed "tagging along" in the lab. I know the insights I've gained will aid me for years to come.

Dr. James King has challenged me continually over the last four years to analyze my thoughts, views, and perspectives. I respect your wisdom and humor.

Katharine Dillingham and Barbara Diener-Phelan have provided an enormous contribution in technical assistance and those tips which only experience can give. Their support has been instrumental in completing this degree.

There are so many other friends and family whose support and confidence in my abilities have made this degree possible. I want to thank everyone, who in small or large ways, have encouraged and supported me in reaching this goal.

And finally, thanks to my God. For You are the Author of all life and I thank you for the privilege of studying Your Creation. When I began this journey, I said I couldn't do it without Your strength and guidance every day. How true that has been! This is truly Your work.
VITA

April 30, 1969 ............................................... Born - Grass Valley, California

1987-1991 ...................................................... Research Assistant, Teaching Assistant
Dr. Paul Madtes, Jr. and Dr. C. Sue Justis
Point Loma Nazarene College
San Diego, California

1991 ............................................................ B.A. Biology/Chemistry, Point Loma
Nazarene College, San Diego, California

1992 ............................................................ Neuroscience Graduate Research Associate
The Ohio State University, Columbus, Ohio

1996 ............................................................ National Institute Health Training Grant
Fellow

PUBLICATIONS


FIELD OF STUDY

Major Field: Neuroscience

Major Study: Anatomy and physiology of neuropeptides in cerebellar circuits
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xvi</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter:</td>
<td></td>
</tr>
<tr>
<td>1. Peptide Localization in the Mouse</td>
<td></td>
</tr>
<tr>
<td>Inferior Olive</td>
<td>11</td>
</tr>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>13</td>
</tr>
<tr>
<td>Results</td>
<td>16</td>
</tr>
<tr>
<td>Discussion</td>
<td>18</td>
</tr>
<tr>
<td>2. Synaptic Localization of Calcitonin Gene-Related Peptide in the Mouse</td>
<td>37</td>
</tr>
<tr>
<td>Inferior Olivary Complex</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>37</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>38</td>
</tr>
<tr>
<td>Results</td>
<td>40</td>
</tr>
<tr>
<td>Discussion</td>
<td>41</td>
</tr>
</tbody>
</table>
3. Physiological Effects of Calcitonin Gene-Related Peptide in the Mouse

Inferior Olive .................................................. 53
  Introduction .................................................. 53
  Materials and Methods .................................. 54
  Results ...................................................... 56
  Discussion ................................................ 59

List of References ........................................... 72
Figure 1 is a diagrammatic representation of the experimental paradigm for the physiology experiments described in Chapter III. A transverse section of the medulla depicts the intermediate level of the inferior olivary complex (IOC) located in the ventral aspect of the brainstem. An enlarged figure of the IOC at that level indicates the major subdivisions including the medial accessory olive (MAO), dorsal accessory olive (DAO), principal olive (PO) and several smaller subdivisions including the ventral lateral outgrowth (vlo), and the ventral and dorsal lamella of the principal olive (vPO and dPO, respectively). The tip of a multibarrel electrode is adjacent to an olivary neuron in the vlo. The center barrel is the recording barrel and the four outer barrels are connected to a current ejector. The outer barrels may contain one or more of the following compounds: glutamate, calcitonin gene-related peptide, calcitonin gene-related peptide (8-37), saline or distilled water.

Figure 2 describes the relationship of a climbing fiber afferent and a Purkinje cell. The structure labeled as (a) is a Purkinje cell body with a dendritic arbor and an axon arising from opposing sides of the neuron. A climbing fiber afferent (b) intertwines around the dendritic arbor of the Purkinje cell. The climbing fiber on the left branches to innervate two Purkinje cells, while the climbing fiber on the right innervates only one neuron in the diagram.

Figure 3 is a series of camera lucida drawings that illustrate the distribution of varicosities immunoreactive (IR) for: A) calcitonin gene-related peptide (CGRP-IR), B) cholecystokinin (CCK-IR), C) corticotropin releasing factor (CRF-IR), D) substance P (Sub P-IR) and E) enkephalin (ENK-IR) in the caudal MAO. CGRP-IR (3A) and CCK-IR (3B) have a dense distribution. CRF-IR (3C) and Sub P-IR (3D) are lightly scattered and ENK-IR (3E) varicosities are focused along the medial, ventral and lateral borders of the nucleus.

Figure 4 is a series of camera lucida drawings that illustrate the distribution of A) CGRP-IR, B) CCK-IR, C) CRF-IR, D) Sub P-IR and E) ENK-IR varicosities.
in the caudal MAO and DAO. CGRP-IR (4A), CCK-IR (4B) and CRF-IR (4C) have a dense distribution in both nuclei, although the CRF-IR density decreases laterally. Sub P-IR (4D) has developed a focus in the lateral pole of the MAO, while immunoreactivity elsewhere remains scattered. ENK-IR (4E) in the DAO is heavy; the distribution in the MAO is the same as seen in Fig. 4E.

Figure 5 is a series of camera lucida drawings that illustrate the distribution of A) CGRP-IR, B) CCK-IR, C) CRF-IR, D) Sub P-IR and E) ENK-IR varicosities in the rostral MAO and DAO, and the principal olive (PO). As seen previously, CGRP-IR (5A), CCK-IR (5B) and CRF-IR (5C) is dense throughout all nuclei. CCK-IR (5B) demonstrates very dense levels of labeling in the vlo, dorsal lamella of the principal olive (dPO) and the lateral pole of the DAO. Sub P-IR (5D) is focused in the vlo and a moderate band in the vPO. ENK-IR (5E) is densest along the medial border, in the vlo, along the ventral and lateral border of the MAO, ventral lamella of the principal olive (vPO) and DAO. Note the complete absence of Sub P-IR (5D) and ENK-IR (5E) in the dPO.

Figure 6 is a series of camera lucida drawings that illustrate the distribution of A) CGRP-IR, B) CCK-IR, C) CRF-IR, D) Sub P-IR and E) ENK-IR in the rostral olive. At this level, the PO and the DAO cannot be morphologically distinguished and are represented as one structure. CGRP-IR (6A), CCK-IR (6B) and CRF-IR (6C) distribution is relatively unchanged in density from previous levels. CRF-IR (6C) is less dense laterally than medially. Sub P-IR (6D) is focused in the vlo and relatively absent from the lateral olive and the MAO. ENK-IR (6E) remains densest medially and ventrally in the MAO, heavy dorsally in an area approximate to the DAO and completely void in laterally in what probably remains of the PO.

Figures 7 is a series of photomicrographs comparing immunolabeling in the only subnuclei densely labeled by all five peptides: the dorsal cap of Kooy (dc) and the ventral lateral outgrowth (vlo). Small and large arrows indicate varying sizes of varicosities for each peptide, which may indicate multiple sources of peptidergic afferents. A) ENK-IR in the vlo; B) CGRP-IR in the vlo; C) CRF-IR in the dc including immunoreactive cell bodies (arrowheads); D) CCK-IR in the dc; E) Sub P-IR in the dc. Varicosities immunoreactive for
ENK (Fig. 7A), CCK (Fig. 7D) and Sub P (Fig. 7E) are very similar in morphology and density of distribution, suggesting possible colocalization of peptides. Fig. 7F is a photomicrograph of the dc of a control section processed in parallel to normal except that it was not exposed to primary antibody. There is no labeling of varicosities in the control section, as can be seen by comparing this to surrounding photomicrographs for any of the peptides. Calibration bar in A is 50 μm and applies to A-E. Calibration bar in F is 50 μm.

Figure 8 is a series of photomicrographs demonstrating immunoreactive foci for each peptide in several subnuclei of the olive. In all figures, large and small arrows indicate varying sizes of immunoreactive varicosities, suggesting multiple sources of peptidergic afferents to the olive. Fig. 8A is a low power micrograph of a section immunostained for ENK and drawn in Fig. 4C. Note the density of varicosities in the DAO and lateral MAO (block arrow). Fig. 8B is a high power micrograph of the lateral MAO taken from the area indicated by the black arrow in Fig. 8A. The blood vessel in the upper left (large arrowhead) corresponds to the one in Fig. 8A. Fig. 8C demonstrates CGRP-IR in the caudal MAO. Note the fibers and the dense distribution of small varicosities. CRF-IR is shown in the dc in Fig. 8D as well as CRF immunolabeled cell bodies (arrowhead). CCK-IR (Fig. 8E) and Sub P-IR (Fig. 8F) varicosities are illustrated in the lateral portion of the caudal MAO. Calibration bar in A is 200 μm. Calibration bar in B is 50 μm and applies to B-F.

Figure 9 is an electronmicrographic montage demonstrating calcitonin gene-related peptide immunoreactivity (CGRP-IR) in the dorsal cap of Kooy. One olivary cell body is heavily labeled, with HRP reaction product almost obscuring the nucleus and organelles in the cytoplasm (*). Adjacent to the cell body, a labeled axon (♀) is present. Small, clear synaptic vesicles can be seen within the axon. No synaptic junction is present within the plane of section. Throughout the micrograph, several profiles containing electron-dense material, mitochondria and vesicle-like structures are present. These characteristics are consistent with that of a dendritic spine. However, since the HRP product obscures the ultrastructure and serial sections were not cut, it is not possible to definitely identify them as such. At the bottom of the micrograph is a longitudinal section through a labeled dendrite (d). The dendrite gives rise to a stalk (st) which may be the source of some of the nearby spines. Microtubules can be seen in the dendrite (arrows), but not in the stalk. To compare the dendrite with an axon cut in cross section (a) near the cell body, the cross sectional
diameter of the dendrite (0.64 μm) is much greater than that of the axon (0.2 μm) and the shape of the axon is much more spherical. Although both the dendrite and the axon contain microtubules (cut longitudinally in the dendrite and in cross section in the axon), the axon does not contain the electron dense material seen in the dendrite. Again, due to the heavy HRP label, identification of the dendrite is difficult.

Figure 10. A) This electron micrograph is from the dorsal cap of Kooy. An olivary neuron is labeled (*) with reaction product over the nucleus and over polyribosomes and rough endoplasmic reticulum (RER) in the cytoplasm. A small axon (a), adjacent to the cell body with a cross sectional diameter of 0.38 μm is postsynaptic to an unlabeled axon terminal. B) This light micrograph demonstrates CGRP-IR climbing fibers (arrows) in the mouse cerebellar cortex. C) CGRP-IR varicosities (arrows) and cell bodies (arrowheads) are visible in a 0.2μm thick section of flat embedded tissue stained with Toluidine Blue from the lateral pole of the caudal MAO. Note that almost every cell body in the field of the micrograph is labeled, suggesting that a majority of cell bodies in the caudal MAO contain CGRP.

