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**PHARMACOLOGICAL CHARACTERIZATION AND DISTRIBUTION OF MULTIPLE
BINDING SITES FOR CALCITONIN GENE-RELATED PEPTIDE AND
HOMOLOGUES**

Denise van Rossum

Department of Pharmacology & Therapeutics

McGill University, Montréal, Canada

November 1994

**A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfilment of the requirements for the degree of Doctor of Philosophy.**

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ABSTRACT

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide that arises from the alternative splicing of the RNA transcript of the calcitonin gene. Both CGRP and its receptors are widely distributed in the peripheral and central nervous systems. Recent data have suggested the existence of at least two major classes of CGRP receptors designated as, CGRP₁ and CGRP₂ receptor subtypes. The evidence suggesting the existence of receptor subtypes is based on the differential potencies in several *in vitro* and *in vivo* bioassays of the C-terminal fragment, CGRP₈₋₃₇ and the linear analogue, [acetamidomethyl-cysteine^{2,7}]CGRP α . The CGRP₁ is particularly sensitive to the antagonistic properties of CGRP₈₋₃₇ whereas the linear analogue acts preferentially as an agonist on the CGRP₂ receptors.

The primary focus of my thesis was therefore to provide novel evidence for the existence of multiple CGRP receptor sites in peripheral and especially brain tissues. First, we studied the binding affinity of CGRP, CGRP₈₋₃₇, the linear analogue [Cys(ACM)^{2,7}]CGRP α and salmon calcitonin (sCT) for [¹²⁵I]hCGRP α binding sites present in the rat brain. Moreover, we developed a novel antagonist radioligand, [¹²⁵I-Tyr]CGRP₈₋₃₇, in an attempt to directly preferentially label the CGRP₁ sites. These approaches confirmed the existence of a CGRP/sCT-sensitive binding site in the nucleus accumbens but not in other regions of the rat brain. Moreover, [¹²⁵I-Tyr]CGRP₈₋₃₇ demonstrated high affinity for all CGRP receptors. Second, we examined the binding affinity of amylin and adrenomedullin, two recently isolated peptides that revealed significant sequence homology to CGRP, for [¹²⁵I]hCGRP α binding sites in rat brain. Results showed the existence of limited cross-reactivity between CGRP, amylin and adrenomedullin as well as the unique distribution of amylin receptor sites in rat brain. Interestingly, sCT also revealed high affinity for this amylin receptor class. This may suggest the existence of distinct CGRP, amylin and adrenomedullin receptors in the rat brain. Third, we investigated the effects of a non-hydrolyzable GTP analogue, Gpp(NH)p, on [¹²⁵I]hCGRP α binding in brain and peripheral tissues in an attempt to define if CGRP receptors are G protein-coupled receptors since cloning has yet to be achieved. High affinity [¹²⁵I]hCGRP α binding was sensitive to Gpp(NH)p (100 μ M) in all tissues studied, although to a different extent, depending on the preparation used. These results suggest that the various CGRP receptor subtypes present in these tissues likely belong to the super-family of G protein-coupled receptors. Finally, on the basis of high densities of CGRP, amylin and sCT receptor sites localize in the rat nucleus accumbens, we evaluated the effects of central administration of CGRP, amylin and sCT on amphetamine-induced

and spontaneous locomotor activities. All three peptides decreased, in a dose-dependent manner, these two neurobehaviors (sCT > amylin > CGRP). Furthermore, bicuculline, a GABA_A receptor antagonist, selectively blocked the locomotor effects of CGRP without affecting those of amylin and sCT. Taken together, the findings of my thesis support the existence of functionally distinct receptors for CGRP and related peptides in the rat brain.

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RÉSUMÉ

Le peptide relié au gène de la calcitonine (CGRP) est un peptide composé de 37 acides aminés résultant de l'épissage alternatif de l'ARN messager du gène de la calcitonine. Le CGRP, ainsi que ses sites de liaison, sont largement distribués dans les systèmes nerveux central et périphérique. De récentes études suggèrent l'existence d'au moins deux classes de récepteurs au CGRP, soit les sous-types CGRP₁ et CGRP₂. Les propriétés pharmacologiques de ces sous-types de récepteurs sont basées sur les puissances relatives du fragment CGRP₈₋₃₇ et de l'analogue linéaire, [acetamidométhylcystéine^{2,7}]CGRP α dans divers essais biologiques *in vitro* et *in vivo*. Les récepteurs de type CGRP₁ sont plus sensibles aux propriétés de l'antagoniste CGRP₈₋₃₇ tandis que l'analogue linéaire agit de façon préférentielle sur le sous-type CGRP₂.

L'objectif premier de ma thèse était donc de fournir des évidences additionnelles quant à l'existence de sous-types de récepteurs au CGRP dans divers tissus et plus particulièrement dans le cerveau. Pour ce faire, nous avons tout d'abord évalué l'affinité comparative du CGRP lui-même, de son analogue linéaire, du fragment CGRP₈₋₃₇ ainsi que de la calcitonine de saumon pour la liaison de l' [¹²⁵I]hCGRP α dans diverses régions du cerveau de rat. De plus, nous avons développé un nouveau radioligand, soit le [¹²⁵I-Tyr]CGRP₈₋₃₇, dans le but de marquer de façon préférentielle les sites de type CGRP₁. Les résultats obtenus ont confirmé l'existence de sites sensibles au CGRP dans diverses régions du cerveau. Dans le noyau accumbens, ces sites sont également sensibles à la calcitonine de saumon. Le [¹²⁵I-Tyr]CGRP₈₋₃₇ a démontré une haute affinité pour les récepteurs au CGRP dans tous les tissus étudiés mais n'a pu révéler de façon distincte un profil de liaison de type CGRP₁ dans ces tissus. En deuxième lieu, nous avons examiné les affinités de l'amyline et de l'adrénomédulline, deux peptides isolés récemment et démontrant une similarité de séquence avec celle du CGRP, pour la liaison de [¹²⁵I]hCGRP α dans diverses régions du cerveau de rat. Les résultats obtenus ont démontré l'existence de réactions croisées limitées entre le CGRP, l'amyline et l'adrénomédulline, supportant l'existence de trois classes de récepteurs pour ces trois peptides. Ces études nous ont aussi permis de révéler la distribution unique d'une classe de récepteurs à l'amyline, dans diverses régions du cerveau de rat; la calcitonine de saumon démontrant également une forte affinité pour cette nouvelle classe de récepteurs. Troisièmement, nous avons investigué les effets d'un analogue stable du GTP, le Gpp(NH)p, sur la liaison de l' [¹²⁵I]hCGRP α , dans le cerveau de rat et certains tissus

périphériques. Cette étude avait pour but de préciser si les différents sous-types de récepteurs au CGRP sont couplés à une protéine G puisque le clonage de ceux-ci n'a pu être accompli jusqu'à maintenant. Les sites de liaison à haute affinité de l'['¹²⁵I]hCGRPα sont sensibles au Gpp(NH)p (100 μM) dans tous les tissus étudiés mais ce, de façon nuancée, dépendamment de la préparation étudiée. Ces résultats suggèrent que les différents sous-types de récepteurs au CGRP retrouvés dans ces tissus appartiennent à la famille des récepteurs couplés à une protéine G. Enfin, sur la base de l'abondance des sites de liaison au CGRP, à l'amyline et à la calcitonine de saumon dans le noyau accumbens, nous avons évalué le profil des effets de l'administration, par voie intracérébroventriculaire, de ces trois peptides sur les comportements locomoteurs spontanés ou induits par l'amphétamine. Chacun de ces peptides diminue, de manière dose-dépendante, l'activité locomotrice spontanée ou induite par l'amphétamine (calcitonine de saumon > amyline > CGRP). De plus, la bicuculline, un antagoniste du récepteur GABA_A, bloque de façon sélective l'effet du CGRP sur l'activité locomotrice puisqu'aucun effet significatif sur les actions de l'amyline et de la calcitonine de saumon n'a pu être observé. Globalement, les résultats de ma thèse appuient l'existence de récepteurs fonctionnellement distincts pour ces divers peptides de la famille du CGRP dans le cerveau de rat.

ACKNOWLEDGMENTS

I simply wish to thank both "families", the one at the Douglas and the one at McGill, that made this experience more enjoyable. I wish to especially thank my supervisor R.Quirion who gave me the chance to fulfill this goal of mine. Although Rémi asks a lot, he gave me a lot back throughout the past four years. Thanks for the "speedy" corrections from all over the world, they were very much appreciated. Thanks to B.Robaire who gave me precious advice throughout my training and especially when oral presentations were requested by the department. Thanks to B.Collier, for the many discussions about life and beer and of course science too because after all, we are supposed to be scientist!

To the crowd and staff from the Department downtown who were always helpful and supportive. Special thanks to Anurag, Daniel H., Ricky, Robert, Steve and Suzanne for their friendship. To the ex, old and new Douglas crowd and staff who all made this place a "unique place"! Thanks to Alain, Annie, Christine, Clarissa, Dajan, Danielle C., Danielle J., Françoise, Hélène, Isabelle, Jacques-André, Jean-Guy, Jim, Joan, Joe, Judes, Kar, Mike (M!), Pascale, Seema, Victor, Wayne and Yvan with whom I shared great moments as well as daily life. To Daniel M., for the many "lively" discussions... To close friends and especially Audrey, Marie-France, Suzanne and Nathalie who gave me support during my numerous McGill years and more! To Alain, for the patience and support throughout these years. Don't worry, good memories stay...

To my parents who were always there for me during the last 28 years... To my numerous family members who kept the spirit alive! To Nicole and Caroline who are happy to know everything about CGRP now... To Uwe, thanks for the past and present. The future will be even better!

A special memory to Trevor who suddenly passed away few months ago. Thanks for your advice and support, for care and professionalism.

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PREFACE

As stated in section B.2 of the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research, McGill University:

Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, **connecting texts, providing logical bridges between the different papers**, are mandatory.

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

Additional material (procedure and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

The following people have contributed to the research included in the present thesis. R. Quirion is my research and thesis supervisor. Hence, he is senior author on most of the manuscripts.

i. Characterization of CGRP₁ and CGRP₂ receptor subtypes

R. Quirion, D. van Rossum, Y. Dumont, S. St-Pierre and A. Fournier (1992) *Ann. NY Acad. Sci.* 657: 88-105. (Chapter 2)

R. Quirion summarized the findings with respect to the pharmacological characterization of CGRP receptors that was pursued in his laboratory since 1988. Original work by Dr. T. Dennis is also included.

D. van Rossum: The original studies that I first completed are included in this report and describe the comparative affinities of CGRP analogs between species (Fig. 2.1) as well as the initial pharmacological characterization of CGRP receptors in rat brain (Figs. 2.2, 2.3, 2.4, 2.5 and Table 2.5).

Y. Dumont is a research assistant in R. Quirion's laboratory. He was involved in the initial pharmacological characterization of the first series of CGRP analogs in peripheral tissues.

S. St-Pierre and A. Fournier are peptide chemists at the INRS-Santé Institute in Pointe-Claire. They were in charge of the synthesis and purification of CGRP and all related peptides.

- ii. Binding profile of a selective calcitonin gene-related peptide (CGRP) receptor antagonist ligand, [¹²⁵I-Tyr]hCGRP₈₋₃₇, in rat brain and peripheral tissues**
D. van Rossum, D.P. Ménard, A. Fournier, S. St-Pierre and R. Quirion
(1994) *J.Pharmacol.Exp.Ther.* 269: 846-853. (Chapter 3)

D.P. Ménard is a post-doctoral fellow in R. Quirion's laboratory. He was technically involved in the development of the radioligand as well as in some of the binding assays performed using membrane preparations.

A. Fournier and S. St-Pierre: see *i*.

- iii. Autoradiographic distribution and receptor binding profile of [¹²⁵I]Bolton Hunter-rat amylin binding sites in the rat brain**
D. van Rossum, D.P. Ménard, A. Fournier, S. St-Pierre and R. Quirion
(1994) *J.Pharmacol.Exp.Ther.* 270: 779-787. (Chapter 4)

D.P. Ménard: see *ii*.

A. Fournier and S. St-Pierre: see *i*.

- iv. **Comparative affinities of human adrenomedullin for [¹²⁵I]hCGRP α and [¹²⁵I]BH-rat amylin specific binding sites in the rat brain**
D. van Rossum, D.P. Ménard, J.K. Chang and R. Quirion
(1994) *Can.J.Physiol.Pharmacol.* (submitted) (Chapter 5)

D.P. Ménard: He participated technically in some of the experiments, especially when *in vitro* receptor autoradiography was performed.

J.K. Chang is a peptides' chemist at Phoenix Pharmaceuticals (USA) who generously provided us with adrenomedullin and related analogs.

- v. **Effect of guanine nucleotides and temperature on calcitonin gene-related peptide receptor binding sites in brain and peripheral tissues**
D. van Rossum, D.P. Ménard and R. Quirion
(1993) *Brain Res.* 617: 249-257 (Chapter 6)

D.P. Ménard: see *ii*.

- vi. **Profile of CGRP-, amylin- and salmon calcitonin-induced depression of locomotion following intracerebroventricular injection**
D. van Rossum, D.P. Ménard and R. Quirion
(Chapter 7)

D.P. Ménard actively participated in both the technical execution and planning of experiments as well as the analysis of all data obtained.

8.3 Claims for originality

The following elements represent original contributions to the pharmacological characterization of CGRP receptors and its related peptides including amylin and adrenomedullin. My thesis provides unique data with respect to the binding profile, transduction mechanism as well as neurobehavioral effects of CGRP and homologues, and their respective receptor classes.

- **Chapter 2:** The characterization of putative CGRP receptor subtypes in peripheral tissues as well as the existence of CGRP/sCT-sensitive sites in the nucleus accumbens have been previously reported. However, the competition profile of CGRP and CGRP receptor subtypes preferring analogs in the CNS was not studied before. In addition, the biological effects of key CGRP analogs in peripheral tissues of the guinea pig and rat were evaluated. This comparison between species was critical in regard to the pharmacological characteristics of the CGRP receptor subtypes and to exclude that CGRP receptor subtypes were only related to species differences. The obtained results clearly revealed the existence of CGRP₁ and CGRP₂ receptors in a given species.

- **Chapter 3:** The radioligand [¹²⁵I-Tyr]¹hCGRP₈₋₃₇ was synthesized and its binding profile characterized. This report includes the time courses of ligand association and dissociation, saturation curves, competition binding profile of several CGRP homologues for [¹²⁵I-Tyr]¹hCGRP₈₋₃₇ binding in brain and peripheral tissues as well as the discrete localization of these binding sites in the rat brain. In fact, [¹²⁵I-Tyr]¹hCGRP₈₋₃₇ represents the first CGRP antagonist radioligand to be developed. It should become a useful tool to distinguish between agonist vs. antagonist binding characteristics of this peptide family.

- **Chapter 4:** Only one earlier report (Beaumont *et al.*, 1993) reported on the existence of specific binding for [¹²⁵I]BH-rat amylin in the rat brain. However, this report focused on binding sites present in the nucleus accumbens as little specific binding was detected in other areas. Our study showed, for the first time, the complete distribution and binding profile of [¹²⁵I]BH-rat amylin in the rat brain. Our study also reports on the competition profile of amylin, salmon calcitonin and hCGRP α for specific [¹²⁵I]BH-rat amylin binding in various brain areas as well as the biological effects of these peptides in two *in vitro* bioassays, namely the atrium (CGRP₁-typical preparation) and vas deferens (CGRP₂-typical preparation). Finally, the binding affinity of amylin for [¹²⁵I]hCGRP α binding sites in the rat brain was also evaluated. Taken together, these findings suggest

the existence of a different class of receptors for amylin as well as demonstrate the rather limited cross-reactivity between CGRP and amylin binding sites in the rat brain.