Figure 11. Two electron micrographs representing dendrites labeled with CGRP-IR in the lateral portion of the caudal MAO. A) A dendrite (*) featuring symmetrically labeled neurotubules is postsynaptic to an unlabeled axon containing ellipsoidal synaptic vesicles. Two more dendritic profiles (X) in the field of view are labeled. Note the absence of the electron dense material present in the dendritic spines in Fig. 1. B) Two dendrites (*) exhibit labeled neurotubules. Both spines are postsynaptic to unlabeled axons containing ellipsoidal vesicles. These photomicrographs suggest that CGRP-IR dendrites may be postsynaptic to axons containing GABA.

Figure 12 demonstrates the effect of CGRP on Quis driven firing rate. A. Application of quisqualate (Quis) at -10nA produces a firing rate of 0.6Hz. At -5nA, the firing rate decreases to 0.4Hz, demonstrating that Quis can elicit a dose-dependent response. CGRP applied at increasing currents of +10, +20, and +30nA produces a dose dependent abolishment of the firing rate induced by Quis. B. The spontaneous firing rate of 0.2-0.4Hz is increased to 1-1.2Hz by the application of -20nA of Quis. Pulses of CGRP at +30nA inhibits the Quis driven activity (firing rate drops to 0.2-0.6Hz). CGRP abolishes spontaneous
firing rate. Quis application is unable to overcome this inhibition (asterisk) until the CGRP is turned off. .......................... 65

Figure 13 demonstrates the ability of CGRP to inhibit Quis induced firing.
A. Spontaneous firing at <0.2Hz is abolished by CGRP application at +40nA. Quis application following CGRP has no effect (asterisk) until the CGRP pulse is terminated. B. Co-application of Glut and Quis increases spontaneous firing to 0.2-0.8 Hz. As seen previously, CGRP application decreases EAA driven firing as well as inhibiting spontaneous activity. When applied before the EAAs, CGRP inhibited their excitatory effects (asterisk). This inhibition continued for the duration of the CGRP pulse. .......................... 67

Figure 14 demonstrate the physiological effects of CGRP (8-37). A. Quis replication at -20 nA produced a firing rate of 0.8Hz in this neuron from the caudal DAO. CGRP (+20nA) had a slight inhibitory effect, although this was not consistent with repeated application. The CGRP, receptor specific antagonist, CGRP (8-37) had a potent inhibitory effect on Quis driven firing, consistently abolishing firing rate. During co-application of Quis and CGRP, short pulses of the antagonist, CGRP (8-37) abolished firing rate as previously seen; however, following the antagonist, the firing rate increased to 1.2 Hz, facilitating the ability of Quis to drive firing rate. B. This neuron from the MAO had no spontaneous firing rate, but was responsive to -30 nA of Quis. Firing rate was inhibited during CGRP application, but following application, was potentiated above that previously seen for Quis alone. CGRP (8-37) had a similar effect on firing rate, also producing a rebound effect. When a pulse of CGRP was applied during Quis and CGRP (8-37), an additive inhibitory effect was visible. .......................... 69

Figure 15 demonstrates the effect of saline control. As seen before, Quis increases spontaneous firing. As a negative control, the solvent for CGRP, saline, was applied with a positive DC current and had a greater inhibitory effect that CGRP. CGRP application delays the ability of Quis to increase firing (asterisk) and this inhibition persisted for the duration of the CGRP pulse. ... 71
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin releasing factor</td>
</tr>
<tr>
<td>ENK</td>
<td>enkephalin</td>
</tr>
<tr>
<td>dc</td>
<td>dorsal cap</td>
</tr>
<tr>
<td>DAO</td>
<td>dorsal accessory olive</td>
</tr>
<tr>
<td>IOC</td>
<td>inferior olivary complex</td>
</tr>
<tr>
<td>MAO</td>
<td>medial accessory olive</td>
</tr>
<tr>
<td>dPO</td>
<td>dorsal lamellae of the principal olive</td>
</tr>
<tr>
<td>vPO</td>
<td>ventral lamellae of the principal olive</td>
</tr>
<tr>
<td>Sub P</td>
<td>Substance P</td>
</tr>
<tr>
<td>vlo</td>
<td>ventral lateral outgrowth</td>
</tr>
</tbody>
</table>
INTRODUCTION

The inferior olivary complex (IOC) has been the subject of scientific investigation as early as Ramon y Cajal's anatomical studies (Ramon y Cajal. 1909; Ramon y Cajal. 1911). The projections of olivary neurons were later found to be climbing fiber (CF) afferents of the cerebellar cortex (Szentagothai and Rajkovits, 1959). As one of two primary afferent systems to the cerebellar cortex, climbing fibers quickly became an important focus of investigation for many cerebellar scientists.

In addition to their role in cerebellar circuits, several features of olivary neurons and the CF afferents they form, make them quite unique within the adult CNS. First, clusters of neurons within the inferior olive are connected via dendro-dendritic gap junctions (Sotelo, Llinas, and Baker, 1974). Second, olivary neurons exhibit spontaneous oscillations in their membrane potential and firing rates (Llinas and Yarom, 1986). Third, the firing rates of olivary neurons are quite low for the CNS, only 1-2 Hz at rest (Armstrong. 1967; Harvey. 1967) and even at maximal stimulation, only 10 Hz (Armstrong, 1974). Fourth, the CF synapse on the Purkinje cells is one of the most powerful excitatory synapses within the adult CNS.

Figure 1 is a diagram of a transverse section from the center of the inferior olivary complex (IOC) in the mouse, showing its location within the brainstem. The diagram also illustrates the experimental design for the extracellular physiology experiments described in
Chapter III. The mammalian inferior olivary complex (IOC) consists of three primary nuclei: the medial accessory olive (MAO), principal olive (PO) and dorsal accessory olive (DAO) (Kooy, 1917). Other subdivisions (some not indicated in the figure) include the dorsal and ventral lamellae of the principal olive (dPO and vPO, respectively), the dorsal cap of Kooy, the beta nucleus in the cat, the ventral lateral outgrowth (vlo) of the PO and the dorsomedialateral cell column (dmcc)(Brodal, 1940).

Olivary neurons in the cat range in size from 20-30 μm in diameter (Sotelo, Llinas, and Baker, 1974). They are multipolar neurons, giving rise to dendrites from any part of the soma (Cadieux, Pomerleau, St-Pierre, and Fournier, 1990). These dendrites branch extensively and the secondary and tertiary branches fold back toward the soma, creating a heavy dendritic arbor surrounding the cell for up to 200 μm (Sotelo, Llinas, and Baker, 1974).

At the ultrastructural level (Sotelo, Llinas, and Baker, 1974; King, 1976), dendritic elements are grouped into synaptic clusters (glomeruli), consisting of 4-10 dendritic spines surrounded by axon terminals containing clear spherical vesicles, pleiomorphic vesicles and/or large dense core vesicles (LDCVs). Astrocytic processes surround the entire structure. The dendritic elements within a synaptic cluster are joined by gap junctions which provide the basis for synchronous activity of olivary neurons. Some gap junctions have been observed outside of synaptic clusters between dendrites (Sotelo, Llinas, and Baker, 1974). The majority of synapses occur as axodendritic synapses within synaptic clusters or on dendritic shafts outside of the clusters. Very few synapses have been seen on cell bodies.
Although this paper will not discuss the afferent projections to the olive in any great detail, one projection in particular is important to discuss. This is the projection from the cerebellar nuclei to the IOC. Most of the GABAergic input to the IOC is from the cerebellar nuclei and this projection is topographically arranged (DeZeeuw, Holstege, Ruigrok, and Voogd, 1990). These GABAergic synapses are often seen near gap junctions within synaptic clusters (glomeruli). A theory proposed by Llinas and Welsh (1993) concludes that the GABAergic input to the synaptic cluster effectively "decouples" olivary neurons, allowing neurons to act independently. This may be a type of feedback circuit, where olivary input to the cerebellar nuclei and cortex results in inhibition within the olive.

The olivary projections to the cerebellum are described in Figure 2. CF projections from the olive are crossed and give off collaterals to the deep nuclear cells of the cerebellum as they travel to the cortex (Beitz, 1976; Chan-Palay, 1977). Each CF intimately wraps around the dendritic tree of the Purkinje cell forming synapses on the proximal, smooth portion of the dendrites (Armstrong, 1974). Each Purkinje cell is innervated by branches of a single CF; each CF innervates 10 - 20 Purkinje cells.

A second primary afferent to the cortex is the mossy fiber (MF) system, arising from various brainstem and spinal cord sources (not shown). MF afferents synapse on granule cell dendrites in the granular layer and the axons of granule cells form parallel fibers (PF) in the molecular layer. Whereas each Purkinje cell receives input from only one CF, approximately 200,000 PF provide weak excitatory input to a single Purkinje cell. Another difference between CF and PF is that PF synapse more distally on Purkinje cell dendrites.
A third system of afferent projections to the cerebellum is the monoaminergic system which consists of a beaded plexus of fibers in the granule, Purkinje and molecular layers.

Purkinje cells are the only cortical neurons with efferent projections leaving the cerebellar cortex. These GABAergic projections synapse on neurons within the deep nuclei of the cerebellum. Thus, firing of olivary neurons produces an increase of firing in the deep nuclei due to collaterals from CF which is followed by a period of inhibition as Purkinje cells excited by CF, release GABA that acts on deep nuclear neurons.

To provide a brief overview of the afferent and efferent connections of the IOC, it is useful to first consider the major functional divisions of the cerebellum: the vestibulocerebellum, spinocerebellum and cerebrocerebellum (Ghez 1991). The vestibulocerebellum is confluent with the flocculonodular lobe. It is concerned with head and eye movements, balance and posture (Ghez 1991). In the cat and rabbit it receives CF projections from the dorsal cap of Kooy, dorsomediolateral cell column (dmcc), ventrolateral outgrowth of the PO, and rostral MAO (Walberg 1980). These areas, in turn, receive afferent projections from vestibular nuclei in the brainstem.

The spinocerebellum is concerned with modification of ongoing motor performance (Ghez 1991). It consists of the anterior lobe and the vermal and intermediate lobules of the posterior lobe. It receives CF afferents from the MAO, DAO, and dPO. These areas of the IOC, especially the MAO and DAO, are often referred to as the "spinal olive". They receive input from the spinal cord, dorsal column nuclei and other areas of the CNS which integrate information from the periphery (Walberg 1980). Although this accounts for only a small portion of its input, the other sources of afferent information are largely unknown.
The cerebrocerebellum is important in planning future movement (Ghez 1991). It consists of the lateral hemispheres of the posterior lobe of the cerebellum and receives CF input from the principal olive, rostral MAO and rostral DAO. These areas of the IOC receive input from sensorimotor areas of the cortex and regions of the midbrain (Walberg 1980).