- **Chapter 5:** Very little is currently known regarding adrenomedullin, its receptor and biological effects as this peptide was isolated just about a year ago. Our study is the first to demonstrate the low affinity of adrenomedullin for either [125 I]hCGRP α or [125 I]BH-rat amylin binding sites in all brain areas investigated suggesting the possible existence of a unique class of receptors for adrenomedullin, a new member of the CGRP peptide family.

- **Chapter 6:** Several earlier studies reported on the sensitivity of [125 I]hCGRP α binding to GTP and its stable analogs in various tissue preparations including the atrium and the cerebellum. The purpose of our study was to compare the sensitivity of [125 I]hCGRP- α binding to a stable GTP analogue (Gpp(NH)p) in tissue preparations enriched with various CGRP receptor subtypes in order to determine if all CGRP receptor subclasses belong to a G protein-coupled receptor type. We have thus confirmed earlier findings in the atrium, a CGRP $_1$ -enriched preparation and in the cerebellum. Furthermore, we have shown that Gpp(NH)p modulated CGRP binding in the vas deferens, a CGRP $_2$ -enriched preparation, and the rat brain (minus cerebellum). Assays were also performed at several incubation temperatures to verify coupling efficacy. These data confirmed that CGRP receptors likely belong to the G protein-coupled receptor super-family, although the potency of Gpp(NH)p was clearly temperature-dependent in these various tissues.

- **Chapter 7:** The existence of high densities of CGRP, sCT and amylin receptor sites in the nucleus accumbens has been established. This nucleus plays a critical role in the modulation of locomotor behaviors. Indeed, the effects of central administration of salmon calcitonin on amphetamine-induced locomotor activity have been previously reported and likely relate to the existence of specific receptors in the nucleus accumbens. We have therefore compared the effects of sCT with those of CGRP and amylin on spontaneous as well as amphetamine-induced locomotor behaviors. Salmon calcitonin and amylin were found to be more potent than CGRP in these two behavioral paradigms. In addition, bicuculline, a GABA $_A$ receptor antagonist, blocked the effects of CGRP whereas not affecting those of sCT and amylin. These results provide evidence for the existence of functionally distinct receptors for these peptides in the modulation of locomotor activity.



CHAPTER 1

Review of the literature

McGill University (1994)



1.1 Calcitonin gene-related peptide and homologues

Calcitonin gene-related peptide (CGRP) is one of the first examples of a neuropeptide to be discovered purely by a molecular approach. Two forms have thus far been isolated, namely the CGRP α and CGRP β (also called CGRP-I and CGRP-II). The family of peptides that CGRP belongs to is still expanding and now includes calcitonin, amylin or islet amyloid polypeptide and adrenomedullin. The present section provides a brief description for each member of this family.

1.1.1 Calcitonin gene-related peptide (CGRP)

1.1.1.1 Alternative splicing process: CGRP α and calcitonin

The calcitonin/CGRP gene is expressed in specific cell types of both the endocrine and nervous systems. The gene is alternatively spliced to yield mRNA encoding calcitonin in thyroid C-cells or the neuropeptide CGRP in a subset of central and peripheral neurons (Amara *et al.* 1982; Rosenfeld *et al.* 1983; Morris *et al.* 1984; Rosenfeld *et al.* 1984). The rat as well as the human calcitonin/CGRP gene consists of six exons (Figure 1.1). The calcitonin mRNA contains the exons 1 to 4 with a poly(A) tail at exon 4 whereas CGRP α includes the exons 1,2,3,5 and 6 with a poly(A) tail at exon 6. Structural comparison of human calcitonin and human or rat CGRP peptides revealed ~25% homology. Interestingly, salmon calcitonin exhibits a greater structural similarity to human CGRP than to human calcitonin (Figure 1.2). The high homology between these peptides suggests that calcitonin and CGRP exons are derived from a common ancestral gene and that the calcitonin/CGRP gene arose from duplication and sequence divergence events (Lips *et al.* 1988). Moreover, the high degree of homology (86 to 89%) between chicken and human CGRP suggests that CGRP is a well conserved, old and important polypeptide. The expression of this gene is regulated by a number of hormones and second messengers including glucocorticoids (Tverberg and Russo, 1992) and cAMP (Wind *et al.* 1993). Leff *et al.* (1987) have proposed that the specific calcitonin splicing is the "default" pathway and that CGRP-specific splicing requires a dominant neuronal trans-acting factor.

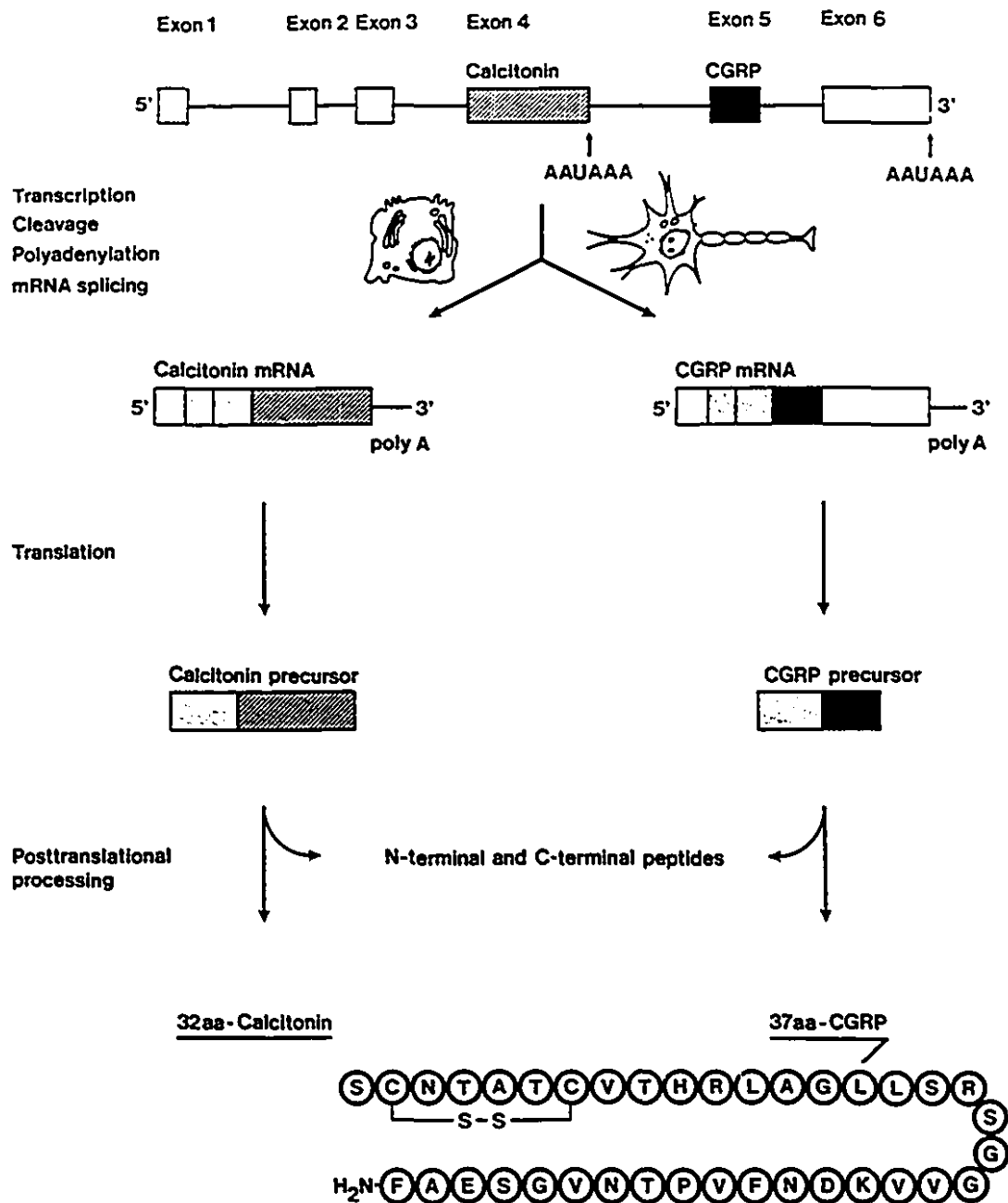


Figure 1.1 Alternative mRNA splicing of the calcitonin gene scheme generating calcitonin and CGRP in a tissue-specific manner.

A good candidate for this factor is a protein component of the spliceosome, SmN, as this protein is expressed in only a limited range of tissues and cell types such as the brain and the heart (Leff *et al.* 1987; Latchman, 1990). The cell types that express SmN are precisely those that are capable of correctly splicing the calcitonin/CGRP transcript to produce CGRP mRNA. However, the inclusion of exon 4 is also a complex event which involves calcitonin-specific uridine branch acceptor, the poly(A) tail of exon 4 as well as a regulatory element within exon 4 (Adema *et al.* 1990; Emeson *et al.* 1989; Bovenberg *et al.* 1989; Cote *et al.* 1992).

1.1.1.2 CGRP β

A mRNA product of a related gene has been identified in rat brain and thyroid to encode the protein precursor of a peptide differing from CGRP α by only one or three amino acids in rat and human, respectively. The mRNA encoding this peptide has been referred to as CGRP β (Amara *et al.* 1985). The gene encoding this second CGRP peptide was isolated from a cosmid library and has been shown to not be subjected to the mechanism of alternative gene expression, as no second calcitonin peptide was produced. Consequently, only the CGRP peptide could be derived from this second gene (Steenbergh *et al.* 1986). In general, the distribution of the hybridization signal for either CGRP α or CGRP β is similar in the central and peripheral nervous systems (Amara *et al.* 1985). However, some evidence suggest that these two CGRP-related peptides and their respective receptors can be differentially distributed in certain tissues such as primary sensory neurons (Mulderry *et al.* 1988), intrinsic enteric neurons (Sternini and Anderson, 1992) and the human hypothalamus (Henke *et al.* 1987). This differential expression pattern suggests a selective regulation of the CGRP genes. Indeed, dibutyryl cAMP predominantly stimulates the expression of CGRP β gene in thyroid carcinoma cell line (Wind *et al.* 1993). Moreover, the levels of mRNA for CGRP α are increased whereas those of the CGRP β mRNA are decreased or unaltered in rat spinal motoneurons that were subjected to either axotomy, crushing or axonal flow blockade (Saika *et al.* 1991; Piehl *et al.* 1993; Katoh *et al.* 1992). These reports all support that these two peptides may be differentially regulated.

It has also been shown that CGRP β can have distinct biological activities including its ability to suppress gastric acid secretion (Beglinger *et al.* 1991; Beglinger *et al.* 1988). Finally, Jansen (1992) showed that the relaxation induced by CGRP α , but not that of

CGRP β , in the guinea pig basilar artery was antagonized by the fragment CGRP₈₋₃₇. These findings could suggest that the α and β forms of CGRP act through different CGRP receptor subtypes although further studies will be needed to confirm this interesting possibility.

1.1.1.3 CGRP conformation and structure-activity relationships

All species variants of CGRP have 37 residues, a C-terminal (phenylalanyl)amide, and a disulfide bridge between positions 2 and 7 (Figure 1.2). Circular dichroism and nuclear magnetic resonance studies have indicated that CGRP has a solvent-dependent tendency to adopt α -helical secondary structure. A significant increase in the α -helical content of rat CGRP, varying from 20% to 60%, in purely aqueous medium over buffer containing 50% trifluoroethanol respectively, has been observed (Lynch and Kaiser, 1988; Manning, 1989; Breeze *et al.* 1991). The use of trifluoroethanol solvent is thought to be consistent with a role for the membrane lipid-water interface in stabilizing an active conformation at the receptor level. Under these conditions, an amphiphilic α -helix conformation between residues 8 to 18, two β -turn structures between residues 17 to 21 and 29 to 34 and a random-coil segment formed by residues 23 to 29 of CGRP have been suggested (Hubbard *et al.* 1991; Saldanha and Mahadevan, 1991). Moreover, the structural characterization of various fragments of hCGRP α such as hCGRP₈₋₃₇, [Cys(ACM)^{2,7}]hCGRP α and hCGRP₁₉₋₃₇ lead to the suggestion that the residues 1 to 7 as well as the disulfide bridge have an important role in the stabilization of the α -helix whereas the C-terminal residues 19 to 37 do not (Mimeault *et al.* 1993).

Essentially no biological activity for trypsin- and chymotrypsin-digested fragments of CGRP has been demonstrated in rat atrial preparations (Tippins *et al.* 1986), bone resorption, blood flow and edema (Zaidi *et al.* 1990), indicating that most of the intact sequence is required for biological activity. Furthermore, both N- and C-terminal regions seem to be important for agonist activity as the acetylation of the N-terminus as well as N-terminal fragments including Ala¹-Arg¹¹, Ala¹-Ser¹⁶ and Ala¹-Arg¹⁸ have revealed a marked reduction in potency (Zaidi *et al.* 1990; Maggi *et al.* 1990). Similarly, the deletion of the C-terminal phenylalaninamide or the C-terminally truncated fragments hCGRP₁₋₃₄, hCGRP₁₋₃₅ and hCGRP₁₋₃₆ have shown marked decrease biological activity (Thiebaud *et al.* 1991; O'Connell *et al.* 1993; Smith *et al.* 1993). Apparently these deletions do not disrupt the conformation of the peptide (O'Connell *et al.* 1993).

The synthetic analogs of chicken and human CGRP were compared for their calcium and phosphate lowering effects (Morita *et al.* 1989) as well as for the formation of cAMP in a pre-osteoblast cell line (Thiebaud *et al.* 1991). The chicken CGRP that differs from the human form at only 4 residues (position 3, 14, 15, 23) showed higher potency than human CGRP in all three assays. Interestingly, the substitution of glycine by an aspartate residue at position 14 in human CGRP confers increased activity, similar to that of chicken CGRP. The respective substitution at the other residues does not significantly affect the activity of human CGRP (Morita *et al.* 1989; Thiebaud *et al.* 1991). These results thus indicate the importance of the aspartate residue at position 14 to exhibit the stronger and prolonged biological activity of chicken vs. human CGRP.

One of the most exciting findings with respect to CGRP structure-activity studies is undoubtedly the potent antagonistic properties of the C-terminal fragment CGRP₈₋₃₇ (Chiba *et al.* 1989). Further details on the pharmacological characteristics of this CGRP fragment are provided in Chapters 2 (2.2.2) and 3. Shorter C-terminal fragments including CGRP₁₂₋₃₇ (Dennis *et al.* 1989), CGRP₁₉₋₃₇ (Rovero *et al.* 1992) and [Tyr⁰]CGRP₂₈₋₃₇ (Chakder and Rattan, 1990; Maton *et al.* 1990) also all showed antagonistic properties but with lower potencies when compared to the CGRP₈₋₃₇ fragment. Hence, Mimeault *et al.* (1991) reported that the threonine residue in position 9 is critical for the maintenance of adequate receptor affinity and antagonistic properties. It thus appears that the amphiphilic α -helix identified between residues 8 to 18 likely plays a major role in the interaction of the molecule with the receptor whereas the N-terminal loop region may principally be involved in triggering the ensuing signal transduction process.

1.1.2 Amylin

The present description will be limited only to the most recent findings as well as some biological effects on peripheral tissues as a more complete description is provided in the Chapter 4 of the present thesis.

Amylin or islet amyloid polypeptide (IAPP) was first isolated from amyloid fibrils of an insulin-secreting human tumor (Westermarck *et al.* 1986; Westermarck *et al.* 1987). It revealed ~50% amino acid sequence homology with CGRP (Fig. 1.2). Amylin is a product of pancreatic β -cells where it is co-localized (Ahrén and Sundler, 1992; Mulder *et al.* 1993) and co-released with insulin in response to glucose (Kanatsuka *et al.* 1989;

Fehmann *et al.* 1990a). It was also found to be a major constituent of the amyloid deposits seen in islets of non-insulin dependent (type 2) diabetic humans and cats (Cooper *et al.* 1987). Furthermore, strong amyloidogenic properties are associated with the human and cat forms of the peptide but not with its rodent counterparts including that of rats and mice (Westermarck *et al.* 1990; Nishi *et al.* 1989). The interspecies variation in the amino acid sequence of residues 25 to 29 of amylin has been shown to play a major role in amyloid deposition in the islets of humans vs. their absence in rats. Secondary structure studies by circular dichroism spectroscopy have revealed fundamental differences in the structures adopted by amylin from human vs. rat species as highly organized α -helical conformation and little structure are observed, respectively (McLean and Balasubramaniam, 1992). In addition, micromolar concentrations of human (but not rat) amylin-induced toxicity have been reported in β -cells of the adult pancreas of rats and humans (Lorenzo *et al.* 1994) as well as in rat primary hippocampal cultures (May *et al.* 1993).

Amylin has been reported to have opposing effects compared to those of insulin. These include decreased basal and insulin-stimulated rates of glycogen synthesis (Leighton and Cooper, 1988) and glucose uptake (Cooper *et al.* 1988; Tabata *et al.* 1992). Increased muscle glycogenolysis, most likely via the modulation of the glycogen synthase and phosphorylase, has also been reported for amylin (Deems *et al.* 1991; Young *et al.* 1993a; Young *et al.* 1993b; Lawrence and Zhang, 1994). Moreover, there are conflicting reports with respect to the direct effects of amylin on insulin secretion as decreased (Wang *et al.* 1993; Silvestre *et al.* 1993), no effect (Nagamatsu *et al.* 1990; Inoue *et al.* 1993) as well as increased insulin secretion (Fehmann *et al.* 1990b) have been reported. Because of its opposing effects to insulin and its amyloidogenic and neurotoxic properties, amylin has been proposed to play a prominent role in the pathogenesis of type 2 diabetes (for recent reviews, see Betsholtz *et al.* 1993; Cooper, 1994). In an attempt to shed light on this issue, Fox *et al.* (1993) developed transgenic mice that overexpress the human amylin gene. However, amyloid deposits were not detected in these animals suggesting that other co-existing abnormalities in type 2 diabetes are required for the formation of amyloid deposits in the islets of Langerhans. The precise role of amylin in the pathology of this disease thus still remain to be fully established.

1.1.3 Adrenomedullin

Chapter 5 includes a full description of this recently isolated peptide. Briefly, adrenomedullin (ADM) is the most recently discovered peptide belonging to the CGRP/calcitonin peptide family revealing ~25% homology with CGRP (Kitamura *et al.* 1993a). It consists of 52 amino acids and has one intramolecular disulfide bond between amino acid residues 16 and 21. Both the mRNA and the peptide, as measured by RNA blot analysis and radioimmunoassay respectively, have been detected in a variety of human and porcine tissues including the adrenal medulla, ventricle, lung and kidney (Kitamura *et al.* 1993b; Ichiki *et al.* 1994; Kitamura *et al.* 1994). Only low amounts of immunoreactive ADM-like peptide(s) have been detected in brain cortex (Ichiki *et al.* 1994).

Similar to CGRP, ADM elicited a potent and long lasting reduction in blood pressure in rats, mainly by inducing a vasodilatation (Ishiyama *et al.* 1993). This effect may be mediated through the activation of a single class of high affinity binding sites for ADM (K_D $1.3 \times 10^{-8}M$) present in vascular smooth muscle cells (Eguchi *et al.* 1994) leading to an increase in cAMP formation (Ishizaka *et al.* 1994). Interestingly, the antagonist hCGRP₈₋₃₇ has been shown to inhibit, in a dose-dependent fashion, the ADM-induced increase in cAMP formation (Eguchi *et al.* 1994). It thus remains to be established if ADM acts through CGRP receptors or unique ADM receptor sites to produce its effects.

human CGRP α	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-NH ₂
human CGRP β	--N-----M--S-----NH ₂
rat CGRP α	S-N-----D-----E--NH ₂
rat CGRP β	S-N-----D-----NH ₂
rat amylin	K-N-----A-Q---NF-VH-SNNFGAILSS-----NTY-NH ₂
salmon calcitonin	CSNLS---LGK-SQE-HKLQT*****YPR--T--GTP-NH ₂
human calcitonin	CGNLS---MLGTYTQDFNKFHT*****-PQ-AI-VG-P-NH ₂
human ADM	G-RFG---TVQK--HQIYQFTDKD-D-VA-RSKI-PGGY-NH ₂
	YRQSMNNFQGLRSF

Figure 1.2 Amino acid sequence of CGRP and related peptides. Amino acids in common to human CGRP α are indicated by dash (-). Spaces inserted to allow for sequence comparison are indicated by a star (★).

1.2 CGRP and the central nervous system

CGRP is one of the most widely distributed peptide in nervous tissues in both vertebrates and invertebrates. The detailed map of the discrete distribution of CGRP mRNA, CGRP immunoreactive cell bodies and nerve fibers as well as CGRP receptor sites have been reported in various species including rat, cat and human brains, and is summarized in the following sections.

1.2.1 CGRP mRNA containing neurons as revealed by *in situ* hybridization

Thus far, few laboratories have mapped the distribution of CGRP mRNA in neurons of the rat central nervous system using *in situ* hybridization histochemistry (Amara *et al.* 1985; Rethelyi *et al.* 1989; Rethelyi *et al.* 1991; Kresse *et al.* 1992). The distribution of neurons expressing CGRP mRNAs is described here according to these reports and summarized in Figures 1.3a, b, c (first column).

The highest densities of neurons expressing the CGRP mRNA have been observed in the rostral part of the lateral hypothalamic area as well as in the lateral portion of the ventral and dorsal parabrachial nuclei. Moreover, high amounts of CGRP mRNA positive neurons have been seen around the subparafascicular nucleus, the posterior thalamic nuclear group, the peripeduncular nucleus and in the lateral subparafascicular nucleus. The parabigeminal nucleus and the lateral superior olive nucleus are also densely labeled. Moderate number of CGRP mRNA positive neurons has been found in the bed nucleus of the stria terminalis, the nucleus of the lateral olfactory tract, the arcuate hypothalamic nucleus, the thalamic ventroposterior nucleus and in the parafascicular nucleus. The lateral lemniscus, the reticular paragigantocellular nucleus, the external cuneate nucleus also contained a moderate number of cell bodies expressing the CGRP mRNA. Few CGRP mRNA expressing neurons are reported in the medial preoptic nucleus, the zona incerta, the ventromedial thalamic nucleus, the ventromedial hypothalamic nucleus as well as the paracentral thalamic nucleus. Few CGRP mRNA labeled cell bodies have been seen in the pontine nuclei, central gray and in the reticular nucleus.

With the exception of the dorsal motor nucleus of the vagus nerve (X), the presence of CGRP mRNA has been reported in all cranial nuclei. However, in the motor nucleus of the trigeminal nerve (V), only a small population of neurons with low levels of

expression is observed. In contrast, the CGRP mRNA signals in most neurons of the facial, hypoglossal and ambiguous nuclei are intense (Rethelyi *et al.* 1989; Rethelyi *et al.* 1991).

Almost all large neurons in the ventral horn of the cervical and lumbar regions of the spinal cord were labeled with the CGRP probe (Rethelyi *et al.* 1989) whereas up to 75% of the motoneurons of the ventral spinal cord (laminae VII-IX) at thoracic level (C5,T8) were found to express CGRP mRNA.

A large number of small and medium size neurons of the rat dorsal root ganglion, at all spinal cord levels (cervical to sacral) have also shown intense hybridization signaling. Sections of the trigeminal ganglion revealed that more than 50% of the perikarya contain CGRP mRNA.

1.2.2 CGRP immunoreactive structures in the brain

The distribution of immunoreactive CGRP in the rat central nervous system has been examined in detail by immunocytochemistry (Rosenfeld *et al.* 1983; Kawai *et al.* 1985; Skofitsch and Jacobowitz, 1985a; Kruger *et al.* 1988; Unger and Lange, 1991; Hares and Foster, 1991; Hökfelt *et al.* 1992). Similar to the CGRP mRNA distribution, CGRP-immunoreactive neurons and fibers are widely but unevenly distributed in the central nervous system, as described in the following sections. The second column of Figures 1.3a, b, and c provides a summary of this description.

1.2.2.1 CGRP-immunoreactive cell bodies

A widespread distribution of CGRP-positive cells has been reported throughout the central nervous system. CGRP-immunoreactive cell bodies are present in various nuclei of the hypothalamus including the preoptic area, the medial preoptic, the periventricular and the anterior hypothalamic nuclei. The perifornical area and the lateral hypothalamus-medial forebrain bundle area as well as in the premammillary nucleus, the medial amygdaloid nucleus, the dentate gyrus of the ventral hippocampal formation, the ventromedial nucleus of the thalamus, the periventricular gray and the parafascicular area, extending laterally over the lemniscus medialis are also enriched with specific CGRP staining. Moreover, CGRP-positive cells are found in the peripeduncular area, ventral to the medial geniculate body, the parabigeminal nucleus as well as in the superior colliculus.

In the hindbrain, positive cells are found in the central portion of the lateral and medial parabrachial areas as well as in the ventral tegmental nucleus, the inferior colliculus, the lateral lemniscus, the superior olive and the nucleus tractus solitari.

All cranial nuclei of the rat brain have shown positive CGRP-immunoreactive cell bodies including the oculomotor (III), trochlear (IV), trigeminal motor (V), facial motor (VII), ambiguus (X) and the hypoglossal (XII) nuclei.

CGRP-labeled somata as well as numerous perisomatic fibers have been reported in various autonomic ganglia such as the ciliary, sphenopalatine, otic, glossopharyngeal-vagal and submandibular ganglia, that compose the cranial postganglionic parasympathetic pathways (Silverman and Kruger, 1989; Hardebo *et al.* 1992). In contrast, only a small proportion (<1%) of sympathetic principal neurons in the stellate and lumbar sympathetic ganglia are positive for CGRP immunoreactive-like peptides. Some of the neurons in the stellate and lower lumbar ganglia, that contain both CGRP and vasoactive intestinal peptide, project to the sweat glands (Landis and Fredieu, 1986). Furthermore, the sympathetic superior cervical ganglion lacks stained somata but exhibits CGRP-immunoreactive perisomatic axons. Such axons are probably of sensory origin as few autonomic parasympathetic preganglionic axons contain CGRP-IR and since in other sympathetic ganglia, similar perisomatic peptidergic nerve terminals are of somatic sensory origin (Silverman and Kruger, 1989).

In human, positive perikarya have also been observed in the hypoglossal, facial and ambiguus nuclei, as well as in dorsal motor nucleus of the vagus and prepositus hypoglossal nucleus. In contrast to the rat, the human superior cervical ganglion cells contain CGRP co-localized with tyrosine hydroxylase-positive cells (Baffi *et al.* 1992).

1.2.2.2 CGRP-immunoreactive fibers

Limited amounts of CGRP fiber terminals can be seen in the olfactory bulb as well as in the medial prefrontal cortex whereas very few fibers are found in most neocortical areas except the ventral sector of the piriform cortex and in the perirhinal cortex. Moderately dense networks of CGRP-positive fibers have been observed in the various thalamic areas, in the septum, the bed nucleus of the stria terminalis, the central amygdaloid nucleus and in the caudal and ventral portion of the striatum. Several hypothalamic nuclei are highly enriched with CGRP-positive fibers, including the medial preoptic area, the periventricular area, the dorsomedial nucleus, the median eminence and

the medial forebrain bundle area. In the lower brainstem, the highest concentrations of fibers have been seen in the superficial layers of the sensory trigeminal areas, with a moderately dense fiber network in the periaqueductal central gray, medial geniculate body, parabrachial nucleus, lateral lemniscus, dorsal part of the interpeduncular nucleus, parabrachial nucleus, superior olive, cochlear nucleus, nucleus tractus solitarius and parts of the vestibular nuclei.

Notable levels of CGRP-like immunoreactive fibers are distributed in the anterior pituitary in the rat (Gon *et al.* 1990). Double-immunostaining experiments proved the nearly complete co-localization with substance P in these nerve fibers (Ju *et al.* 1993).

CGRP fiber terminals are heavily concentrated in laminae I-II and in the reticulated region of lamina V of the dorsal horn of the rat spinal cord (Gibson *et al.* 1984; Skofitsch and Jacobowitz, 1985a). The CGRP-containing axons are largely unmyelinated or small-diameter myelinated and constitute almost 30% of the primary afferent axons of the Lissauer's tract, the major afferent input to the superficial laminae of the dorsal horn (Gibson *et al.* 1984; Levine *et al.* 1993). Furthermore, over 80% of spinal afferent neurons supplying visceral structures including urogenital tract and upper gastro-intestinal tract are CGRP-immunoreactive (Su *et al.* 1986). In these and other systems, CGRP-containing primary afferent fibers are frequently found around blood vessels. CGRP-immunoreactive fibers have also been noted around the central canal and in a number of motoneurons of the ventral horn (Gibson *et al.* 1984; Skofitsch and Jacobowitz, 1985a).

In the human brainstem, CGRP-immunoreactive fibers are concentrated in dorsal horn (laminae I-III), spinal trigeminal nucleus, nucleus of the solitary tract and principal sensory trigeminal nucleus (Unger and Lange, 1991). A moderately dense fiber network can also be seen in the locus coeruleus as well as in the parabrachial nucleus. Few scattered CGRP-immunoreactive fibers are found in the central periaqueductal gray and in the pars reticulata of the substantia nigra and the medial and lateral lemnisci. In the ventral horn of the human cord, CGRP-IR is concentrated in lamina IX, especially α -motoneurons (Unger and Lange, 1991), confirming findings obtained earlier in other species (Gibson *et al.* 1984). A few positively stained fibers and nerve cells have also been seen in the cuneate and gracilis nuclei (Unger and Lange, 1991). High levels of CGRP immunoreactive-like peptide (as measured by RIA) are reported in the locus coeruleus (Tiller-Borcich *et al.* 1988), pituitary gland (Tschopp *et al.* 1985), the *substantia gelatinosa* and the trigeminal nerve (Skofitsch and Jacobowitz, 1985b).

1.2.3 Distribution of CGRP receptor sites

Several studies have demonstrated the anatomically discrete distribution of binding sites for CGRP in the central nervous system in various species, including rat and human (Tschopp *et al.* 1985; Henke *et al.* 1985; Inagaki *et al.* 1986; Sexton *et al.* 1986; Kruger *et al.* 1988; Dennis *et al.* 1991; Wimalawansa and El-Kholy, 1993). These binding sites have a discrete distribution (Figure 1.3a, b, and c; third column) that is distinct from those previously reported for other neuropeptide binding sites.