Interest in the olive has continued as investigators tried to understand its role in cerebellar circuits and motor output. Ito et al. (Ito, Sakurai, and Tongroach, 1982; Ito, 1993) have proposed that CF modify the responsiveness of the Purkinje cells to parallel fiber input through a mechanism known as long term depression (LTD). Another theory, proposed by Thompson et al. (1993) suggests that the cerebellum is vital for motor learning.

A third theory proposes that there is no motor learning in the cerebellum (Llinas and Welsh, 1993). Rather, CF input is important in motor coordination. In this model, it is the group of neurons in the deep cerebellar nuclei acted upon by CF and Purkinje cells and the timing of the excitation or inhibition that are critical in the coordination of movement.

Any neuroactive chemical which alters the responsiveness of olivary neurons or their electrotonic coupling via gap junctions, has the potential to alter Purkinje cell firing and cerebellar function. It is our hypothesis that neuropeptides modulate the physiological activity of olivary neurons. The focus of this study is on the peptide calcitonin gene-related peptide (CGRP) which we found to have an extensive distribution in the olivary complex. We propose that the peptide modulates olivary firing rate by altering Ca$^{2+}$ channels in both the dendrites and soma of olivary neurons. CGRP is a 37-amino acid peptide produced by
alternative splicing of the calcitonin gene (Amara, Jonas, Rosenfeld, Ong, and Evans, 1982). It is found throughout the body in multiple organ systems, and although its function in many of these systems has been studied, its actions in the CNS are relatively unknown.

As a first step in testing this hypothesis anatomical studies were carried out to determine its distribution and synaptic relationship and physiological studies were initiated to determine if the peptide had any effect on olivary firing rate. Three experimental techniques were used in this thesis: 1) immunohistochemistry at the light microscopic level to localize CGRP-immunoreactive varicosities within olivary nuclei, 2) immunohistochemistry at the electron microscopic level to localize CGRP with respect to synaptic structures, and 3) extracellular unit physiology to determine the effect of CGRP on olivary firing rate and its interaction with excitatory amino acids (EAAs) within the olive. Data from these experiments demonstrate that CGRP is densely distributed throughout the IOC and is localized in both axons, and dendritic processes. Its action on olivary neurons is primarily suppressive, although it less frequently works to potentiate the response to EAAs. Thus, CGRP may occupy a unique role in the mouse inferior olive. The present findings suggest that this peptide is not only released from afferent terminals, but also from olivary dendrites.
Figure 1 is a diagrammatic representation of the experimental paradigm for the physiology experiments described in Chapter III. A transverse section of the medulla depicts the intermediate level of the inferior olivary complex (IOC) located in the ventral aspect of the brainstem. An enlarged figure of the IOC at that level indicates the major subdivisions including the medial accessory olive (MAO), dorsal accessory olive (DAO), principal olive (PO) and several smaller subdivisions including the ventral lateral outgrowth (vlo), and the ventral and dorsal lamella of the principal olive (vPO and dPO, respectively). The tip of a multibarrel electrode is adjacent to an olivary neuron in the vlo. The center barrel is the recording barrel and the four outer barrels are connected to a current ejector. The outer barrels may contain one or more of the following compounds: glutamate, calcitonin gene-related peptide, calcitonin gene-related peptide (8-37), saline or distilled water.
Figure 2 describes the relationship of a climbing fiber afferent and a Purkinje cell. The structure labeled as (a) is a Purkinje cell body with a dendritic arbor and an axon arising from opposing sides of the neuron. A climbing fiber afferent (b) intertwines around the dendritic arbor of the Purkinje cell. The climbing fiber on the left branches to innervate two Purkinje cells, while the climbing fiber on the right innervates only one neuron in the diagram.
CHAPTER 1

PEPTIDE LOCALIZATION IN THE MOUSE INFERIOR OLIVE

INTRODUCTION

The inferior olivary complex (IOC) is the sole source of climbing fibers (CF) to the cerebellar cortex (Armstrong, 1974). Several theories have been proposed to define the role of olivocerebellar circuitry in controlling motor activity. One theory proposes that climbing fibers modify the ability of Purkinje cells to respond to input derived from parallel fibers, a second source of afferents to Purkinje cells (Ito, 1993). A second theory suggests a primary role for olivary neurons in directly controlling the dynamic properties of motor activity (Llinas and Welsh, 1993). Although the theories vary, one common concept between them is that the output of the cerebellum is highly regulated by the input it receives from climbing fibers originating in the inferior olivary complex. Thus, any change in the firing rate or responsiveness of olivary neurons may have a significant effect on cerebellar output and subsequently on motor activity.
Neuropeptides are a class of neuroactive chemicals shown to modulate neuronal firing rate in several systems (Hokfelt, 1991). They may act by altering membrane potential or by modulating ion channels to bring about their effects (Hokfelt, 1991). Not only do they act independently to change firing rate, but they also alter the responsiveness of a neuron to a classic neurotransmitter (Hokfelt, 1991).

This paper begins a series of investigations to determine if peptides modulate physiological activity within the inferior olivary complex (IOC). Several of the peptides analyzed in this paper have been individually identified in the IOC of different species in separate studies (Petrusz, Merchenthaler, and Maderdrut 1985; Vanderhaeghen 1985; King, Ho, and Bishop, 1989; Bishop and Ho, 1984; Palkovits, Brownstein, and Vale, 1983; Kawai et al. 1985; Skofitsch and Jacobowitz, 1985). In comparing results, there are species differences with respect to the density and distribution of these peptides. Due to these species differences, it is important to verify peptide localization in the mouse IOC before physiological studies can begin. Immunohistochemistry was employed to determine if five neuropeptides known to be present and functional in cerebellar circuitry, namely: cholecystokinin (CCK), calcitonin gene-related peptide (CGRP), corticotropin releasing factor (CRF), enkephalin (ENK) and substance P (Sub P) (King, Cummings, and Bishop, 1992; Bishop, 1990; Bishop, 1991; Bishop, 1995) were present in the IOC. Further, the differential distribution of these peptides were analyzed and compared. These data will serve to define the anatomical substrate(s) for ongoing studies in the laboratory which are focusing on defining the physiological role of selected peptides in the IOC.
This study was carried out in the mouse for several reasons. First, due to the availability of several disease models in mice, an increasing amount of research is being conducted in this species. Second, there are several spontaneous mutations in cerebellar circuitry in this species which will allow us to better address the role of peptides in the system in future studies. Finally, the mouse has been used extensively in transgenic studies, some of which involve alterations of cerebellar circuits. However, before a disease or altered genetic process can be addressed, a foundation of normal mouse anatomy and physiology must be established.

MATERIALS AND METHODS

Immunohistochemical procedure

Adult C57BL/6J mice were deeply anesthetized with sodium pentobarbital (7mg/100g) given intra peritoneally and perfused through the aorta with 0.9% saline followed sequentially by 4% paraformaldehyde in phosphate buffer (pH 6.5) and 4% paraformaldehyde in borate buffer (pH 11). The brain was removed and stored in phosphate buffer plus 20% sucrose overnight. 50μm transverse sections of the cerebellum and brainstem were cut on the freezing microtome or vibratome (CRF only). The sections were placed in a primary antibody against CGRP (Amersham, diluted 1:2500), CRF (Incstar, 1:500), ENK (1:5000), CCK (1:5000) or Sub P (1:1000) The last three were kindly provided by Dr. Raymond Ho. All primary antibodies were raised in rabbit and sections were incubated for 36-48 hours with constant agitation. Sections were then sequentially incubated at room temperature in sheep anti-rabbit IgG (1:500) and rabbit peroxidase-anti-peroxidase (1:500). For sections incubated
in CCK, CGRP, ENK or Sub P, the HRP reaction was catalyzed with 0.05% 3,3'dianinobenzidine tetrahydrochloride (DAB) to which 0.006% H$_2$O$_2$ had been added. For processing of tissue incubated in CRF, the HRP reaction was catalyzed using the glucose oxidase-DAB-nickel method (Shu, Ju, and Fan, 1988). Sections were mounted on clean glass slides, dehydrated through a graded series of alcohol, cleared in xylene, and coverslipped. A minimum of three cases were analyzed for each peptide.

Absorption and non-primary antibody control experiments

Two types of control experiments were conducted. Specificity of primary antibodies for their peptide has been previously addressed in absorption control experiments carried out in this laboratory using the same immunohistochemical technique and antibodies employed in this study for CCK (King and Bishop, 1990), CRF (Cummings, Elde, Ells, and Lindall, 1983), ENK (King, Ho, and Bishop, 1986), and Sub P (Bishop and Ho, 1984). Specificity studies for CGRP were carried out by the commercial source (Amersham, personal communication). To control for nonspecific staining by the secondary antibody, sections were incubated in the antibody diluent (PBS with 0.3% Triton X-100) without the primary antibody. The rest of the processing was carried out as described above.
Analysis of peptide distribution

Every section through the IOC was analyzed for the presence of peptide-immunoreactive varicosities. Representative sections were selected and the distribution of immunolabeled varicosities was mapped under an oil immersion lens at 63X with the use of a drawing tube and selected sections were photographed.

Kooy's terminology

Kooy (1917) has previously described the morphology and nuclear groups of the inferior olive of the mouse and we have used his terminology in the present study. Kooy identified the medial accessory olive (MAO), dorsal accessory olive (DAO), dorsal lamella of the principal olive (dPO) and ventral lamella of the principal olive (vPO). We have also included more recently defined subnuclei including the dorsal cap of Kooy (dc) and ventral lateral outgrowth (vlo) in our description because the morphology of these nuclei in the mouse is similar to what has been described in other species (Foster and Peterson, 1986; Saint-Cyr and Courville 1980). In the present study, the PO is used as a landmark to differentiate the caudal and rostral aspects of the IOC. Any portion of the MAO or DAO in a section not containing the PO is considered caudal. If the PO is present, the MAO or DAO are considered the rostral aspects of these nuclei. Likewise, the dorsomedial tip of the IOC is referred to as the dc caudally, and the vlo rostrally.
For our analysis of peptide localization in the inferior olive, we plotted the distribution of each peptide on four representative transverse sections (Figs 3-6). Each figure depicts the distribution of the five peptides at a specific transverse level of the IOC. Fig. 3 is at the level of the caudal IOC, and Fig. 6 is at the rostral pole. Figs. 4 and 5 are at two intermediate transverse levels of the nuclear complex.

RESULTS

Caudal MAO

All five peptides are present throughout the IOC, however, they have unique patterns of distribution and density at each level. The peptides labeled varicosities that ranged in size from approximately 1 μm to 5 μm. Varicosities immunolabeled by CGRP (Fig. 3A, 4A, 8C), CCK (Fig. 3B, 4B, 8E) or CRF (Fig. 3C, 4C) are densely distributed throughout the nucleus. However, the density of distribution of CRF at the most caudal levels of the MAO (Fig. 3C, 4C) is not as great as for the other peptides. Varicosities immunolabeled for Sub P are sparsely scattered in the caudal MAO (Fig. 3D) except for a focus in the lateral pole (Fig. 4D, 8F). A few scattered ENK labeled varicosities are observed medially, whereas numerous ENK-labeled varicosities are distributed laterally in the nucleus (Fig. 3E, 4E).

All five peptides immunolabeled varicosities within the dorsal cap of Kooy, as demonstrated in the camera lucida drawings (Figs. 3, 4) and photomicrographs (Figs. 7A-E, 8D).
Caudal DAO

Varicosities immunolabeled for CGRP (Fig. 4A), CCK (Fig. 4B), CRF (Fig. 4C) and ENK (Fig. 4E) are densely distributed throughout the DAO. In contrast, Sub P immunolabeled varicosities are lightly scattered in the nucleus (Fig. 4D).

Principal Olive

The dorsal and ventral lamellae contain numerous varicosities immunolabeled for CGRP (Fig. 5A), CCK (Fig. 5B) and CRF (Fig. 5C). The dorsal lamella is completely devoid of Sub P (Fig. 5D) or ENK (Fig. 5E) labeling. However, the ventral lamella contains a focus of labeling for these latter two peptides in the medial aspect (Fig 5D,E). Immunolabeled varicosities for all five peptides are present in the ventrolateral outgrowth (Fig 5,6).

Rostral MAO

Numerous varicosities immunoreactive for CGRP (Fig 5A, 6A), CCK (Fig. 5B, 6B) and CRF (Fig. 5C, 6C) are present in the rostral MAO. Sub P immunolabelling is sparse and confined almost entirely to the lateral aspect of the nucleus (Fig. 5D, 6D). ENK immunolabeled varicosities are heavily distributed along the ventral and lateral borders of the nucleus (Fig. 5E, 6E, 8A,B) as seen caudally.

Rostral DAO

All peptides except Sub P have an extensive distribution in the rostral DAO (Figs. 5, 6A,B,C,E). Only scattered Sub P profiles (Figs. 5D, 6D) are present in the nucleus.
Analysis of control sections

A photomicrograph of a control section, processed without primary antibody, is shown in Fig. 7F. This is a high magnification photomicrograph of the dorsal cap of Kooy. When compared to other photomicrographs in Fig. 7 which contain positive immunolabeling in the dc and vlo, no immunolabeled varicosities are observed in the control section.

DISCUSSION

Neuropeptides are important signaling molecules for cellular communication in the nervous system (Hokfelt, 1991). Their colocalization with other peptides, monoamines or amino acids suggest multiple interactions within functionally distinct subclasses of neurons. Based on data derived in this study, several peptides have been identified within the mouse IOC. Of the five peptides examined CCK, CGRP and CRF exhibit dense immunolabeling throughout the IOC, whereas ENK and Sub P are more restricted in the location and density of their distribution. In the absence of colchicine, CRF is the only peptide in our study which demonstrated immunolabeled cell bodies (Figure 7C, 8D) throughout the IOC. The presence of CRF in olivary cell bodies, as discussed previously, has been confirmed with in situ hybridization studies in the IOC (Yamano and Tohyama, 1994) and immunohistochemistry which has demonstrated CRF-immunolabeled climbing fibers in the cerebellum of the adult mouse (Overbeck and King 1995). In our studies, no other peptide was present in olivary cell bodies. This has also been confirmed by either immunohistochemistry or in situ hybridization for CGRP in the mouse (Yamano and
Tohyama, 1994; Morara, Sternini, Provini, and Rosina, 1995); Sub P (Warden and Young III, 1988) and ENK (Bloch, Popovice, Chouham, and Kowalski, 1986) in the rat; and CCK (Savasta, Palacios, and Mengod, 1990) in the human.

Species Differences

As previously mentioned, immunohistochemical analysis has demonstrated significant differences in peptide distribution between species. In comparing the results presented in this paper with other studies, these differences are maintained. For example, no evidence of CGRP-immunolabeled varicosities are present in the rat inferior olive (Kawai et al. 1985; Skofitsch and Jacobowitz, 1985). This is in sharp contrast to the dense distribution of this peptide reported in the present paper for the mouse. Also in sharp contrast to our results, CCK in the rat immunolabeled varicosities only in the PO (Vanderhaeghen 1985). CRF-IR fibers in the rat (Sakanaka, Shibasaki, and Lederis, 1987) and monkey (Foote and Cha, 1988) inferior olive are scattered throughout the nuclei with the heaviest distribution in the vlo. These data are similar to our results in the mouse for caudal and rostral levels of the IOC. In contrast to our results where density of fibers increases in intermediate levels, there is an absence of labeling in the PO of the monkey (Foote and Cha, 1988) and fibers are dense only in the dorsomedial bend of the DAO and medial portion of the rostral MAO in the rat (Sakanaka, Shibasaki, and Lederis, 1987).

In the rat, Substance P-immunolabeled varicosities are primarily found in the DAO and caudal MAO; only light labeling was observed in the dorsal cap and PO (Bishop and Ho, 1984). These results differ from those in the mouse where labeling is observed in the dorsal
cap, ventral lateral outgrowth and vPO. In the opossum inferior olive ENK has been identified in terminals that are especially numerous in the PO, DAO, the lateral aspect of the rostral MAO, and the dorsal cap of Kooy (King, Ho, and Bishop, 1989). In contrast, in the rat, ENK positive varicosities are moderately distributed throughout the nucleus (Petrusz, Merchenthaler, and Maderdrut 1985). Our results in the mouse demonstrating ENK-immunolabeled profiles in the lateral pole of the MAO, DAO, dc and part of the PO more closely resemble results seen in the opossum.

In general, neuropeptides are present in afferent terminals within the IOC of several species, indicating a consistent role for these putative neuromodulators in mammalian neural transmission. Differences in the density or location of peptides within olivary subnuclei suggest that the different neuropeptides may influence specific components of olivary circuitry across species.

Origin of Peptide Containing Afferents to the Olive

Several studies have identified numerous sources of afferent projections to the various nuclei of the IOC in species such as the cat and opossum (Martin, Culberson, Laxon, Linauts, Panneton, and Tschismaadia 1980; Saint-Cyr and Courville 1980). These afferent sources are very consistent across species; however, no studies have been conducted in the mouse to confirm the data on the sources of afferents. Although there is overlap of afferent systems in some regions of the IOC, there are also sources unique to each region. The extensive distribution of many of the peptides crosses afferent boundaries, suggesting multiple sources of these peptidergic afferents to the olive (Figs. 3-6).
In a separate series of immunohistochemical experiments (Petrusz, Merchenthaler, and Maderdrut 1985; Vanderhaeghen 1985; Sakanaka 1992; Inagaki 1992; Daikoku and Hisano 1992) cell bodies containing different peptides were identified in the brainstem and spinal cord in several species, including rat and cat. Several of these nuclei have been shown to be sources of afferents to the IOC. For example, CCK, CRF, CGRP, ENK and Sub P all have been identified in cell bodies within regions of the spinal cord which project to the caudal MAO and throughout the DAO (Petrusz, Merchenthaler, and Maderdrut 1985; Vanderhaeghen 1985; Sakanaka 1992; Inagaki 1992; Daikoku and Hisano 1992). Thus one or more of the peptidergic terminals seen in these olivary nuclei could be of spinal origin.

In the brainstem, nuclei which give rise to afferents to the IOC also contain various peptidergic neurons. ENK- and Sub P-labeled cell bodies are present in the medullary reticular formation, pontine reticular formation, hypoglossal nucleus, raphe nuclei, spinal trigeminal nucleus, nucleus of the solitary tract, superior colliculus and periaqueductal gray (Petrusz, Merchenthaler, and Maderdrut 1985; Sakanaka 1992). In addition to ENK and Sub P, CCK labeled somata also were observed in the medullary reticular formation (Vanderhaeghen 1985), CRF in the nucleus of the solitary tract (Daikoku and Hisano 1992), CGRP in the superior colliculus (Inagaki 1992), and CGRP and CRF in the periaqueductal gray (Inagaki 1992; Daikoku and Hisano 1992). ENK labeled cells have been identified in the nucleus fastigius of the cerebellum and the subparafascicular nucleus (Petrusz, Merchenthaler, and Maderdrut 1985). The vestibular complex demonstrated cell bodies labeled by Sub P or CRF (Sakanaka 1992; Daikoku and Hisano 1992). Finally, CCK labeled cell bodies have been identified in the nucleus gracilis (Vanderhaeghen 1985).
From these data, it is probable that several of these brainstem nuclei may be the source(s) of one or more of these peptides found within the IOC. It is also clear that sources of afferents to the inferior olive may contain cell bodies with several peptides. The periaqueductal gray, in particular, contains cell bodies labeled for CRF, Sub P, ENK and CGRP. Only CCK does not label any cells in this area. This could explain the overlap in distribution of several peptides. Double label studies need to be conducted to conclusively identify the sources of each peptide.

Colocalization

It is very likely peptides are colocalized with other neurotransmitters such as acetylcholine, catecholamines or GABA. Because of the similarity in the morphology and the overlap in the distribution of labeled varicosities, it is also possible that several peptides may be colocalized in the same terminal, as discussed above; although conclusive proof of this hypothesis requires further study. For example, all five peptides label larger profiles in the dorsal cap of Kooy and vlo (large arrows, Fig. 7A-E). The varicosities labeled by CRF (Fig. 7C) are of a slightly different morphology, being more round than those labeled by the other peptides (Fig. 7A,B,D,E). However, the varicosities labeled by ENK (Fig. 7A), CGRP (Fig. 7B), CCK (Fig. 7D) and Sub P (Fig. 7E) are all very similar in morphology and distribution.