High levels of [125 I]hCGRP binding have been reported in the nucleus accumbens, ventral striatum and tail of the caudate putamen, central and basolateral nuclei of the amygdala, superior and inferior colliculi, molecular and Purkinje cell layers of the cerebellum and the inferior olive. Moderate levels of labeling have been seen in the mammillary body, habenula, substantia nigra, medial geniculate nuclei, central gray, laterodorsal tegmental nucleus, pontine nuclei, reticular formation, locus coeruleus and the vestibular nuclei. The dorsal motor nucleus of the vagus (X), nucleus cuneatus lateralis and nucleus of the solitary tract also show moderate levels of CGRP binding sites. In contrast, most of the cortical areas, the hippocampal formation, the thalamus and most of the hypothalamic nuclei exhibit relatively low levels of specific CGRP binding sites.

In the rat spinal cord, high densities of [125 I]hCGRP binding sites have been observed in laminae I and X and the medial portion of laminae III and IV, as well as in the intermediolateral and intermediomedial nuclei. The *substantia gelatinosa* (lamina II) contains relatively lower densities of [125 I]hCGRP binding sites whereas the ventral horn is globally not enriched with specific CGRP labeling (Tschopp *et al.* 1985; Inagaki *et al.* 1986; Yashpal *et al.* 1992; Rossler *et al.* 1993).

The distribution of [125 I]hCGRP binding sites in the human and rat brains is mostly similar. However, the inferior olive nuclei and the cerebellum in man contain higher densities of CGRP binding as compared to the rat. Similarly, the arcuate nucleus of the human medulla oblongata exhibits an abundance of binding sites (Tschopp *et al.* 1985; Inagaki *et al.* 1986). In contrast, the rat nucleus accumbens is enriched with CGRP labeling whereas the human is not as well endowed with CGRP labeling (Dennis *et al.* 1991)

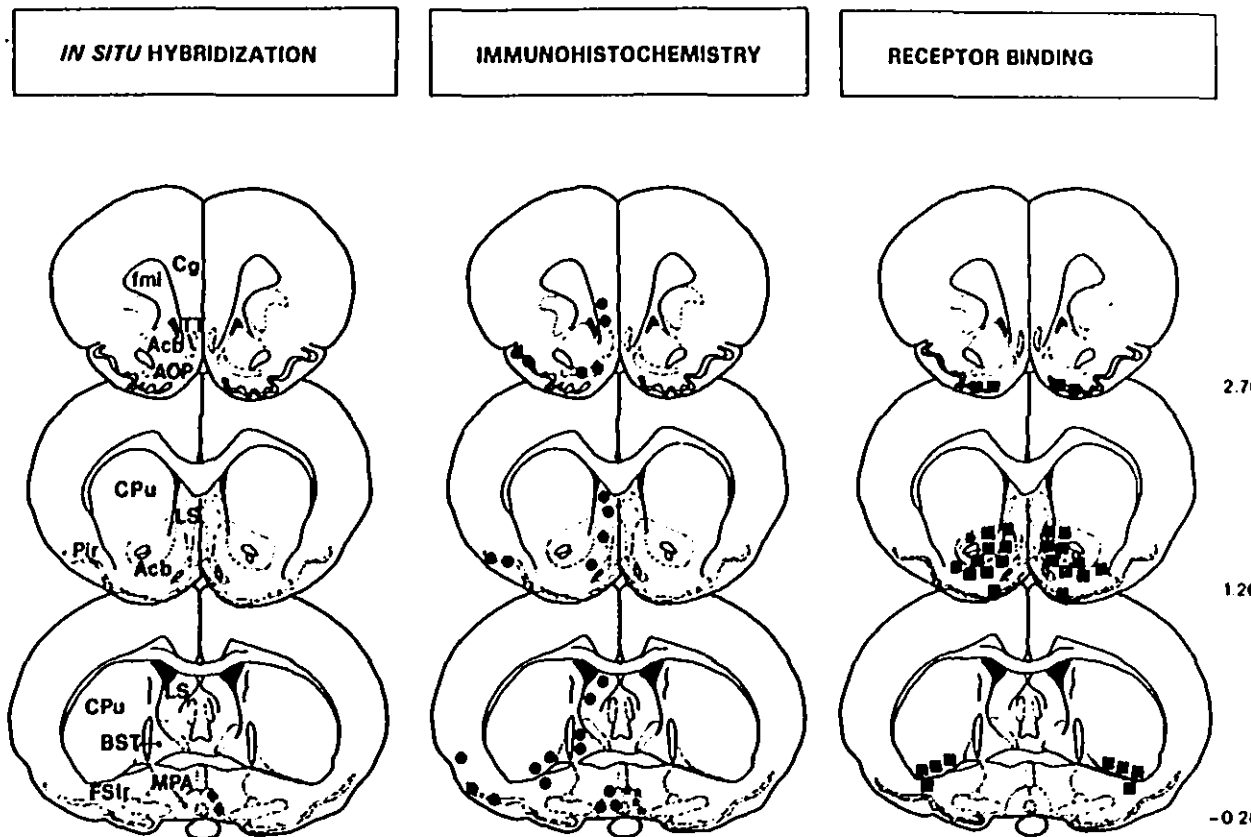


Figure 1.3a Summary of CGRP mRNA (◆, first column), CGRP-like immunoreactive fiber tract (●, second column), CGRP-like immunoreactive cell body (★, second column) and CGRP binding sites in rat brain (■, third column). *Abbreviations:* Acb, accumbens nucleus; AOP, anterior olfactory nucleus; BST, bed nucleus of the stria terminalis; Cg, cingulate cortex; CPu, caudate putamen (striatum); fmi, forceps minor of the corpus callosum; FSlr, fundus striati; LS, lateral septal nucleus; MPA, medial preoptic area; Pir, piriform cortex; TT, tenia tecta.

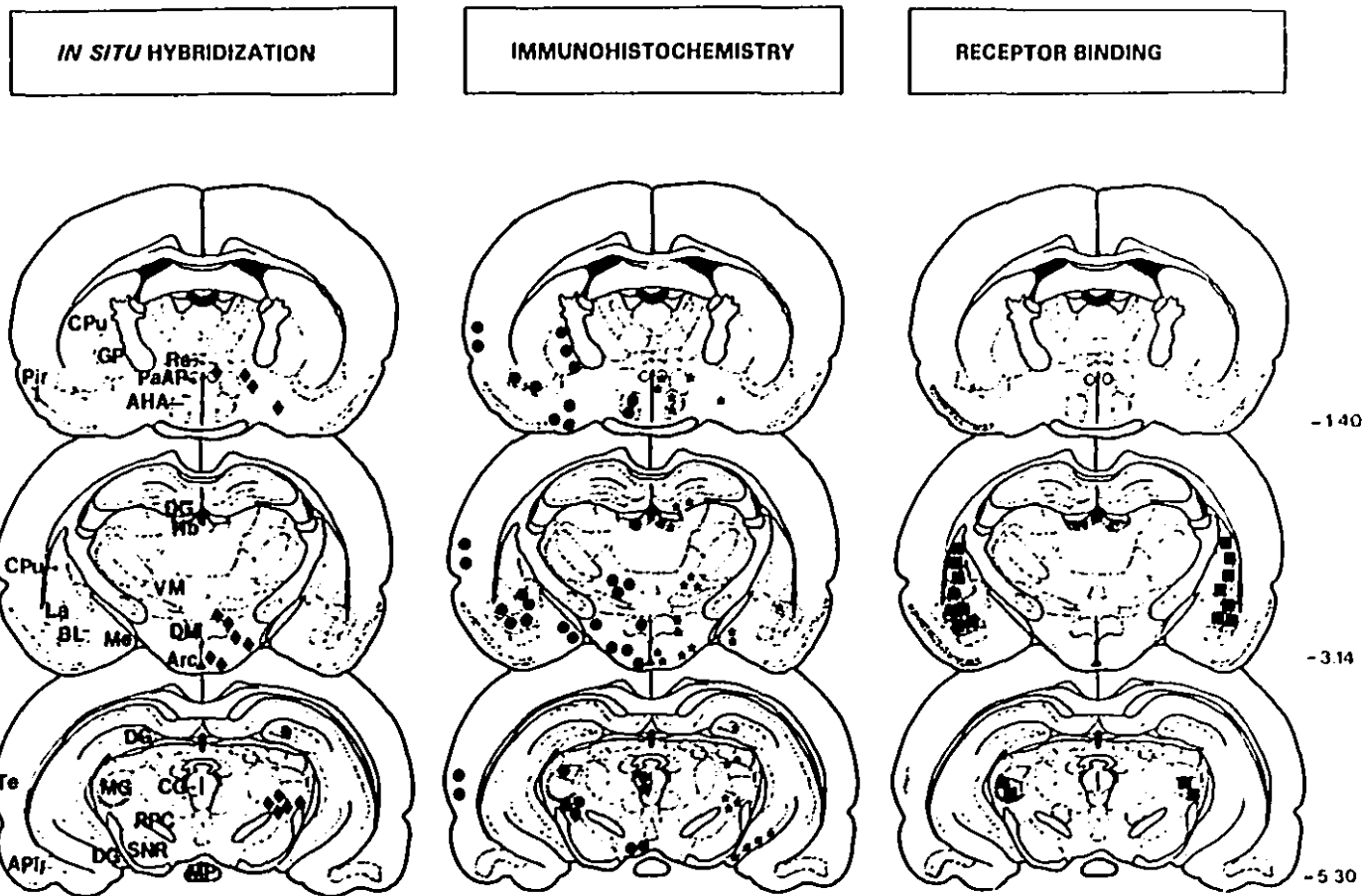


Figure 1.3b See legend Figure 1.3a. *Abbreviations:* AHA, anterior hypothalamic area; APir, amygdalopiriform transition area; Arc, arcuate hypothalamic nucleus; BL, basolateral amygdaloid nucleus; CG, central periaqueductal gray; CPU, caudate putamen (striatum); DG, dentate gyrus; DM, dorsomedial hypothalamic nucleus; GP, globus pallidus; Hb, habenular; La, lateral amygdaloid nucleus; Me, medial amygdaloid nucleus; MG, medial geniculate nucleus; MP, medial mammillary nucleus; PaAP, paraventricular hypothalamic nucleus; Pir, piriform cortex; Re, reuniens thalamic nucleus; RPC, red nucleus; SNR, substantia nigra; Te, temporal cortex; VM, ventromedial thalamic nucleus.

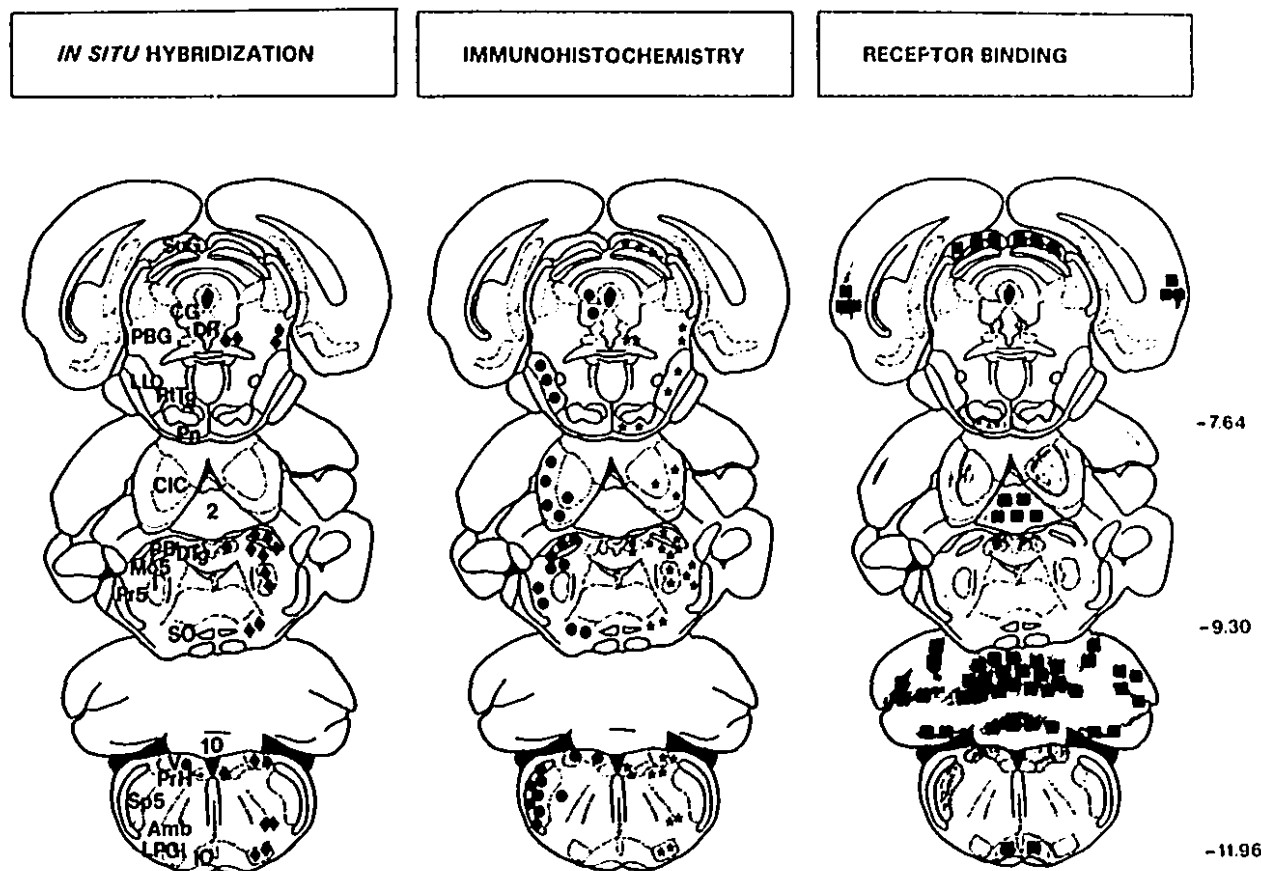


Figure 1.3c See legend Figure 1.3a. *Abbreviations:* 2, cerebellar lobules; 10, cerebellar lobules; Amb, ambiguus nucleus; CG, central periaqueductal gray; CIC, central nucleus of the inferior colliculus; DR, dorsal raphe nucleus; DTg, dorsal tegmental nucleus; IO, inferior olive; LL, lateral lemniscus; LPGi, lateral paragigantocellular nucleus; Mo5, motor trigeminal nucleus; PB, parabrachial nuclei; PBG, parabrachial nucleus; Pn, pontine nuclei; Pr5, principal sensory trigeminal nucleus; PrH, prepositus hypoglossal nucleus; RITg, reticulotegmental nucleus of the pons; SO, supraoptic nucleus; Sp5, spinal trigeminal nucleus; SuG, superficial gray layer of the superior colliculus; Ve, vestibular nuclei.

1.2.4 Mismatches

Generally, the widespread distribution of CGRP mRNA synthesizing neurons, as revealed by *in situ* hybridization, is in agreement with the localization of the reported CGRP-immunoreactive cells. However, no CGRP mRNA has been detected in the dentate gyrus as well as in various regions of the amygdaloid body whereas numerous CGRP-immunoreactive cells have been found in these regions. Moreover, *in situ* hybridization studies did not correlate with findings of CGRP immunoreactivity in the inferior colliculus, pedunclopontine, tegmental and medullary raphe nuclei. In contrast, *in situ* hybridization data revealed the presence of CGRP mRNA expression in the nucleus of the lateral olfactory tract whereas no CGRP-immunoreactive cells have been reported in this area.

Similarly, the distribution of CGRP binding sites corresponds rather well with that of CGRP-immunoreactive perikarya and fibers. However, apparent mismatches are also seen. For example, in the thalamus and hypothalamus, two areas enriched with CGRP fibers, only few binding sites have been reported. Moreover, in the inferior olive complex and the molecular layer of the cerebellar cortex, little or no immunoreactivity for CGRP has been found in adult brain whereas very high densities of CGRP binding sites have been located in these two structures (Kruger *et al.* 1988). On the other hand, transient expression of CGRP-like immunoreactivity has been observed in the olivo-cerebellar fibers during development suggesting a role for CGRP in the cerebellum at least at the neonatal stages in the rat (Morara *et al.* 1989; Provini *et al.* 1992). In agreement, CGRP immunoreactive fibers were reported to be mainly confined to terminal-like structures that apposed Purkinje cells at the rat neonatal stages. However, species differences exist in regard to CGRP-like immunoreactivity in cerebellar afferents. Indeed, positive CGRP-like staining in the cat cerebellar cortex is localized in mossy fibers arising from neurons located in the lateral reticular, external cuneate and inferior vestibular nuclei, as well as from the basilar pons (Sugimoto *et al.* 1988; Bishop, 1992).

In human, the density of [125 I]hCGRP binding sites has been reported to be high in the ventral part of the spinal cord whereas correspondingly low levels of endogenous CGRP have been quantified except in lamina IX (α -motoneurons) (Unger and Lange, 1991). In contrast, the pituitary gland is devoid of [125 I]hCGRP binding but contains high concentrations of CGRP-like peptides (Tschopp *et al.* 1985). Finally, differences are seen

between rat and human CGRP levels in subcortical nuclei such as striatum and amygdala as high and low CGRP levels have been seen, respectively (Tiller-Borcich *et al.* 1988).

Overall, the respective distribution of CGRP mRNA, CGRP-like immunoreactivity and CGRP binding sites, throughout the brain, are in rather good agreement. The source of some of the minor discrepancies may include the sensitivity of the various assays as well as the use of human CGRP probes in the rat brain. However, the role of CGRP in areas such as in the adult cerebellum in which major differences have been observed remains to be clarified.

1.2.5 Fiber pathways containing CGRP

CGRP-immunoreactive structures are widely distributed in the brain suggesting the involvement of this peptide in various brain functions, especially in specific sensory, motor and integrative systems. The main CGRP pathways are described in the following sections and summarized in Figure 1.4. For an extensive review on immunohistochemical localization and functional aspects of CGRP in peripheral sensory branches, see Ishida-Yamamoto and Tohyama (1989).

A group of primary olfactory fibers that terminate in the glomerular layer of the olfactory bulb express CGRP-like immunoreactivity, suggesting that CGRP may play a role in olfaction. Accordingly, CGRP binding sites have been observed in the olfactory system including in the mitral and plexiform cell layers of the bulb and in the accessory and anterior olfactory nuclei. In addition, olfactory-related areas such as the diagonal band, olfactory tubercle, lateral and basolateral amygdaloid nuclei and the primary olfactory, periamygdaloid and entorhinal cortices have been reported to contain few CGRP binding sites. Interestingly, when applied to cultured mouse olfactory bulbs, CGRP has been shown to increase the number of tyrosine hydroxylase-expressing neurons *in vitro* (Denis-Donini, 1989). On the other hand, although a few CGRP-stained receptor cells have been observed in some regions of the epithelium, the peptide was hardly detectable within most olfactory receptor neurons during development (Baker, 1990), as well as in response to lesions capable of increasing the number of maturing receptor cells (Biffo *et al.* 1990). It thus remains unclear if CGRP is directly involved in the differentiation of dopaminergic olfactory bulb neurons (Finger and Böttger, 1992).

The numerous CGRP-immunoreactive fibers in the caudal portion of the caudate-putamen seem to be of extrinsic origin, as few if any CGRP-immunoreactive perikarya are

present in this region (Inagaki *et al.* 1990). By the use of immunohistochemistry, selective knife cuts, retrograde tracer and electrolytic lesions, the main origin of the CGRP-thalamostriatal projection to the caudal portion of the caudate-putamen has been located within the posterior thalamus with a caudal extension toward the ventral and medial borders of the medial geniculate nucleus (Kubota *et al.* 1991). In addition, a few CGRP neurons located in the thalamus project to central amygdaloid subnuclei (Yasui *et al.* 1991). However, most of CGRP terminals projecting to central amygdaloid subnuclei originate from cell bodies in the parabrachial nucleus of the pons. It has been suggested that the overlap between acoustic and somesthetic inputs in the posterior thalamus, and the projection from this region to the amygdala and the striatum is the anatomical substrate for the formation of learned associations between acoustic stimuli and pain (Yasui *et al.* 1991). Indeed, similar to the interruptions of the pathways connecting the posterior thalamus to the amygdala and striatum, it has been observed that bilateral destruction in the region of the medial geniculate nucleus or of the amygdala and striatum prevents the changes in autonomic activity and behavior elicited by acoustic conditioned stimuli (Inagaki *et al.* 1990). It is thus likely that the CGRP-immunoreactive projections from the posterior intralaminar complex to the amygdala and striatum may play a role in the mediation of these autonomic and behavioral responses to acoustic stimuli. In support of this hypothesis, CGRP has been reported to be involved in the facilitation of learning and memory processing as it enhances the acquisition, consolidation and retrieval of a passive learning task (Kovács and Telegdy, 1992a). The effects of CGRP on learning could be blocked by pre-treatment with either a serotonergic, β -adrenergic or opioid receptor antagonists (Kovács and Telegdy, 1992a; Kovács and Telegdy, 1992b; Kovács and Telegdy, 1994), suggesting that CGRP modulates several systems that are involved in these behavioral effects.

CGRP-containing cells have been detected in the superior olivary nucleus, in the area medial to the medial geniculate body and at a lower density, in the lateral lemniscus. In addition, positive fibers have been detected in the inferior colliculus and the lateral lemniscus suggesting a role for CGRP in audition although no direct functional data are available at present.

Following the injection of a retrograde tracer into the cochlea, the labeling of CGRP neurons has been observed in the ipsilateral lateral superior olive nucleus (Silverman and Kruger, 1989; Simmons and Raji-Kubba, 1993). Most of these fibers have been found in the inner spiral bundle under the inner hair cells and form synaptic contacts

with afferent terminals on the inner hair cells (Ohno *et al.* 1993). In addition, the presence of a cholinergic/CGRP vestibular efferent system that feeds back on the primary sensory fibers and receptor hair cells in the cochlea has been reported (Ohno *et al.* 1991; Ohno *et al.* 1993). Indeed, injection of a retrograde tracer into the vestibular end-organs labeled CGRP cells located just dorsolateral to the genu of the facial nerve that corresponds to one of the three main origins of this vestibular efferent system. Furthermore, CGRP and acetylcholine have been shown to coexist in neurons of the facial nerve projecting to the vestibular end-organs (Ohno *et al.* 1991). The functional interactions between CGRP and acetylcholine in this system are still unclear as excitatory and inhibitory functions for CGRP and acetylcholine respectively, have been reported (Sewell and Starr, 1991; Ohno *et al.* 1991). Thus, the precise role of CGRP-immunoreactive fibers innervating cochlear as well as vestibular sensory receptor cells is not apparent.

CGRP is expressed in various neurons associated with taste including, sensory fibers endings in taste buds and the central projections of these fibers terminating in the rostral part of the nucleus of the solitary tract as well as in the relay system originating from the parabrachial nucleus and projecting to the thalamic nucleus and in cortical areas (e.g. posterior agranular insular area) involved in taste pathways. Most motor neurons in the hypoglossal nucleus (XII) are also CGRP-positive. Similarly, CGRP binding sites have been observed in the multisynaptic gustatory pathway including in the nucleus of the solitary tract, the parabrachial nuclei, the central amygdaloid nucleus, the ventromedial posterior nucleus of the thalamus and the insular cortex. The presence of both CGRP-like immunoreactivity and CGRP binding sites in the olfactory and gustatory systems suggests that this peptide may have a functional effect in ingestive behavior. Indeed, intracerebroventricular administration of CGRP has been reported to produce a decrease in food intake (Krahn *et al.* 1984; Tannenbaum and Goltzman, 1985; Jolicœur *et al.* 1992). Some areas of the brain known to be involved in feeding such as the lateral and paraventricular nuclei of the hypothalamus and the perifornical and zona incerta areas, all contain low to moderate levels of CGRP binding sites. As illustrated in Figure 1.4, the lateral portion of the dorsal parabrachial area contains a significant amount of CGRP neurons that project to the ventromedial hypothalamic nucleus that is considered as one of the major satiety center (Swanson, 1987). The lesion of the lateral portion of the parabrachial area, that comprises CGRP and CCK expressing neurons, induced hyperphagia and obesity whereas its stimulation caused hyperglycemia (Nagai *et al.* 1987). Moreover, the inhibition of gastric acid secretion has been observed following

intracerebroventricular injection of CGRP (Taché *et al.* 1984a; Lenz *et al.* 1984; Kraenzlin *et al.* 1985). Gastric acid secretion appears to be influenced by the lateral and ventromedial regions of the hypothalamus as well as the central amygdaloid nucleus (Skofitsch and Jacobowitz, 1992), supporting a role for CGRP in these pathways.

As described earlier, CGRP is present throughout the parabrachial nucleus and in the caudal and intermediate part of the nucleus of the solitary tract, suggestive of its role in the relay of visceral sensory information from the vagus and glossopharyngeal nerve (Sykes *et al.* 1994). This pathway appears to mostly arise from the parabrachial and peripeduncular nuclei and to project to various areas including the lateral hypothalamus, the central nucleus of the amygdala, caudal parts of the caudate-putamen and globus pallidus, the lateral septal nucleus and bed nucleus of the stria terminalis and to layer III of the agranular insular and perirhinal cortices (Rosenfeld *et al.* 1992; Takahashi *et al.* 1994). The presence of CGRP in vagal sensory afferents has been implied as a result of the marked reductions in CGRP-like immunoreactivity seen in the nucleus of the solitary tract and area postrema following unilateral nodose ganglionectomy in the cat (Torrealba, 1992). In addition to the CGRP-containing cells in the inferior ganglia of the vagal nerve, glossopharyngeal nerve geniculate ganglion also terminates in the medial part of the nucleus of the solitary tract. A number of CGRP neurons have been located in the caudal portion of the nucleus of the solitary tract suggesting their potential association with vagal and glossopharyngeal nerve afferents. This relationship could relate to the effects of CGRP on cardiovascular functions including baroreceptor reflexes.

A high quantity of CGRP-immunoreactive neurons are located in the parabrachial area, an important relay center for the processing of autonomic-related information such as cardiovascular, respiratory and sleep regulations. The lateral parabrachial nucleus is particularly implicated in the regulation of cardiovascular functions and projects to a variety of forebrain structures including the central nucleus of the amygdala and bed nucleus of the stria terminalis (Ju, 1991). Neurotensin- and CGRP-containing neurons have been described in these pathways (Shinohara *et al.* 1988). Recently, Harrigan *et al.* (1994) showed that over 35% of the corticotropin releasing factor neurons in the central amygdaloid nucleus are contacted by CGRP terminals that mainly originate from the parabrachial nucleus. Consistent with the anatomical distribution, microinjections of CGRP into the central nucleus of the amygdala elicit increases in arterial blood pressure and heart rate in the rat (Nguyen *et al.* 1986). Moreover, CGRP neurons that have been identified within the parabrachial area are in contact with serotonergic and non-

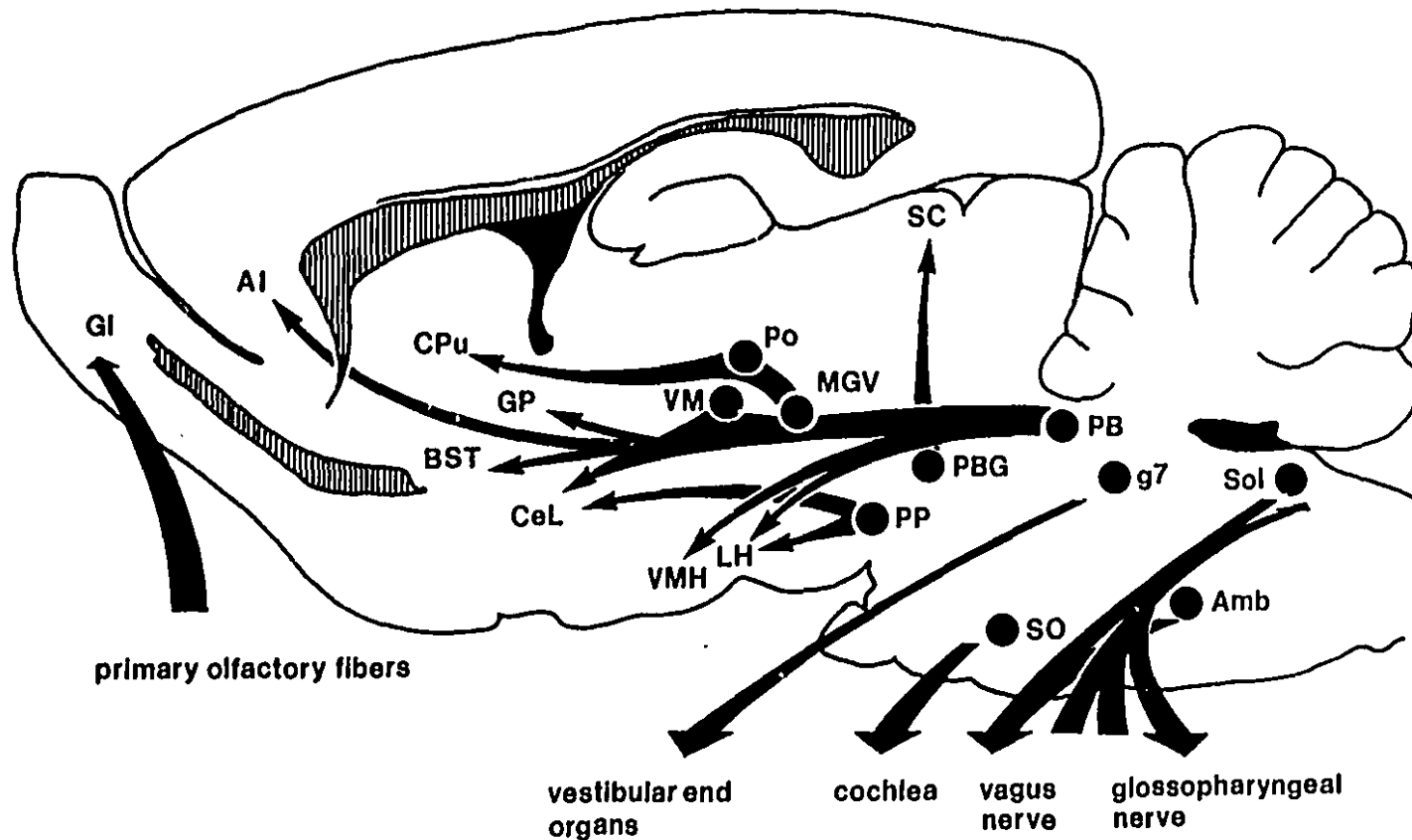


Figure 1.4 Summary of the main CGRP-containing pathways shown on a sagittal section diagram of the rat brain. *Abbreviations:* AI, agranular insular cortex; Amb, ambiguus nucleus; BST, bed nucleus of the stria terminalis; CeL, central amygdaloid nucleus; CPu, caudate putamen (striatum); g7, genu of the facial nerve; Gl, glomerular layer of the olfactory bulb; GP, globus pallidus; LH, lateral hypothalamic area; MG, medial geniculate nucleus; PB, parabrachial nuclei; PBG, parabigeminal nucleus; Po, posterior thalamic nuclear group; PP, peripeduncular nucleus; SC, superior colliculus; Sol, nucleus of the solitary tract; SO, superior olive; VM, ventromedial thalamic nucleus; VMH, ventromedial hypothalamic nucleus.

serotonergic neurons within the dorsal raphe (Petrov *et al.* 1992). The latter are implicated in the modulation of autonomic functions.