Another similarity between peptide distribution is in the "smaller" varicosities labeled by CGRP (small arrows, Fig. 8C), CRF (Fig. 8D) and CCK (Fig. 8E). All three lightly label small varicosities similar in appearance and in their density of distribution. These varicosities are labeled throughout the olive by CGRP and CCK (Figs. 3-6). The distribution of small
CRF-IR varicosities is more limited to the intermediate levels of the olive, but is fairly dense there (Figs 4C, 5C). Double-labeling experiments at light and electron microscopic levels are needed to determine possible colocalization(s) of neuropeptides with each other as well as with other neurotransmitters.

Receptors

Several studies have been conducted to localize peptide receptors within the CNS using immunohistochemistry or autoradiography for receptor binding, or in situ hybridization for the receptor mRNA (van Rossum, Menard, Fournier, St-Pierre, and Quirion, 1994; Nakaya, Kaneko, Shigemoto, Nakanishi, and Mizuno, 1994). Data indicate that CGRP receptors in the rat and CCK receptors in the human brain are densely distributed throughout the IOC (van Rossum, Menard, Fournier, St-Pierre, and Quirion, 1994; Dietl, Probst, and Palacios, 1987). This correlates with the immunohistochemical data presented in this paper. Sub P receptor-like immunohistochemistry is moderately dense in the MAO of the adult rat, but very weak in the PO and DAO (Nakaya, Kaneko, Shigemoto, Nakanishi, and Mizuno, 1994). This is also consistent with our data which demonstrate some Sub P varicosities in the MAO, but some focal points in the PO as well. In situ hybridization in the rat for CRF receptor mRNA results in sparse labeling (Potter et al. 1994). Based on the dense distribution of peptide in the mouse inferior olive, it is surprising not to see a greater amount of mRNA for the receptor. In situ hybridization for opioid receptors did not demonstrate signal in the inferior olive (George et al. 1994). This contrasts data in this paper which suggests that ENK is present in the MAO, DAO and portions of the PO (Fig.4).
Conclusions

The number of peptides present in the olive, as well as their dense and complex distributions, suggest that these neuroactive compounds play an important role in modulating the activity of olivary neurons. As neuromodulators, peptides often act by slowly depolarizing or hyperpolarizing the membrane (Hokfelt, 1991). The peptides described in this study could act independently to alter firing rate or through interactions with other neurotransmitters. Modulation of olivary output by neuropeptides will have a significant influence on the cerebellar circuitry and ultimately on control of motor activity.
Figure 3 is a series of camera lucida drawings that illustrate the distribution of varicosities immunoreactive (IR) for: A) calcitonin gene-related peptide (CGRP-IR), B) cholecystokinin (CCK-IR), C) corticotropin releasing factor (CRF-IR), D) substance P (Sub P-IR) and E) enkephalin (ENK-IR) in the caudal MAO. CGRP-IR (3A) and CCK-IR (3B) have a dense distribution. CRF-IR (3C) and Sub P-IR (3D) are lightly scattered and ENK-IR (3E) varicosities are focused along the medial, ventral and lateral borders of the nucleus.
Figure 3
26
Figure 4 is a series of camera lucida drawings that illustrate the distribution of A) CGRP-IR, B) CCK-IR, C) CRF-IR, D) Sub P-IR and E) ENK-IR varicosities in the caudal MAO and DAO. CGRP-IR (4A), CCK-IR (4B) and CRF-IR (4C) have a dense distribution in both nuclei, although the CRF-IR density decreases laterally. Sub P-IR (4D) has developed a focus in the lateral pole of the MAO, while immunoreactivity elsewhere remains scattered. ENK-IR (4E) in the DAO is heavy; the distribution in the MAO is the same as seen in Fig. 4E.
Figure 5 is a series of camera lucida drawings that illustrate the distribution of A) CGRP-IR, B) CCK-IR, C) CRF-IR, D) Sub P-IR and E) ENK-IR varicosities in the rostral MAO and DAO, and the principal olive (PO). As seen previously, CGRP-IR (5A), CCK-IR (5B) and CRF-IR (5C) is dense throughout all nuclei. CCK-IR (5B) demonstrates very dense levels of labeling in the vlo, dorsal lamella of the principal olive (dPO) and the lateral pole of the DAO. Sub P-IR (5D) is focused in the vlo and a moderate band in the vPO. ENK-IR (5E) is densest along the medial border, in the vlo, along the ventral and lateral border of the MAO, ventral lamella of the principal olive (vPO) and DAO. Note the complete absence of Sub P-IR (5D) and ENK-IR (5E) in the dPO.
Figure 5
Figure 6 is a series of camera lucida drawings that illustrate the distribution of A) CGRP-IR, B) CCK-IR, C) CRF-IR, D) Sub P-IR and E) ENK-IR in the rostral olive. At this level, the PO and the DAO cannot be morphologically distinguished and are represented as one structure. CGRP-IR (6A), CCK-IR (6B) and CRF-IR (6C) distribution is relatively unchanged in density from previous levels. CRF-IR (6C) is less dense laterally than medially. Sub P-IR (6D) is focused in the vlo and relatively absent from the lateral olive and the MAO. ENK-IR (6E) remains densest medially and ventrally in the MAO, heavy dorsally in an area approximate to the DAO and completely void in laterally in what probably remains of the PO.
Figure 6

32
Figures 7 is a series of photomicrographs comparing immunolabeling in the only subnuclei densely labeled by all five peptides: the dorsal cap of Kooy (dc) and the ventral lateral outgrowth (vlo). Small and large arrows indicate varying sizes of varicosities for each peptide, which may indicate multiple sources of peptidergic afferents. A) ENK-IR in the vlo; B) CGRP-IR in the vlo; C) CRF-IR in the dc including immunoreactive cell bodies (arrowheads); D) CCK-IR in the dc; E) Sub P-IR in the dc. Varicosities immunoreactive for ENK (Fig. 7A), CCK (Fig. 7D) and Sub P (Fig. 7E) are very similar in morphology and density of distribution, suggesting possible colocalization of peptides. Fig. 7F is a photomicrograph of the dc of a control section processed in parallel with normal except that it was not exposed to primary antibody. There is no labeling of varicosities in the control section, as can be seen by comparing this to surrounding photomicrographs for any of the peptides. Calibration bar in A is 50μm and applies to A-E. Calibration bar in F is 50μm.
Figure 8 is a series of photomicrographs demonstrating immunoreactive foci for each peptide in several subnuclei of the olive. In all figures, large and small arrows indicate varying sizes of immunoreactive varicosities, suggesting multiple sources of peptidergic afferents to the olive. Fig. 8A is a low power micrograph of a section immunostained for ENK and drawn in Fig. 8E. Note the density of varicosities in the DAO and lateral MAO (block arrow). Fig. 8B is a high power micrograph of the lateral MAO taken from the area indicated by the black arrow in Fig. 8A. The blood vessel in the upper left (large arrowhead) corresponds to the one in Fig. 8A. Fig. 8C demonstrates CGRP-IR in the caudal MAO. Note the fibers and the dense distribution of small varicosities. CRF-IR is shown in the dc in Fig. 8D as well as CRF immunolabeled cell bodies (arrowhead). CCK-IR (Fig. 8E) and Sub P-IR (Fig. 8F) varicosities are illustrated in the lateral portion of the caudal MAO. Calibration bar in A is 200μm. Calibration bar in B is 50μm and applies to B-F.
CHAPTER 2
SYNAPTIC LOCALIZATION OF CALCITONIN GENE-RELATED PEPTIDE
IN THE MOUSE INFERIOR OLIVARY COMPLEX

INTRODUCTION

The inferior olivary complex is the sole source of climbing fiber (CF) afferents to the cerebellar cortex (Armstrong, 1974). Olivocerebellar axons exert a powerful excitatory effect on Purkinje cells in the cerebellar cortex and are a critical component in determining cerebellar output and function. Because of the powerful effect of the CF synapse on Purkinje cells in the cerebellar cortex, any neuroactive chemical altering the firing rate of olivary neurons will influence Purkinje cell firing and ultimately cerebellar function. Neuropeptides are one class of neuroactive chemicals known to alter neuronal firing rate for a prolonged time period (Hokfelt, 1991).

In a previous study (Gregg and Bishop, 1997), several neuropeptides were found to be localized in the mouse IOC, including enkephalin (ENK), corticotropin releasing factor (CRF), cholecystokinin (CCK), substance P (Sub P), and CGRP. Data from this study indicate that CGRP immunoreactivity (CGRP-IR) labels two distinct sizes of varicosities in the mouse IOC. The small type is heavily distributed throughout the caudal to rostral extent of the IOC in all nuclei, whereas the larger CGRP-IR varicosity is restricted to the dorsal cap.
of Kooy (dc), ventral lateral outgrowth (vlo) and several restricted foci in the caudal MAO, PO and DAO. Extracellular unit recordings in the IOC indicate that CGRP is inhibitory both to the spontaneous firing rate and the excitatory effects of excitatory amino acids (EAAs), but that it also potentiates the response to EAAs following CGRP application (Gregg and Bishop 1995).

The purpose of this study is to gain further insight into the role of CGRP in olivary circuitry by investigating the synaptic relationships of the two types of CGRP-IR profiles in electron micrographs. Of particular interest was the distribution of CGRP labeled profiles relative to synaptic clusters.

MATERIALS AND METHODS

Immunohistochemistry and electron microscopy

Fourteen adult C57BL/6J mice were perfused with saline followed by 4% paraformaldehyde in Sorensen's phosphate buffer containing 0.2-0.5% glutaraldehyde and in some cases, 0.4% sodium metabisulfide. 60μm transverse sections of the brainstem were cut on a vibratome, rinsed in phosphate buffer and incubated in 0.3% Triton X-100 for 15-60 min. Sections were rinsed again and placed in a solution containing a rabbit antibody against CGRP (1:1000 or 1:2500, Amersham) overnight at 4°C with constant agitation. Pre-embedding immunohistochemistry followed a previously described protocol modified for free floating sections (Stemberger, 1979). Briefly, sections were rinsed, incubated in sheep anti-rabbit antibody (1:500), rinsed again and placed in rabbit peroxidase-anti-peroxidase (1:500) with constant agitation. The tissue was processed using the glucose oxidase reaction (Shu,
Ju, and Fan, 1988) at 4°C with constant agitation. Sections were examined under a Zeiss light microscope to localize reaction product. Sections were then serially incubated in 1% osmium tetroxide, dehydrated through a series of graded concentrations of acetone and embedded in Spurr's resin. The 60μm flat embedded sections were examined with a light microscope to confirm immunolabeling. Photographs were taken of each block and the sections were drawn with the aid of a camera lucida attachment before the flat embedded sections were glued to plastic blocks. Flat embedded sections from all levels of the IOC were represented; however, the present fine structural analysis primarily focuses on the dorsal cap of Kooy, caudal MAO (cMAO), rostral MAO (rMAO), and ventral lamellae of the principal olive (vPO). Ultrathin gold or silver sections were cut with a glass knife using an LKB ultramicrotome. Sections were stained with uranyl acetate and lead citrate, and examined with a Philips CM12, 60kv TEM. Sections from a total of 51 blocks representing 14 mice were examined.

**Colchicine Studies**

To ascertain the presence of CGRP-IR in olivary cell bodies, two mice were anesthetized with avertin (0.2cc/10g) and given an injection of colchicine (2 μl of 1mg/1ml saline) directly into the brainstem to impede axonal transport. 18 hours later, mice were again anesthetized, perfused and processed for immunohistochemistry as described above.

**Olivocerebellar axon analysis in the cerebellum**

Two mice were perfused with either Zamboni's fixative or 4% paraformaldehyde and 60μm sagittal sections of the cerebellum were cut and processed for CGRP-IR as described
above. After processing, sections were mounted on glass slides, dehydrated through an alcohol series and coverslipped. The cerebellar cortex was examined under the light microscope for the presence of CGRP-IR labeled olivocerebellar afferents.

RESULTS

CGRP-IR in Axons within the Olivary Complex

CGRP-IR was observed only rarely in axonal profiles filled with synaptic vesicles (n=8) (Figs. 9; 10A). The cross sectional diameter of these axons was small (0.5-1μm), they contain mitochondria as well as small, clear vesicles, and only one was contained within the glial boundaries of a synaptic clusters. Although labeled axons were in close proximity to dendritic profiles and cell bodies (Figs 9 and 10A), no synaptic junctions have been identified.

CGRP-Immunoreactivity in Olivary Dendrites

Numerous olivary dendrites contained CGRP-immunoreactivity (CGRP-IR) (n=69). The cross sectional diameter of all dendritic profiles ranged in size from 0.54-0.75μm; however, differences in ultrastructure suggest two populations of labeled dendritic profiles. One population contained mitochondria, smooth endoplasmic reticulum and microtubules, in a pattern consistent with the defined ultrastructure of dendrites (Fig 11A,B). The second population contained mitochondria, smooth endoplasmic reticulum, and electron dense material (s in Fig. 9). No microtubules were visible in this second population (s in Fig. 9), suggesting that these profiles are dendritic spines (n=50). Both populations were often
postsynaptic to unlabeled synaptic terminals (s in Fig 9; * in Fig. 11A,B). Dendritic elements of either type with CGRP-IR were not observed within the glial boundaries of synaptic glomeruli.

CGRP-IR in the Soma and Axon Terminals of Olivary Neurons

Two mice were injected with colchicine to confirm the presence of CGRP in cell bodies as seen in light microscopic analysis. Light microscopic examination of thick flat-embedded sections (0.2μm thick) revealed numerous CGRP labeled cell bodies (arrowheads, Fig. 10C). At the ultrastructure level, CGRP-IR was present over the nucleus and around polyribosomes in the cytoplasm (Figs. 9, 10A). CGRP in olivary neurons was further confirmed by analyzing light microscopic sections of cerebellar cortex processed for CGRP-IR. Numerous varicosities were found in the molecular and Purkinje cell layers (arrows, Fig. 10B). Their size and distribution suggest they are climbing fibers, adding support to our findings that olivary cell bodies produce CGRP.

DISCUSSION

Peptide Localization in Axons Within the Olivary Complex

Olivary neurons have several unique features including an oscillatory firing pattern, a maximum firing rate of only 10Hz (Llinas and Yarom, 1981b; Llinas and Yarom, 1981a), and electrotonic coupling via gap junctions, allowing multiple neurons to fire synchronously.
(Llinas and Yarom, 1986). These properties permit populations of olivary neurons to send action potentials to longitudinal strips of Purkinje cells in the cerebellum (Llinas and Welsh, 1993).

There are several sources of afferent input to the inferior olivary complex (Martin, Culberson, Laxon, Linauts, Panneton, and Tschismaadia 1980). Excitatory input is derived from spinal cord sources or nuclei near the mesodiencephalic junction. The greatest source of inhibitory input to the olive is derived from the deep cerebellar nuclei. This GABAergic input is thought to uncouple olivary dendrites by blocking transmission through the gap junctions (Sotelo, Llinas, and Baker, 1974). This allows individual olivary neurons to fire independently; thus activating fewer Purkinje cells in the cerebellar cortex.

The fine structural location for these events is the synaptic cluster (glomerulus), a synaptic configuration unique to the inferior olivary complex (IOC). Synaptic clusters (glomeruli) have previously been described as a core of dendritic spines coupled by gap junctions and surrounded by synaptic terminals (Sotelo, Llinas, and Baker, 1974; King, 1976). Dendritic spines in the IOC are not as simple as the knob-like structures seen on Purkinje cell dendrites or in the cerebral cortex. Rather, spines in the olive have one stalk emanating from the dendrite which gives rise to several varicose-like enlargements ("spiny appendages"). Each synaptic cluster may contain several interdigitating spiny appendages from multiple neurons (King 1980). At the ultrastructural level, dendritic spines in the olive may contain mitochondria, smooth endoplasmic reticulum and an electron-dense material, but they do not contain microtubules as do dendritic shafts.
The synaptic terminals surrounding the dendritic spines in a synaptic cluster either contain ellipsoidal synaptic vesicles; small, clear, spherical vesicles; or large dense core vesicles (LDCVs) and small, clear vesicles (King 1980; Sotelo, Llinas, and Baker, 1974). The former are considered to be the GABAergic terminals from the deep cerebellar nuclei (DeZeeuw, Hostege, Ruigrok, and Voogd, 1986) responsible for uncoupling olivary neurons, while the latter are thought to be excitatory inputs from the mesodiencephalic junction (DeZeeuw, Hostege, Ruigrok, and Voogd, 1986).

Data in this study indicate one CGRP-IR axon within a synaptic cluster. This axon contained small, clear spherical vesicles. Therefore, CGRP may play a minor role in controlling the electrotonic coupling of olivary dendrites.

In addition to the synaptic cluster, synapses also occur on dendritic shafts, but rarely on the cell soma. One more ultrastructural configuration to mention is the dendritic thicket, a group of several dendrites adjacent to each other, but not coupled by gap junctions. Unlike synaptic clusters, these dendrites are not surrounded by a glial sheath (Sotelo, Llinas, and Baker, 1974).

Neuropeptide Release and Localization of other Neuropeptides within Axons

Neuropeptide release is calcium dependent (Hokfelt, 1991; Santicioli, Del Bianco, Tramontana, Geppetti, and Maggi, 1992), and it has been suggested that peptides may be released at classically defined synapses, or possibly at other sites distant from synaptic junctions (Hokfelt, 1991). This study has demonstrated that CGRP-IR appears in axons containing small clear vesicles located outside synaptic clusters. The presence of CGRP-IR
in these axons is consistent with its proposed role as a neuromodulator. Double-label studies in the mouse indicate that CGRP containing afferents to the caudal IOC may arise from the locus coeruleus and/or subparafascicular nucleus (Peltier, Gregg, King, and Bishop 1996). Thus, it is likely that the CGRP-IR axons seen in the present study originate from one of these two nuclei. Other possible sources include the spinal cord or deep cerebellar nuclei.

Several neuropeptides have been localized to either synaptic terminals in other species. In the opossum, ENK-IR was observed in synaptic terminals that contain small, clear vesicles which synapsed on dendritic profiles both inside and outside the glial boundaries of synaptic clusters (King, Ho, and Bishop, 1989). In addition, ENK-IR also was localized in terminals that contained both large dense-core vesicles (LDCVs) and small, clear vesicles, as well as in terminals containing only small, clear vesicles within the plane of section. Both of these latter ENK-IR terminals containing ENK were located within synaptic clusters. Therefore, it has been proposed that ENK plays a role in modulating electrotonic coupling of olivary dendrites.

**Peptide Localization in Dendrites**

CGRP-IR was frequently localized within dendritic profiles. Like the distribution of CGRP-IR in axons, all labeled CGRP-IR dendrites were detected outside the glial boundaries of synaptic clusters. However, in our EM studies, two populations of dendritic profiles were observed based on their ultrastructure. One population shown in Fig. 11A,B are likely dendrites due to the presence of microtubules, while the other is thought to be dendritic spines. It might be argued that the first population represents preterminal axons that do not
contain synaptic vesicles. An axon at this stage may be represented by (a) in Fig. 9. As can be seen, the microtubule arrangement is similar to that of the dendrites in Fig. 11A,B; however, the diameter of the axon is much smaller than that of the dendrites; the latter are more comparable to synaptic boutons with synaptic vesicles. Therefore, it is possible, although, unlikely, that some of these profiles are axonic in origin.

The second population does not appear to contain microtubules, but only spherical membrane bound structures and electron dense material. These profiles likely represent dendritic spines. Although the cross sectional diameter of the two populations are equal, their cytological features are quite distinct.

**Functional Roles for CGRP in the Inferior Olive**

Only one of the labeled axons and none of the labeled dendrites seen in this study were contained within a synaptic cluster. Therefore, CGRP may play a minor role in regulating electrotonic coupling mediated by gap junctions. Physiological data indicate that CGRP has an inhibitory influence on olivary firing activity (Gregg and Bishop 1995). CGRP receptor binding studies have reported a high density of binding sites for CGRP in the inferior olive (van Rossum, Menard, Fournier, St-Pierre, and Quirion, 1994), and in fact it has one of the highest densities of CGRP receptors seen in the CNS in the rat.