Finally, CGRP neurons are found throughout the parabrachial nucleus that projects essentially to the superior colliculus. Concomitantly, the high density of CGRP binding sites in the superficial layers of the superior colliculus and its low densities in the lateral geniculate nucleus suggest its involvement in the visual system.

1.2.6 CGRP-induced behavioral changes

The central administration of CGRP produces a unique profile of neurobehavioral effects, including the reduction of the frequency and amplitude of spontaneous growth hormone secretory pulses (Tannenbaum and Goltzman, 1985), hyperthermia (Jolicoeur *et al.* 1992), catalepsy (Jolicoeur *et al.* 1992; Clementi *et al.* 1992) and reduced motor activity (Jolicoeur *et al.* 1992; Clementi *et al.* 1992). Furthermore, CGRP increases haloperidol-induced catalepsy and decreased apomorphine-induced hypermotility (Clementi *et al.* 1992). Although these behaviors are known to be related to dopamine, no significant change of striatal dopamine or DOPAC concentrations have been observed after the central administration of CGRP (Clementi *et al.* 1992; Drumheller *et al.* 1992). However, the direct administration of CGRP into the rat ventral tegmental area resulted in a dose-related selective increase in dopamine utilization in the medial prefrontal cortex but not in other mesocortical, mesolimbic or striatal dopaminergic terminal field regions (Deutch and Roth, 1987). These effects of CGRP on dopamine turnover are likely to be indirect as the density of [¹²⁵I]hCGRP binding sites in the nucleus accumbens was not modified following the injection of 6-hydroxydopamine into the ventral tegmental area, hence suggesting that CGRP binding sites are not located on the dopaminergic nerve terminals (Masuo *et al.* 1991). Moreover, Orazzo *et al.* (1993) detected large CGRP immunoreactive neurons in the ventral tegmental area that are not tyrosine hydroxylase immunoreactive. In contrast, CGRP-like immunoreactivity has been observed in dopaminergic neurons of the A11 cell group. These dopaminergic neurons are located in the periventricular gray matter at the border between the hypothalamus and the mesencephalon and project to the spinal cord (Orazzo *et al.* 1993).

Following an iontophoretic application, CGRP predominantly depresses neuronal firing in the rat forebrain (Twery and Moss, 1985). Likewise, central injections of CGRP are effective in inhibiting nociceptive responses in the rat by inhibiting the neuronal

thalamic firing evoked by peripheral noxious mechanical stimuli (Pecile *et al.* 1987). In agreement with a modulator role of CGRP on these neurons, primary afferent CGRP fibers make synaptic contacts with spinothalamic tract cells in laminae I-V (Carlton *et al.* 1990). It is also known that central administrations of CGRP produce antinociception in the mouse as measured in the hot plate and formalin tests while being ineffective in the tail flick test (Welch *et al.* 1988; Candeletti and Ferri, 1990).

Finally, the intracerebroventricular administration of CGRP produces concentration-dependent elevations of mean arterial pressure and heart rate by inducing a prompt rise in plasma noradrenaline levels (Fisher *et al.* 1983; Kuo *et al.* 1994). On the other hand, CGRP has been reported to inhibit electrically stimulated-evoked release of noradrenaline in rat hypothalamic slices, partially by acting through dihydropyridine-sensitive Ca^{++} channels (Tsuda *et al.* 1992).

1.2.7 CGRP and motoneurons: development and functions

Immunocytochemical detection of CGRP shows that this peptide is present in motoneurons in embryonic chicks and rats as well as in the adult (Gibson *et al.* 1984; Villar *et al.* 1989; Forger *et al.* 1993). Motoneurons themselves synthesize CGRP that is then transported to the neuromuscular junction (Popper and Micevych, 1989). CGRP coexists with acetylcholine in the nerve terminals of the rodent neuromuscular junction (Takami *et al.* 1985). In rat phrenic nerve-hemidiaphragm preparations, CGRP increases, in a dose-dependent manner, the twitch contraction induced by nerve or transmural stimulation with a concomitant increase in cAMP levels (Uchida *et al.* 1990). However, CGRP, by itself, has no effect in such a preparation (Takami *et al.* 1985).

Several reports suggest that CGRP might play an active role in the formation of a functional synapse at the level of the neuromuscular junction. The time course of appearance of CGRP-immunoreactive motoneurons parallels that of the formation of neuromuscular synapses during chick development (New and Mudge, 1986; Villar *et al.* 1989) and high affinity CGRP binding sites have been observed in chick myotubes in culture (Jennings and Mudge, 1989) and chick skeletal muscle membrane preparations (Roa and Changeux, 1991). Several effects of CGRP on striated muscle have also been reported including the induction of an increased level of surface acetylcholine receptors in cultured chick myotubes mainly by enhancing the rate of synthesis and insertion of new acetylcholine receptors into the plasma membrane (Fontaine *et al.* 1986; New and Mudge,

1986), an increase in the rate of desensitization of nicotinic receptors produced by a decrease of the acetylcholine-activated channel opening frequency (Mulle *et al.* 1988) and a regulation of the phosphorylation state of the nicotinic acetylcholine receptor in rat myotubes (Miles *et al.* 1989). All these effects are likely mediated by increased levels of intracellular cAMP. Furthermore, daily treatment with CGRP suppresses the disuse-induced terminal sprouting without altering the increases in transmitter release in the rat neuromuscular junction (Tsujimoto and Kuno, 1988). More recently, CGRP was shown to enhance the postsynaptic response at developing neuromuscular junctions by increasing the burst duration of acetylcholine channels in 1-day-old *Xenopus* nerve-muscle cultures (Lu *et al.* 1993). This CGRP-induced potentiation of the acetylcholine response was mediated by protein kinase A. However, this potentiating effect of CGRP may occur only during an early phase of synaptic development as no effect of CGRP on acetylcholine channel burst duration was observed in 3-day old culture (Lu *et al.* 1993). Finally, CGRP has been reported to inhibit the insulin-stimulated synthesis of glycogen and to stimulate glycolysis in mammalian skeletal muscles (Leighton and Cooper, 1988). Taken together, these findings suggest that CGRP most likely play a role in the formation, maintenance and normal functioning of the neuromuscular junction.

There is evidence to suggest that the levels of CGRP may be influenced by axotomy of motoneurons. Sciatic nerve section or facial nerve crushing in rat induces a transient increase in the levels of immunoreactive CGRP and CGRP mRNA in axotomized motoneurons (Arvidsson *et al.* 1990a; Piehl *et al.* 1993; Saika *et al.* 1991). This increase could indicate that CGRP is important for the acute survival of severed motoneurons, or for the early stages of regeneration by exerting a trophic action on the damaged motoneurons. In the same way, blockade of axonal flow increases the expression of CGRP α mRNA in motoneurons (Kato *et al.* 1992). Interestingly, levels of CGRP in motoneurons are also sensitive to deafferentation. Low thoracic spinal cord transection which deprives lumbar motoneurons of supraspinal inputs results in decreased CGRP-like immunoreactivity (Marlier *et al.* 1990). In contrast, following neurotoxin-induced denervation of bulbospinal raphe neuronal inputs, in which CGRP is co-localized with serotonin (Arvidsson *et al.* 1991; Arvidsson *et al.* 1990b), increases in CGRP-like immunoreactivity have been observed in the ventral thoracolumbar cord (Fone, 1992). Globally, these studies suggest the existence of supraspinal influence on the expression of CGRP in the motoneurons.

1.2.8 CGRP and sensory neurons

CGRP-immunoreactive cells constitute 40 to 50% of dorsal root ganglia neurons (Gibson *et al.* 1984; Ju *et al.* 1987; Levine *et al.* 1993). Primary somatosensory systems containing CGRP, originating from the trigeminal ganglion, terminate in the spinal trigeminal nucleus and those from the dorsal root ganglion project to the dorsal horn of the spinal cord and the dorsal column nuclei (Kruger *et al.* 1989; Pohl *et al.* 1990; Villar *et al.* 1991; Alvarez *et al.* 1993). Dorsal rhizotomy induces CGRP depletion (85%) within the ipsilateral dorsal zone of the spinal cord (Pohl *et al.* 1990) whereas CGRP mRNA and immunoreactivity are increased in the dorsal root ganglia. These increases are likely due to the effects of nerve growth factor supplied from the peripheral target (Inaishi *et al.* 1992). Moreover, the expression of CGRP was reduced in the dorsal root ganglia in response to peripheral axotomy (Villar *et al.* 1991; Inaishi *et al.* 1992).

Since a neonatal capsaicin treatment destroys a large proportion of primary afferent nerves, it is not surprising that it also significantly reduces the level of CGRP in the dorsal horn of the spinal cord (Gibbins *et al.* 1987a; Pohl *et al.* 1990). While CGRP-positive cells are mainly sensory neurons with fibers of slow conduction velocities (unmyelinated C and small myelinated A δ fibers), 10 to 15% of CGRP-positive cells have been identified as A α / β neurons. At the ultrastructural level, CGRP-labeled varicosities form asymmetric synapses on dendritic spines or neuronal somata and rarely establish glomerular synaptic complexes in cat and monkey (Traub *et al.* 1990; Alvarez *et al.* 1993; Henry *et al.* 1993; Ribeiro da Silva, 1994). These synaptic glomeruli are thought to play an important role in sensory transmission. It also appears that the CGRP content in primary afferent neurons is largely independent of the nature of the innervated target tissue (Levine *et al.* 1993; Hoheisel *et al.* 1994).

In several instances, CGRP has been shown to coexist with other peptides in sensory afferents (Ju *et al.* 1987; Gibbins *et al.* 1987a; Zhang *et al.* 1993). For example, CGRP has been found with up to three other peptides (substance P, cholecystokinin, dynorphin) in the same neuron of the guinea pig dorsal root ganglia (Gibbins *et al.* 1987a). CGRP- and substance P-like immunoreactivity are even co-localized in the same large dense-core vesicle in dorsal root ganglia neurons (Gulbenkian *et al.* 1986). Numerous and morphologically different neuronal structures of the human trigeminal sensory system contain CGRP-like immunoreactivity. Co-existence with substance P occurs in about 25% of these CGRP-immunoreactive neurons (Quartu *et al.* 1992). Finally, more than 75% of

CGRP-like immunoreactive sensory fibers associated with the epidermis have also been shown to contain somatostatin (Gibbins *et al.* 1987b).

The localization of CGRP in small dorsal root ganglion neurons and in the major sites of termination of nociceptors suggests that CGRP may participate in nociceptive transmission. Amongst the many peptides that are located in dorsal root ganglion neurons, CGRP provides one of the best examples of a neuromodulator, in the sense of a molecule that exerts limited effects by itself, and yet dramatically potentiates the effects of others such as substance P. For example, Biella *et al.* (1991) reported that concentrations of CGRP that by themselves had little or no consistent effect, markedly potentiated the excitatory action of substance P or a noxious stimulation in the rat dorsal horn neurons *in vivo*. Likewise, intrathecal administration of CGRP in the rat cord, while not evoking a caudally-directed scratching-biting behavior suggestive of nociceptive effect, potentiates this behavior following the intrathecal administration of either substance P (Wiesenfeld-Hallin *et al.* 1984) or somatostatin (Wiesenfeld-Hallin, 1986). Although the mechanism by which CGRP can potentiate the effects of substance P is not clear, there is evidence that CGRP may retard the enzymatic degradation of substance P (Le Greves *et al.* 1985; Le Greves *et al.* 1989; Mao *et al.* 1992). CGRP has also been shown to enhance the release of substance P (Oku *et al.* 1987) and more recently, of excitatory amino acids (Kangrga *et al.* 1990) from primary afferent fibers, hence possibly leading to the increased activity of these transmitters.

Furthermore, intrathecal administration of CGRP has been reported to facilitate the spinal nociceptive flexor reflex in rats (Xu *et al.* 1990) whereas at low doses, an attenuation of the facilitation of the tail flick reflex induced by either substance P or noxious cutaneous stimulation was reported by Cridland and Henry (1989). Consistent with the notion that CGRP is contributing to nociceptive processing in the dorsal horn, Morton and Hutchison (1989) using the antibody microprobe technique, found that noxious thermal, mechanical, or electrical stimulation evokes the release of CGRP in the superficial dorsal horn. Moreover, the intrathecal administration of antisera to CGRP has an analgesic effect on thermal and mechanical noxious stimuli (Kawamura *et al.* 1989). Accordingly, iontophoretically applied CGRP produces a slower onset but prolongs excitation of nociceptive dorsal horn neurons *in vivo* (Miletic and Tan, 1988). *In vitro* studies have also demonstrated that CGRP produces a slow depolarization by a direct action on nociceptive dorsal horn cells (Ryu *et al.* 1988a). In addition, CGRP increases Ca^{++} conductance in rat dorsal root ganglion cells (Ryu *et al.* 1988b; Oku *et al.* 1988) and

in cat parasympathetic ganglia (Nohmi *et al.* 1986). In contrast, no pain-related behavioral effects have been measured following the intrathecal injection of CGRP in mice (Gamse and Saria, 1986; Welch *et al.* 1989).

Noxious sensory stimuli also evoke adrenal and autonomic responses that remain to be better characterized. Microinjections of CGRP into laminae I-II of the trigeminal caudalis subnucleus increased significantly the secretion of catecholamines from the adrenal glands, the adrenal blood flow and vascular conductance as well as the mean arterial pressure and heart rate consistent with an activation of nociceptive mechanisms (Bereiter and Benetti, 1991). In contrast, injection of CGRP into the deeper magnocellular laminae (V-VI) of the same nucleus had no effect on cardiovascular function, although a decrease in the adrenal secretion of catecholamines has been observed (Bereiter and Benetti, 1991).

Taken together, CGRP plays a role in the transmission of nociception in the rat spinal cord likely as both a neuromodulator and a neurotransmitter. The precise mechanism of interactions with other nociceptive neurotransmitters in the spinal cord such as substance P, glutamate and opioids remains to be fully determined.

1.3 Biological activities in other systems

1.3.1 CGRP effects on cardiovascular system

CGRP-like immunoreactivity has been reported in various species including human, to be broadly distributed within the cardiovascular system consisting in a dense peripheral sensory network innervating the arteries, veins and heart (Mulder *et al.* 1985; McCulloch *et al.* 1986; Edvinsson *et al.* 1987a; Edvinsson *et al.* 1987b; Franco Cereceda *et al.* 1987). In the heart, the atrial myocardium contains the highest density of CGRP fibers. Moreover, the trigeminal ganglion consists of a large number of CGRP-immunoreactive nerve cell bodies. Some of these neurons project to cerebral arteries as following unilateral section of the trigeminal nerve, a pronounced reduction in the levels of CGRP and substance P has been observed in the middle cerebral arteries (McCulloch *et al.* 1986). Several studies have shown that CGRP and substance P co-exist in a sub-population of sensory neurons innervating the cerebral circulation (Lundberg *et al.* 1985; McCulloch *et al.* 1986; Gibbins *et al.* 1987a). Furthermore, there is ultrastructural evidence for the co-localization of CGRP and substance P in large granular vesicles in trigeminal ganglion cells and in peripheral nerve fibers around blood vessels (Gulbenkian *et al.* 1986; Gulbenkian *et al.* 1990). Similar to the distribution of CGRP-like immunoreactivity, specific binding sites for CGRP have been detected in heart and blood vessels (Sigrist *et al.* 1986; Coupe *et al.* 1990; Knock *et al.* 1992). Receptor autoradiography with [125 I]CGRP revealed the presence of specific sites in the intima and media of the aorta, the coronary arteries and the heart valves (Sigrist *et al.* 1986). In membrane homogenates of the rat heart, specific [125 I]CGRP binding is highest in the atria as only small amounts have been detected in ventricles.