Although our data indicate that the greatest concentration of CGRP is outside the synaptic clusters, it is important to localize receptors with regard to synaptic clusters as well, for it is the location of the receptor that determines the function of the peptide. One feature of olivary physiology is the presence of dendritic calcium currents which create an after-
depolarization following the sodium spike of an action potential (Llinas and Yarom, 1981b; Llinas and Yarom, 1981a). Release of CGRP from central terminals of dorsal root ganglion neurons induced by electrical field stimulation has been shown to be calcium dependent (Santicioli, Del Bianco, Tramontana, Geppetti, and Maggi, 1992). This raises the possibility that the calcium influx in olivary dendrites may be sufficient to induce CGRP release from dendrites. One study in the dorsal horn of the spinal cord of rat demonstrated that NP-Y was packaged into vesicles in the constitutive pathway following axotomy of dorsal root ganglion neurons (Zhang, Aman, and Hokfelt, 1995). Thus, it is possible for neuropeptides to be packaged in "non-traditional" cytoplasmic pathways in the nervous system. In non-neuronal cells, CGRP has an autocrine/paracrine effect (Segond et al. 1996; Bracq, Clement, Pidoux, Moukhtar, and Jullienne, 1994). Because of its physiological actions in the IOC (Gregg and Bishop 1995) we suggest that release of CGRP from olivary dendrites may occur through regulated secretion. We propose that CGRP may play a unique role in the IOC of the mouse through release from olivary dendrites.
Figure 9 is an electronmicrographic montage demonstrating calcitonin gene-related peptide immunoreactivity (CGRP-IR) in the dorsal cap of Kooy. One olivary cell body is heavily labeled, with HRP reaction product almost obscuring the nucleus and organelles in the cytoplasm (*). Adjacent to the cell body, a labeled axon (†) is present. Small, clear synaptic vesicles can be seen within the axon. No synaptic junction is present within the plane of section. Throughout the micrograph, several profiles containing electron-dense material, mitochondria and vesicle-like structures are present. These characteristics are consistent with those of dendritic spines. However, since the HRP product obscures the ultrastructure and serial sections were not cut, it is not possible to definitely identify them as such. At the bottom of the micrograph is a longitudinal section through a labeled dendrite (d). Calibration bar represents 1 μm.
Figure 9
Figure 10. A) This electron micrograph is from the dorsal cap of Kooy. An olivary neuron is labeled (*) with reaction product over the nucleus and over polyribosomes in the cytoplasm. The structure labeled as (a) is a small dendrite with a cross sectional diameter of 0.38 μm; it is postsynaptic to an unlabeled axon terminal. Calibration bar represents 1 μm.

B) This light micrograph demonstrates CGRP-IR climbing fibers (arrows) in the mouse cerebellar cortex. Calibration bar represents 50 μm and applies to C. C) CGRP-IR varicosities (arrows) and cell bodies (arrowheads) are visible in a 0.2μm thick section of flat embedded tissue stained with Toluidine Blue from the lateral pole of the caudal MAO. Note that almost every cell body in the field of the micrograph is labeled, suggesting that a majority of cell bodies in the caudal MAO contain CGRP.
Figure 11. Two electron micrographs representing dendrites labeled with CGRP-IR in the lateral portion of the caudal MAO. A) A dendrite (*) featuring symmetrically labeled neurotubules is postsynaptic to an unlabeled axon containing ellipsoidal synaptic vesicles. Two more dendritic profiles (‡) in the field of view are labeled. Calibration bar represents 1 μm. B) Two dendrites (*) exhibit labeled neurotubules. Both spines are postsynaptic to unlabeled axons containing ellipsoidal vesicles. These photomicrographs suggest that CGRP-IR dendrites may be postsynaptic to axons containing GABA.
CHAPTER 3

PHYSIOLOGICAL EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE
IN THE MOUSE INFERIOR OLIVE

INTRODUCTION

The inferior olivary complex (IOC) is the source of climbing fiber (CF) afferents to the cerebellum. Several theories have been proposed to define the role of olivocerebellar circuitry in controlling motor activity. One theory proposes that climbing fibers modify the ability of Purkinje cells to respond to input derived from parallel fibers, a second source of afferents to the Purkinje cells (Ito, 1993). A second theory suggests a primary role for olivary neurons in directly controlling the dynamic properties of motor activity (Llinas and Welsh, 1993). Although these two theories vary, one common concept between them is that the Purkinje cells are highly regulated by input they receive from climbing fibers originating in the inferior olivary complex. Thus, any change in the firing rate or responsiveness of olivary neurons may have a significant effect on cerebellar output and subsequently on motor activity.

The purpose of this study is to investigate how the peptide calcitonin gene-related peptide (CGRP) effects the physiological activity of neurons in the mouse inferior olive. Previous studies have shown that the mouse IOC contains a dense distribution of CGRP-
immunoreactive varicosities that are present primarily in dendrites of olivary neurons (Gregg, Bishop, and King, 1997). CGRP is a 37-amino acid peptide present throughout the body and CNS (Rosenfeld et al. 1983). It is active in the immune, cardiovascular, and gastrointestinal systems as well as the peripheral nervous system (PNS) (Rosenfeld et al. 1983). Although the peptide and its receptor(s) have been rigorously studied in many of these areas, the investigation of its roles in the CNS has only begun. Studies in the dorsal horn of the spinal cord indicate that CGRP depolarizes and increases firing rate through modulation of a Ca\(^{2+}\) current (Ryu, Gerber, Murase, and Randic, 1988). However, in the cerebellum (Bishop, 1995) and cerebral cortex (Elaagouby, Yuste, and Tank 1994), CGRP inhibits firing rate through an unknown mechanism. Due to the long time course of its actions and its ability to alter neuronal responsiveness to neurotransmitters, CGRP is classified as a neuromodulator (Hokfelt, 1991). The approach of this study is to use an in vitro slice preparation of the mouse IOC to determine if CGRP has a physiological effect on olivary neurons.

MATERIALS AND METHODS

Slice Preparation

Adult animals were decapitated, their brains quickly removed and placed in cold, oxygenated modified artificial cerebral spinal fluid (MaCSF) consisting of 248mM sucrose, 5mM KCl, 1.25mM KH\(_2\)PO\(_4\), 1.15mM MgSO\(_4\), 2.5mM CaCl\(_2\), 28mM NaHCO\(_3\) and 12mM glucose. The MaCSF uses sucrose instead of NaCl to prevent osmotic shock to neurons initially traumatized by the slice procedure. The brain stem was blocked and sectioned in the transverse plane at 200-400\(\mu\)m on a vibrating slicer. Tissue sections were collected and
placed in a holding chamber containing MaCSF or an interface slice chamber (Medical Systems) containing normal aCSF (with 124mM NaCl in place of sucrose) at 33°C. Slices were left in the moist oxygenated environment of the slice chamber for 30-60 min. before recording was initiated to allow equilibration to the aCSF and to temperature.

**Recording Electrodes**

Multibarrel electrodes were used for extracellular recordings. One barrel was filled with 4M NaCl for purposes of recording neural activity. A second barrel was filled with 2M KCl which functioned in an automatic balancing circuit. This eliminated the possibility that observed responses were the result of the applied current rather than the substance being tested. The remaining barrels were filled with combinations of the following substances: 0.2M aspartate (ASP), pH 7.5; 5.0mM quisqualate (Quis), pH 7.5-9.0; 0.2M glutamate (Glut), pH 7.5-9.0; 0.1mM calcitonin gene-related peptide (CGRP, Bachem, Inc.), pH 6.0 and 0.1mM CGRP (8-37), pH 6.0. During advancement of the electrode, retaining currents of at least 10 nA of appropriate polarity were applied to prevent leakage of the various chemicals. All electrodes contained CGRP with some combination of amino acids and all compounds were administered with varying currents to establish a dose-response relationship. Results were recorded on a strip chart recorder, as well as on computer disks and VCR tape. Multiple trials were carried out to determine that results were consistent. Asp, Quis and Glut were ejected with a negative DC current. CGRP and CGRP (8-37) were ejected with a positive DC current.
RESULTS

Activity of neurons in a slice preparation

Each upward deflection on the chart recordings shown in Figures 12-15 represents the number of spikes fired by an olivary neuron over a 5 second period. The spontaneous activity of most olivary neurons in our slice preparation is 0.2-0.8 Hz (Figs. 12B; 13A,B; 15). Occasionally there is no spontaneous activity (Fig. 14B), but neurons will respond to excitatory amino acids (EAAs) (Fig. 14B). These levels of activity are in general agreement with observations of Armstrong (1974) for other species. Preliminary in vivo recordings in the mouse IOC have indicated a spontaneous firing rate of 1-2 Hz (personal observation). This suggests that damage to afferent and efferent processes through sectioning may decrease the spontaneous activity seen in the slice.

Effects of EAAs on spontaneous firing rate

In all cases (n=9), the excitatory amino acids (EAAs) increased firing rate from spontaneous levels to 0.8-2 Hz (Figs. 12B; 13B; 14B; 15). In addition, in some cases Quis induced the neuron to fire more regularly. Several EAAs including glutamate (Glut), quisqualate (Quis) and aspartate (Asp) were examined for their potency. All were roughly equal in their ability to increase firing rate. However, in our experience, Quis was the most consistent in this effect. EAA mediated excitation of olivary neurons was immediate (Fig. 12B; 13B; 14B; 15), indicating that Quis is most likely acting on ligand-gated ion channels.
Effect of CGRP on spontaneous firing rate

CGRP decreased the spontaneous firing rate in every neuron tested (n=8) (Figs. 12B; 13A,B). It was not uncommon for CGRP to completely abolish firing (Fig. 12B). Occasionally, the cell overcame the inhibition induced by CGRP application but in the majority of cells, the neuron was able to recover only after CGRP was turned off or Quis was applied to the neuron (Figs. 12B; 13A,B).

Effect of CGRP on EAA-induced activity

When EAA were applied in the presence of CGRP there was a delay to onset or complete suppression of EAA-induced excitation (n=6) (asterisk, Figs.12B; 13A,B; 15). In some cases, Quis was eventually able to overcome the inhibition, even in the continued presence of CGRP, and firing rates recovered to those recorded when Quis was applied alone. In other cases, only upon termination of CGRP application was Quis able to increase firing to previous levels (Fig.12B, 13A,B; 15).

Effects of the CGRP antagonist CGRP (8-37)

CGRP (8-37) is an antagonist at the type 1 CGRP receptor, where it has been shown to block neuronal responses to CGRP (Yamaguchi et al. 1988). CGRP (8-37) did not block olivary neurons from responding to CGRP. In several cases, CGRP (8-37) in the mouse IO actually potentiated the ability of CGRP to inhibit firing rate (n=3), or inhibited firing on its own (n=5). These results are seen in Figures 14A,B. In all cases (n=5), CGRP (8-37) was more potent and consistent in its inhibitory effects on Quis driven activity than CGRP itself.
In several cases (Fig. 13B; 14A,B), Quis driven activity was potentiated following application of CGRP or CGRP (8-37) (n=3).