CGRP is one of the most potent vasodilating substance known thus far. Indeed, following intravenous infusion of as little as femtomolar concentrations, this peptide induces profound vascular response in various species, including human (Brain *et al.* 1985; Girgis *et al.* 1985; Marshall *et al.* 1986b; Struthers *et al.* 1986; Gardiner *et al.* 1989). Besides producing a decrease in vascular resistance, CGRP increases the rate and force of contraction of the heart (Tippins *et al.* 1984; Gennari and Fischer, 1985; Marshall *et al.* 1986a; Wang and Fiscus, 1989). However, during infusion of low doses of CGRP (0.06 nmol/h) in conscious rats, decrease in carotid, renal, mesenteric vascular resistance have

been observed whereas the mean arterial pressure was not significantly affected. Higher doses of CGRP (6.0 nmol/h) were required to decrease the mean arterial pressure and increase the heart rate (Gardiner *et al.* 1989; Gardiner *et al.* 1990b). In addition, the atrial contractile effects of CGRP were not significant when the atria preparations were previously treated with CGRP indicating that desensitization to CGRP occurred (Sigrist *et al.* 1986; Fisher *et al.* 1988). This is in contrast to the vasodilatory effects of CGRP that are not accompanied by tachyphylaxis (Franco Cereceda, 1991).

CGRP also potentiates the local effects of inflammatory agents including histamine, leukotriene B₄, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), platelet-activating factor (PAF), bradykinin and substance P (Brain and Williams, 1985; Hughes and Brain, 1991; Cruwys *et al.* 1992), essentially through its vasodilating properties. On the other hand, CGRP has also been shown to modulate immune cells such as T-helper cells (Wang *et al.* 1992), B cells (McGillis *et al.* 1993) and neutrophils (Richter *et al.* 1992). In addition to its direct effects on B cells and neutrophils, CGRP may mediate indirect influences via an inhibitory action on T-helper cells that subsequently modulate B cells and neutrophils (Wang *et al.* 1992).

1.3.1.1 Clinical applications

Despite considerable debate concerning the pathophysiology of migraine, there is general agreement that the cranial vessels play some role either in the pathogenesis or in the expression of the migraine syndrome (Edvinsson, 1991). The trigeminal innervation of the cerebral circulation with sensory fibers provides the substrate for pain of intracranial origin. Substance P and CGRP are believed to be the main two sensory neuropeptides in the cerebral circulation. In patients with an attack of migraine, CGRP levels were elevated in the cranial circulation but not in peripheral blood. No significant changes have been reported for either substance P or neuropeptide Y levels (Goadsby *et al.* 1990; Goadsby and Edvinsson, 1993). The specific elevation of only CGRP in patients suffering from migraine potentially provides a link between the basic anatomical and physiological observations on the trigeminal vascular system and migraine. More recently, Wahl *et al.* (1994) reported the involvement of both CGRP and nitric oxide in the first and transient increase in cerebral blood flow in cats. These findings are in agreement with a potential role of CGRP and nitric oxide in the genesis of migraine.

CGRP and substance P contained in the trigeminovascular system are also believed to be involved in the vasomotor events occurring after subarachnoid haemorrhage. A large increase in cerebrospinal fluid content of both peptides were detected 30 min after the induction of a subarachnoid haemorrhage (Tran Dinh *et al.* 1994; Juul *et al.* 1994). Interestingly, infusion of CGRP improved, with no adverse effects, the neurological deficits after intracranial aneurysm surgery for subarachnoid haemorrhage in 9 of 15 patients compared to 2 of 15 patients after placebo infusion (Johnston *et al.* 1990; European CGRP in Subarachnoid Haemorrhage Study Group, 1992). These observed effects of CGRP in this group of patients strongly support that it would be worthwhile to further pursue this avenue forward the clinical use of CGRP like molecules in the treatment of subarachnoid haemorrhage.

In patients with chronic congestive heart failure, the short term or prolonged infusions of CGRP had beneficial effects with an increased cardiac output and lowered blood pressure without change in heart rate (Shekhar *et al.* 1991; Gennari *et al.* 1990; Dubois-Randé *et al.* 1992). Acute myocardial infarction is another situation where there is a demand for counteracting vasoconstriction. A significant increase (almost 2-fold) in plasma CGRP-like immunoreactivity was seen in patients suffering from acute myocardial infarction suggests the potential release of CGRP in response to myocardial ischemia or constriction of peripheral vessels (Mair *et al.* 1990). Accordingly, CGRP-like immunoreactivity release was evoked by low pH and lactic acid in the guinea pig heart (Franco Cereceda *et al.* 1989; Franco-Cereceda *et al.* 1993). Moreover, CGRP has been shown to decrease vascular resistance and increase renal blood flow in a rat model of reversible renal ischemic insult (Bergman *et al.* 1994). Overall, these results suggest potential beneficial role(s) of CGRP in various ischemic conditions.

Serum concentrations of CGRP measured in patients with untreated mild to moderate essential hypertension was not significantly different to CGRP concentrations measured in controls (Schifter *et al.* 1991). It thus seems that CGRP is not directly involved in the development of essential hypertension. On the other hand, CGRP might be responsible for changes in peripheral vasculature in response to increased plasma volume such as in pregnancy and in haemodialyzed patients. Indeed, the plasma levels of CGRP were significantly increased in normal pregnancy in contrast to a dramatic decrease (~50%) in patients with pregnancy-induced hypertension (Stevenson *et al.* 1986; Taquet and Uzan, 1992). Similarly, plasma CGRP concentrations were positively correlated with fluid excess being significantly higher in 5 patients with severe fluid overload (Odar-Cederloef

et al. 1991). Thus by causing vasodilatation, CGRP might be an important defense against the more serious consequences of increased plasma volume including oedema, hypertension and increased cardiac workload.

1.3.2 CGRP effects on gastrointestinal tract

CGRP-like immunoreactivity is present in varying concentrations throughout the mammalian gastrointestinal tract, with highest amounts found in the stomach (Sternini *et al.* 1987; Mulderry *et al.* 1988; Green and Dockray, 1988). The CGRP-positive fibers arise from a set of neurons in the myenteric and submucous plexuses as well as from sensory neurons whose cell bodies are located in the nodose and dorsal root ganglia (Sternini *et al.* 1987). Up to 95% of spinal afferent neurons innervating the stomach contain CGRP immunoreactivity whereas less than 10% of vagal afferent neurons innervating the stomach are positive for CGRP (Green and Dockray, 1988; Su *et al.* 1987). Likewise, systemic capsaicin treatment in adult or neonatal rats result in an almost complete loss of CGRP immunostaining in the stomach (Su *et al.* 1987; Sternini *et al.* 1987; Varro *et al.* 1988). However, in contrast to CGRP α , CGRP β is still present in the intestines of capsaicin-treated rats suggesting that distinct neuronal populations, i.e. sensory neurons and enteric autonomic neurons, could preferentially express CGRP α or CGRP β , respectively (Mulderry *et al.* 1988). In agreement with an extrinsic site of synthesis of intestinal CGRP α , the analysis of CGRP α or CGRP β mRNAs revealed that only CGRP β mRNA was present in the intestine where it is localized to enteric neurons. On the other hand, both peptides mRNAs have been detected in the dorsal root ganglia (Mulderry *et al.* 1988). Specific CGRP receptor binding sites have also been reported throughout the gastrointestinal tract (Maton *et al.* 1988; Gates *et al.* 1989).

In past years, the use of capsaicin has demonstrated the importance of sensory innervation in the regulation of gastric mucosal blood flow, gastric motility and gastric acid secretion (Holzer, 1988; Raybould *et al.* 1990; Holzer *et al.* 1991a). Similar effects were measured following either peripheral or central administration of CGRP including suppression of acid secretion (Taché *et al.* 1984b; Hughes *et al.* 1984; Lenz *et al.* 1984; Kraenzlin *et al.* 1985) and inhibition of gastric motor functions (Jurgen Lenz, 1988; Raybould *et al.* 1988; Taché *et al.* 1991). Interestingly, CGRP β but not CGRP α could inhibit gastric acid secretion in humans (Beglinger *et al.* 1988). The protection of the

gastric mucosa against damage induced by a variety of factors such as ethanol, following intragastric application of capsaicin, is essentially associated with a marked rise of gastric mucosal blood flow (Holzer *et al.* 1991b). This increase in blood flow is thought to be essentially induced by CGRP. Accordingly, CGRP has been shown to modulate the gastric blood flow (Bauerfeind *et al.* 1989; Gardiner *et al.* 1990a; Holzer and Guth, 1991). Furthermore, the capsaicin-induced gastroprotection from ethanol injury is inhibited by the CGRP receptor antagonist, CGRP₈₋₃₇ (Lambrecht *et al.* 1993) as well as by a monoclonal CGRP antibody (Peskar *et al.* 1993). These findings thus support an important role for CGRP in capsaicin-induced gastric protection.

It appears that CGRP mediates its effects through various mechanisms and possibly through multiple receptor subtypes (Evangelista *et al.* 1992). For example, the inhibition of gastric emptying is thought to be primarily through the activation of the sympathoadrenal axis as adrenalectomy or celiac ganglionectomy abolishes the actions of CGRP (Raybould, 1992), whereas the central action of CGRP to inhibit acid secretion does not involve sympathetic outflow but is mainly mediated through a decreased vagal outflow to the stomach (Lenz *et al.* 1985; Taché *et al.* 1984a). In addition, the mechanisms by which peripheral CGRP injection inhibits acid secretion are still not well defined but likely partially involve an increase in somatostatin release since the intravenous injection of somatostatin antibody significantly blocked the effects of CGRP (Dunning and Taborsky, 1987; Holzer and Lippe, 1992). Another possibility could be the modulation of the cholinergic inputs as a decrease in acetylcholine release by CGRP has been reported in the small intestine and antral mucosal fragments (Schwörer *et al.* 1991; Ren *et al.* 1992). In the latter however, the inhibitory effects of CGRP on cholinergic discharge are mediated through the increased release of somatostatin (Ren *et al.* 1992). The precise mechanisms by which CGRP exerts its effects following peripheral injections thus remain to be clarified.

The functional implications of CGRP-containing sensory neurons in decreasing acid secretion as well as by increasing gastric blood flow resulting in gastric protection might thus provide new target for the development of novel therapeutic strategies in the treatment of, for example, gastric ulcers.

1.4 Receptor characterization

1.4.1 CGRP receptors

The binding characteristics of CGRP receptors have been studied in a variety of tissues. The existence of high affinity binding sites for CGRP have been reported in the brain (Yoshizaki *et al.* 1987; Dennis *et al.* 1990) including the cerebellum (Nakamuta *et al.* 1986; Yoshizaki *et al.* 1987; Chatterjee and Fisher, 1991) as well as in various peripheral organs such as heart (Yoshizaki *et al.* 1987; Dennis *et al.* 1990; Chatterjee *et al.* 1991), liver (Nakamuta *et al.* 1986), spleen (Nakamuta *et al.* 1986; Dennis *et al.* 1990), skeletal muscle (Takamori and Yoshikawa, 1989; Jennings and Mudge, 1989; Roa and Changeux, 1991), lung (Mak and Barnes, 1988; Umeda and Arisawa, 1989b) and lymphocytes (Umeda and Arisawa, 1989a; McGillis *et al.* 1993). Apparent affinity (K_D) values varied from 9 pM in whole brain (Yoshizaki *et al.* 1987) to 6.3 nM in skeletal muscle (Takamori and Yoshikawa, 1989). The tissue studied appears to be the main source of variation in K_D values as the species had little influence (for example, Wimalawansa and El-Kholy, 1993).

Currently, little is known about the molecular structure of CGRP receptors as cloning has yet to be reported. Cross-linking studies have been used alone, or in combination with various enzymatic digestions, to provide insights into the structure of the CGRP receptors in various tissue preparations. The first purification of a CGRP receptor from human placenta revealed a membrane-bound receptor of an estimated molecular weight of 240 kD composed of multiple 62 to 68 kD subunits (Foord and Craig, 1987). Thereafter, single binding component without apparent multi-units was isolated from the porcine spinal cord (70 kD; Sano *et al.* 1989), cultured rat vascular smooth muscle cells and bovine endothelial cells (60 kD; Hirata *et al.* 1988), guinea pig gastric smooth muscle and pancreatic acinar cells (57 kD; Honda *et al.* 1993), rat cerebellum (67 kD; Chatterjee *et al.* 1993) and rat lung (64 kD; Bhogal *et al.* 1993). Recently, Stangl *et al.* (1993) solubilized CGRP binding proteins from the cerebellum, brainstem, spinal cord, liver and spleen each having apparent molecular masses varying from 68 to 90 kD. An enzymatic N-deglycosylation converted all these CGRP labeled binding proteins into a common solubilized protein of 44 kD (Stangl *et al.* 1993).

In contrast to these results, other studies have reported the existence of more than one cross-linked molecular weight band in several tissues including, porcine coronary arteries (90 and 70 kD; Sano *et al.* 1989), rat atrium (120 and 70 kD; Sano *et al.* 1989), porcine kidney (30, 58 and 78 kD; Aiyar *et al.* 1991), rat liver (70 and 44 kD; Chantry *et al.* 1991) and skeletal muscle (110 and 70 kD; Chantry *et al.* 1991). In the human cerebellum, two CGRP binding proteins of 50 and 13.7 kD have been identified by Dotti-Sigrist *et al.* (1988) whereas three specifically labeled binding proteins with apparent molecular weights of 60, 54 and 17 kD have been identified by Stangl *et al.* (1991). The latter study also revealed that the solubilized proteins are glycosylated since further treatment with endoglycosidase converted the 60 and 54 kD to 46 and 41 kD binding components, respectively (Stangl *et al.* 1991). It remains to be demonstrated if these various proteins correspond to differential post-translational processing of the same protein or to multiple CGRP binding moieties. Indeed, only receptor cloning will provide definite answers in this regard. However, the comparative potencies of CGRP and analogs in various *in vitro* and *in vivo* functional assays have led to the suggestion of the existence of multiple classes of CGRP receptors (Dennis *et al.* 1989; Dennis *et al.* 1990; Stangl *et al.* 1993; for review, see Quirion *et al.* 1992; Poyner, 1992). A detailed description of the pharmacological characteristics of these proposed receptor subtypes is given in Chapter 2 of this thesis.

1.4.2 Calcitonin receptors

Similar to the characterization of CGRP receptors by covalent cross-linking techniques, purified calcitonin receptors from rat kidney membrane preparations revealed apparent molecular masses of 70, 40 and 33 kD (Bouizar *et al.* 1986). More recently, Lin *et al.* (1991) reported the sequence of the porcine calcitonin receptor following cDNA cloning of calcitonin receptors from a porcine kidney epithelial cell line. Subsequently, calcitonin receptor sequences have been identified from human ovarian carcinoma cell line (Gorn *et al.* 1992), the rat hypothalamus (Sexton *et al.* 1993; Njuki *et al.* 1993) and the rat nucleus accumbens (Albrandt *et al.* 1993). Hydropathy plots of the receptors predicted amino acid sequence revealed multiple hydrophobic regions that could generate 7 transmembrane spanning domains, similar to other G protein-coupled receptors (Lin *et al.* 1991; Goldring *et al.* 1993). CGRP and amylin showed no or little affinity for these calcitonin receptors except for the renal porcine calcitonin receptor on which amylin has

similar efficacy as salmon and porcine calcitonin in stimulating the production of cAMP (Sexton *et al.* 1994).