**Saline and distilled water controls**

In several experiments as a negative control, one barrel of the multibarrel electrode was filled with saline, the solvent for the EAAs and CGRP. Fig. 15 demonstrates that saline had a greater inhibitory effect on Quis driven firing than CGRP. Distilled water was also tested as a possible solvent for the drugs; however, it had the same effect on Quis driven activity as saline (data not shown). The effects of saline and distilled water on firing rate are probably related to ejection of Na⁺ ions from saline and H⁺ ions from water upon application of a positive current to the barrel, the current used for ejection of CGRP. The Na⁺ and H⁺ ions may change the osmolarity and pH of the extracellular fluid and possibly the binding conditions for receptors. In previous in vivo work done in this laboratory, saline controls had no effect on firing rate (personal observation). It is likely that in in vivo experiments, the intact circulatory system and glia can compensate for even slight variances in the pH or ion composition of the CSF so that these effects are never seen. Therefore, it seems that the inhibitory effects of the controls are a slice phenomena. CGRP ejection produces a lesser degree of inhibition because the osmolarity of the solution is greater. Because CGRP and the negative controls did not exhibit the same level of inhibition, it is assumed that the CGRP effect is real.
DISCUSSION

CGRP-immunoreactivity has been identified in axons and dendritic profiles within the inferior olive of the mouse (Gregg, Bishop, and King, 1997). Localization of a neuropeptide within a dendritic profile of a neuron in the CNS has not been previously documented. It is not clear whether CGRP is released from olivary dendrites; however, if it is, it will have a unique role in modulating olivary physiology. The possible mechanisms can be better understood by first considering basic components of olivary neuron physiology.

Oscillations in firing rate and membrane potential of olivary neurons

The oscillatory firing pattern of olivary neurons is highly dependent on dendritic and somatic Ca\(^{2+}\) currents (Llinas and Yarom, 1981a; Llinas and Yarom, 1981b). Depolarization of the dendrites opens a high threshold calcium channel and the Ca\(^{2+}\) influx activates a Ca\(^{2+}\)-dependent K\(^+\) current. The K\(^+\) efflux produces a long lasting after hyperpolarization (AHP) of approximately 100ms, limiting the maximum firing rate of an olivary neuron to 10Hz. As the AHP spreads to the cell body, a somatic calcium inactivation is removed allowing influx of Ca\(^{2+}\) into the soma where it depolarizes the neuron and initiates another action potential. This somatic Ca\(^{2+}\) inactivation is very sensitive to membrane potential. Its inactivation is more rapidly removed when the neuron is hyperpolarized resulting in a firing rate of up to 10 Hz. In contrast when the cell is depolarized, firing levels do not exceed 4-6 Hz.
CGRP in the inferior olive

As noted in our results, CGRP appears to have three effects: a) it inhibits spontaneous activity, b) it inhibits EAA driven activity, and c) it delays the excitatory effect of EAAs. The ability of CGRP to alter neuronal responsiveness to a neurotransmitter is consistent with a neuromodulatory role for CGRP. CGRP may inhibit olivary firing rate by enhancing Ca\textsuperscript{2+} influx into the dendrites. This in turn would facilitate opening of the Ca\textsuperscript{2+} - dependent K\textsuperscript{+} channels, and the formation of a higher amplitude or more prolonged AHP. Another possible site of action is at the soma. CGRP may prevent membrane potential from hyperpolarizing thus blocking removal of the somatic calcium inactivation and thus depolarization of the neuron. Although CGRP also has an inhibitory effect in the cerebellar cortex of the cat (Bishop, 1995) and in the cerebral cortex (Elaagouby, Yuste, and Tank 1994), there is currently no indication as to its mechanism of action in these tissues.

CGRP (8-37) in the inferior olive

CGRP has been shown to bind to three types of receptors defined as type 1, type 2 and a calcitonin receptor. CGRP (8-37) is a specific antagonist for the type 1 receptor. It has no agonist properties even at micromolar concentrations. Both CGRP and CGRP (8-37) have agonist effects on the calcitonin receptor. In our study, CGRP (8-37) did not block the effects of CGRP, suggesting that CGRP is acting at a receptor other than the CGRP\textsubscript{1} receptor. However, CGRP (8-37) had physiological effects of its own, exerting more potent and consistent inhibition of Quis driven firing than CGRP itself. In LLC-PK\textsubscript{1} kidney cells, human CGRP (8-37) acts as an agonist at a calcitonin receptor to activate adenylate cyclase.
(Chiba et al. 1989), similar to the agonist effects seen in this study. The dorsal horn of the spinal cord appears to contain a population of CGRP type 1 receptors due to the ability of CGRP (8-37) to inhibit hyperexcitability induced by noxious stimuli of the periphery, a phenomenon thought to be mediated in part by CGRP (Neugebauer, Rumenapp, and Schaible, 1996). CGRP in the dorsal horn of the spinal cord in cat (Miletic and Tan, 1988) and rat (Ryu, Gerber, Murase, and Randic, 1988) induces a prolonged depolarization and increased firing rate of dorsal horn neurons, quite unlike the inhibition seen in our results, suggesting the presence of functionally distinct receptors.

To conclude, data indicate that the olive contains a dense population of CGRP binding sites (van Rossum, Menard, Fournier, St-Pierre, and Quirion, 1994). Due to the inability of CGRP (8-37) to inhibit CGRP, we conclude that olivary CGRP receptors do not belong to the type 1 class of receptor. Furthermore, based on the ability of CGRP (8-37) to act as an agonist of CGRP, we suggest that a calcitonin receptor may be present in the inferior olive.

Directions for further studies

It has been previously shown that CGRP is present in afferent terminals in the IOC (Gregg, Bishop, and King, 1997). The data presented in this paper further indicate that CGRP serves a physiological function in the IOC by altering the firing rate of olivary neurons. The next step is to investigate the effect of CGRP on membrane potential and ionic conductances to further understand its role in olivary neural transmission.
SUMMARY

The data presented in this dissertation emphasize three points about CGRP in the inferior olive. First, at the light microscopic level, CGRP-immunoreactive profiles are densely distributed throughout all olivary subnuclei. Second, at the ultrastructural level, CGRP-immunoreactivity is present within olivary dendrites, dendritic spines and cell bodies as well as axons. Third, CGRP inhibits spontaneous firing rate and the excitatory effects of Quis; these effects may be mediated by a calcitonin receptor.

Double label studies in the inferior olive of the mouse indicate that CGRP contained in axons may represent afferents arising from the locus coerules or nucleus subparafascicularis. These sources may use CGRP to modulate the responsiveness of olivary neurons in regards to information about the physiological state of an organism. The data in this dissertation also demonstrate that CGRP is produced by olivary cell bodies and transported to both dendrites and olivocerebellar axons within the cerebellar cortex.

CGRP may have multiple effects in the inferior olive depending on the location of receptors. If receptors are located on afferent terminals, CGRP released from these afferent terminals or olivary dendrites could act on these receptors to facilitate or inhibit neurotransmitter or neuromodulator release.
CGRP could also act at receptors present on olivary dendrites or cell bodies to inhibit the excitatory state of the neuron. If CGRP is released from afferent terminals, it could act as a neuromodulator; if released from dendrites or dendritic spines, CGRP could act in an autocrine or paracrine manner. In the IOC, Ca\(^{2+}\) currents are involved in generating the afterhyperpolarization as well as a low threshold spike in the soma. CGRP modulation of either of these Ca\(^{2+}\) currents could result in a slowing of olivary activity. For example, CGRP could prolong the duration of the afterhyperpolarization or block the removal of the Ca\(^{2+}\) inactivation at the soma. Future studies include investigating the effects of CGRP on these Ca\(^{2+}\) currents through the use of intracellular recordings.

CGRP is the first peptide to be localized to both afferent terminals and dendrites within the olivary nucleus. This suggests complex interactions between olivary neurons and their afferent sources.
Figure 12 demonstrates the effect of CGRP on Quis driven firing rate. A. Application of quisqualate (Quis) at -10nA produces a firing rate of 0.6Hz. At -5nA, the firing rate decreases to 0.4Hz, demonstrating that Quis can elicit a dose-dependent response. CGRP applied at increasing currents of +10, +20, and +30nA produces a dose dependent abolishment of the firing rate induced by Quis. B. The spontaneous firing rate of 0.2-0.4Hz is increased to 1-1.2Hz by the application of -20nA of Quis. Pulses of CGRP at +30nA inhibits the Quis driven activity (firing rate drops to 0.2-0.6Hz). CGRP abolishes spontaneous firing rate. Quis application does not recover from this inhibition (asterisk) until the CGRP is turned off.
Figure 13 demonstrates the ability of CGRP to inhibit Quis induced firing. A. Spontaneous firing at $<0.2$Hz is abolished by CGRP application at $+40nA$. Quis application following CGRP has no effect (asterisk) until the CGRP pulse is terminated. B. Co-application of Glut and Quis increases spontaneous firing to 0.2-0.8 Hz. As seen previously, CGRP application decreases EAA driven firing as well as inhibiting spontaneous activity. When applied before the EAAs, CGRP inhibited their excitatory effects (asterisk). This inhibition continued for the duration of the CGRP pulse.
Figure 13
Figure 14 demonstrates the physiological effects of the CGRP antagonist, CGRP (8-37). A. Quis application at -20 nA produced a firing rate of 0.8 Hz in this neuron from the caudal DAO. CGRP (+20 nA) had a slight inhibitory effect, although this was not consistent with repeated application. The CGRP$_{1}$ receptor specific antagonist, CGRP (8-37) had a potent inhibitory effect on Quis driven firing, consistently abolishing firing rate. During co-application of Quis and CGRP, short pulses of the antagonist, CGRP (8-37) abolished the firing rate as previously seen; however, following application of the antagonist, the firing rate increased to 1.2 Hz. B. This neuron from the MAO had no spontaneous firing rate, but was responsive to -30 nA of Quis. Firing rate was inhibited during CGRP application, but following application, was potentiated above that previously seen for Quis alone. CGRP (8-37) had a similar effect on firing rate, also producing a rebound effect. When a pulse of CGRP was applied during Quis and CGRP (8-37), an additive inhibitory effect was visible.
Figure 15 demonstrates the effect of saline control. As seen before, Quis increases spontaneous firing. As a negative control, the solvent for CGRP, saline, was applied with a positive DC current and had a greater inhibitory effect that CGRP. CGRP application delays the ability of Quis to increase firing (asterisk) and this inhibition persisted for the duration of the CGRP pulse.
Figure 15
REFERENCES


DeZeeuw, C.I., Holstege, J.C., Ruigrok, T.J.H. and Voogd, J. (1990) Mesodiencephalic and cerebellar terminals end upon the same dendritic spines within the glomeruli of the cat and rat inferior olive: an ultrastructural study using a combination of $^{3}$H]leucine and WGA-HRP anterograde tracing. Neuroscience 34, 645-655.


Harvey, R.J. (1967). *The Spino-Olivo-Cerebellar pathway of the cat* (PhD Thesis), Australian National University,