Although the deduced amino acid sequences of the cloned calcitonin receptors suggest some characteristics of the G protein-coupled receptors such as the presence of seven hydrophobic regions, the calcitonin receptors do not show significant sequence similarity (<12%) with previously reported members of this superfamily. On the other hand, a significant degree of homology (26 to 51%) was observed with the recently cloned parathyroid hormone and parathyroid hormone-related peptide (Juppner *et al.* 1991), as well as with secretin (Ishihara *et al.* 1991) and vasoactive intestinal peptide (Ishihara *et al.* 1992) receptors. Hence, these receptors may constitute members of an emerging subfamily of G protein-coupled receptors. One of the most striking divergence resides in the third cytoplasmic loop (between helices V and VI) that is markedly shorter compared to corresponding regions of other adenylate cyclase-coupled receptors (Lin *et al.* 1991; Goldring *et al.* 1993). This segment of the receptor is thus implicated in the capacity to interact with specific G proteins. Indeed, the calcitonin receptors have demonstrated the capacity to couple to G proteins as well as to induce two independent signal transduction pathways (adenylate cyclase and phospholipase C) through presumably interacting with distinct G proteins (Chakraborty *et al.* 1991; Chabre *et al.* 1992; Force *et al.* 1992; Goldring *et al.* 1993). These findings further support the unique properties of this new subfamily of G protein-coupled receptors that may also include the CGRP receptors considering the relative sensitivity of CGRP binding sites to guanine nucleotides (see section 1.5.1 and Chapter 6 for a detailed discussion)

1.5 Transduction mechanisms

1.5.1 G protein coupling

Evidence from biochemical and pharmacological studies suggests that the CGRP receptors belong to the family of G protein-coupled receptors. For example, various reports have described the effect of GTP or its analogs on [125 I]CGRP binding affinity (e.g., Chatterjee *et al.* 1991; Roa and Changeux, 1991). Under these conditions, a reduction in agonist affinity is predicted in the case of G protein-coupled receptors as guanine nucleotides induce the dissociation of G proteins from the receptor and hence promotes the formation of a lower affinity state of the receptor. Accordingly, CGRP binding affinity has been shown to be sensitive to the addition of GTP or related analogs in a variety of tissue preparations. We have also investigated the effect of a non-hydrolyzable GTP analogue, Gpp(NH)p, on CGRP binding affinity in the cerebellum, brain, atrium and vas deferens membrane preparations (van Rossum *et al.* 1993). These results as well as findings by other laboratories are extensively described and discussed in Chapter 6. In addition, CGRP has been reported to activate muscarinic K^+ channels in rat atrial cells (Kim, 1991) and to enhance Ca^{2+} currents in nodose ganglion neurons (Wiley *et al.* 1992) via a G protein as these effects were pertussis toxin-sensitive. Similarly, CGRP has been shown to increase transmembrane Ca^{++} currents in guinea pig myocytes and bull frog atria via a G protein since the CGRP effects were potentiated in the presence of GTP γ S (Ono and Giles, 1991). More recently, following the solubilization of the CGRP receptors from the rat cerebellum and Western analysis of receptor preparations with antisera against a fragment of the $G_{s\alpha}$ subunit, Chatterjee *et al.* (1993) provided direct evidence for the association of these receptors with $G_{s\alpha}$. It is thus highly likely that CGRP receptors belong to the G protein-coupled receptors family, at least in some tissues such as cerebellum, skeletal muscle and cardiac myocytes.

1.5.2 Second messengers

Activation of adenylate cyclase and/or increases in intracellular cAMP induced by CGRP have been reported in many tissue preparations including the rat liver (Yamaguchi *et al.* 1988), rat and guinea pig heart cells (Wang and Fiscus, 1989; Fisher *et al.* 1988;

Ishikawa *et al.* 1988), rat skeletal muscle (Andersen and Clausen, 1993), astrocyte cultures (Lazar *et al.* 1991) and human neuroblastoma and gliomas (Van Valen *et al.* 1990; Robberecht *et al.* 1994). The relaxant effects of CGRP on various smooth muscle preparations such as the rat thoracic and abdominal aorta (Fiscus *et al.* 1991; Gray and Marshall, 1992a; Wang *et al.* 1991), rat intracerebral arterioles (Edwards *et al.* 1991), porcine coronary arteries (Kageyama *et al.* 1993) as well as the guinea pig gastric muscle cells (Chijiwa *et al.* 1992) also appear to be partly or fully mediated through raises in cAMP. The precise mechanism underlying the relaxation of smooth muscle by intracellular cAMP production remains to be elucidated as cGMP is generally believed to be the major second messenger involved in vasorelaxation. Indeed, CGRP has been shown to induce both endothelium-independent and endothelium-dependent vasorelaxation. Reports on CGRP-induced relaxation indicate that in several tissue preparations such as in cat middle cerebral artery (Edvinsson *et al.* 1985), rat mesentery (Li and Duckles, 1992; Amerini *et al.* 1993), rat skin microvasculature (Ralevic *et al.* 1992) and rat aorta (Ishikawa *et al.* 1993), the effects of CGRP are endothelium-independent. In contrast in the latter tissue, CGRP effects have also been reported to be endothelium-dependent (Brain *et al.* 1985; Fiscus *et al.* 1991; Gray and Marshall, 1992a; Gray and Marshall, 1992b). In agreement with CGRP endothelium-dependent effects, inhibitors of nitric oxide synthase such as N^G-nitro-L-arginine methyl ester (L-NAME) antagonizes CGRP-induced vasorelaxation of the rat aorta (Fiscus *et al.* 1991; Gray and Marshall, 1992a). In addition, L-NAME antagonizes CGRP-induced hypotension in conscious rats (Abdelrahman *et al.* 1992) and the anti-ulceric activity of CGRP in the rat (Clementi *et al.* 1994), suggesting that some effects of CGRP appear to be mediated through the induction of nitric oxide synthesis and hence possibly cGMP.

Moreover, CGRP has been shown to act on K⁺_{ATP} in rabbit arterial smooth muscle cells (Nelson *et al.* 1990; Quayle *et al.* 1994). However, it appears that CGRP does so indirectly, through the elevation of cAMP levels that stimulates protein kinase A, that in turn leads to the activation of K⁺_{ATP} channels (Quayle *et al.* 1994). In addition, glibenclamide, a blocker of K⁺_{ATP} channels, antagonizes CGRP-induced hypotension in rabbit (Andersson, 1992). It thus appears that CGRP-mediated vasorelaxation involves multiple second messengers including cAMP, nitric oxide-cGMP and potassium channels. The precise role of cAMP remains unclear with respect to the relaxant effects of CGRP. In the rat aorta, CGRP-induced increase in cAMP levels has been shown to not play a direct role in vasorelaxation. While the presence of nitric synthase inhibitors blocked the

relaxant effects of CGRP, it did not alter the increase in cAMP levels (Marshall, 1992; Gray and Marshall, 1992a). The stimulation of adenylate cyclase in rat aorta thus probably precedes the activation of nitric oxide synthase or is simply not directly involved in this cascade of events.

CGRP has also been shown to increase Ca^{2+} inward currents in guinea pig atria (Ohmura *et al.* 1990; Ono and Giles, 1991), rat vas deferens (Nakazawa *et al.* 1992) and rat nodose neurons (Wiley *et al.* 1992) as well as to elevate cytosolic Ca^{2+} in guinea pig cardiac myocytes (Gill *et al.* 1992). In contrast, CGRP attenuated both voltage-activated calcium and sodium currents in cortical neurons from rat through an increase of cAMP (Zona *et al.* 1991).

In macrophages, CGRP has been reported to increase intracellular cAMP content and to enhance the activity of the Na-H exchanger. The latter was shown to be dependent at least in part on the activity of protein kinase C (Vignery *et al.* 1991). These results suggest that CGRP receptors might be directly coupled to multiple signaling pathways to regulate macrophage functions. However, these two pathways might also interact and complement each other. Indeed, CGRP has been shown to stimulate phosphoinositide turnover in skeletal muscle most likely through the CGRP-induced increased levels of cAMP as this effect was mimicked by other cAMP-mobilizing agents such as forskolin (Laufer and Changeux, 1989). In a similar way, CGRP has been reported to increase intracellular cAMP levels in human ocular ciliary epithelial cells (Crook and Yabu, 1992). This increase is apparently modulated by protein kinase C since the interaction of the latter with CGRP receptors prevents further increase of cAMP levels.

Overall, it is thus likely that CGRP can activate various transduction pathways. The activation of multiple intracellular pathways might be due to the existence and stimulation of various CGRP receptor subtypes coupled to different transduction mechanisms or to the close interaction between the different second messengers pathways measured, since cross-talk between these is now a well established phenomenon. However, the clear interactions between the second messengers involved here, namely cAMP with phospholipase C-inositol phosphates-protein kinase C, or cAMP with nitric oxide-cGMP remain to be fully clarified.

1.6 Objectives of the thesis

Over the last decade, a wide spectrum of biological actions has been reported for CGRP and related homologues including calcitonin, amylin and adrenomedullin. Surprisingly, little is known about the biochemical and pharmacological characteristics of the receptors mediating these specific effects. To our knowledge, no study has thus far reported the cloning of the receptors for the peptides of this family except for a few forms of the calcitonin receptor (see 1.4.2). Based on the differential potency of various CGRP analogs, recent data have suggested the existence of multiple receptor classes for CGRP in peripheral tissues. Hence, the primary objective of my thesis was to provide additional and novel evidence for the existence of CGRP subtypes in peripheral and to a greater extent, central nervous system preparations. The characterization of the binding profiles of CGRP analogs and related homologues in peripheral tissues as well as in the rat brain was therefore studied first. We also investigated the nature of the putative transduction mechanisms coupled with various CGRP receptor subtypes. Finally, we evaluated the effects of CGRP, amylin and calcitonin on locomotor behaviors in an attempt to functionally differentiate these receptors in the rat brain. Taken together, these approaches enable us to better define the pharmacological characteristics of CGRP receptor subtypes as well as to provide evidence for the existence of brain amylin and adrenomedullin receptors.

CHAPTER 2

Characterization of CGRP₁ and CGRP₂ receptor subtypes

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2.0 Preface to Chapter 2

As summarized in the review of the literature, a wide spectrum of biological actions have been described for CGRP. These effects are essentially mediated through specific CGRP receptor sites. However, relatively little is known about the structural characteristics of the CGRP receptors (see section 1.4.1). By evaluating the pharmacological properties of numerous CGRP fragments and analogs in several peripheral tissue preparations, the existence of multiple CGRP receptor sites has been proposed. In addition, the distinct neurobehavioral profiles of CGRP, its fragments and analogs support the existence of multiple CGRP receptor sites in the rat brain. The primary objective of this initial study was thus to investigate the distribution of putative CGRP receptor subtypes in the rat brain. To do so, we first investigated the relative affinity of hCGRP₈₋₃₇ (CGRP₁-preferential affinity antagonist) and [acetamidomethyl-cysteine^{2,7}]hCGRP α (CGRP₂-preferential affinity agonist) for [¹²⁵I]hCGRP α binding sites in various areas of the brain. The pharmacological characteristics reported thus far for the putative CGRP receptor subtypes are also described and discussed in this chapter.

2.1 Introduction

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide generated from the alternate tissue-specific processing of the calcitonin gene mRNA (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). Various forms (α and β) of CGRP have been characterized from human tissues (Morris *et al.*, 1984; Amara *et al.*, 1985; Steenbergh *et al.*, 1985). In the periphery, CGRP is co-localized and co-released with calcitonin in the parafollicular C cell of the thyroid gland (Grunditz *et al.*, 1986), with substance P in sensory nerves (Wiesenfeld-Hallin *et al.*, 1984) and with acetylcholine in motoneurons (Takami *et al.*, 1985). In the brain, CGRP-like immunoreactivity is broadly distributed and especially concentrated in hypothalamic areas and some brainstem nuclei (Skofitsch and Jacobowitz, 1985a).

A variety of biological effects have been claimed for CGRP, including the modulation of nicotinic receptor activities at the neuromuscular junction (Miles *et al.*, 1989; New and Mudge, 1986) and of substance P in inflammation (Ohlen *et al.*, 1988), a reduction of gastric acid secretion (Hughes *et al.*, 1984; Okimura *et al.*, 1986), peripheral blood vessel dilation (Brain *et al.*, 1985), cardiac acceleration (Tshikawa *et al.*, 1988; Sigrist *et al.*, 1986), a regulation of calcium metabolism (Grunditz *et al.*, 1986) and insulin secretion (Hermansen and Ahren, 1990), an increase in body temperature, and a decrease in food intake (Dennis *et al.*, 1990; Krahn *et al.*, 1984; Jolicoeur *et al.*, 1992).

In spite of the various biological actions induced by CGRP, relatively little is known concerning the characteristics of its receptors (Sigrist *et al.*, 1986; Lynch and Kaiser, 1988; Tippins *et al.*, 1986; Sexton *et al.*, 1988; Umeda and Arisawa, 1989; Dotti-Sigrist *et al.*, 1988; Henke *et al.*, 1987). Consequently, a few years ago, our group undertook the development of selective agonists and antagonists which prove to be most useful for the identification of the existence of CGRP receptor subtypes (Dennis *et al.*, 1990; Dennis *et al.*, 1989; Dennis *et al.*, 1991; Mimeault *et al.*, 1991). The various criteria used to classify these receptor classes are reviewed here.

2.2 Results

2.2.1 Profile of [Cys(ACM)^{2,7}]hCGRP α as a receptor subtype selective agonist

Early studies had revealed that the mid-portion of the CGRP molecule, made up of amino acid residues 8 to 18 most likely adopted an amphiphilic alpha helical structure which was essential for the interaction of hCGRP α with its receptors (Lynch and Kaiser, 1988; Tippins *et al.*, 1986). Moreover, it was shown that the integrity of the disulfide bridge between residues 2 and 7 was important for the maintenance of adequate receptor affinity and biological activity in atrial preparations (Tippins *et al.*, 1986; Seifert *et al.*, 1985). Consequently, we first attempted to characterize in detail the importance of the disulfide bridge in a variety of binding assays and bioassays. Among the various cyclic and linear analogs produced (Dennis *et al.*, 1989) the linear analog [acetamidomethyl-Cys^{2,7}]hCGRP α ([Cys(ACM)^{2,7}]hCGRP α) was found to be of special interest.

As shown in Table 2.1, while the binding affinities of this analogue are rather similar in all the tissues tested, its biological activity is tissue-specific. For example, while

Table 2.1 Profile of the linear analogue [Cys(ACM)^{2,7}]hCGRP α in various binding and bioassay preparations.

Binding assays	K _i (nM)	Bioassays	EC ₅₀ (nM)
Rat brain	2.67	Guinea pig left atrium	>710
Rat spleen	6.70	Guinea pig right atrium	>710
Guinea pig deferens	—	Rat vas deferens	76
Guinea pig atrium	—	Mouse aorta	150
		Hyperthermia (icv)	potent agonist

Note: Values taken from Jolicoeur *et al.* (1992) and Dennis *et al.* (1989) and unpublished results. The K_i is calculated according to the formula $K_i = IC_{50} / (1 + F/K_d)$ in which the IC₅₀ represents the concentration of analogue needed to compete for 50% of specifically bound [¹²⁵I]hCGRP α ; F is the concentration of radioligand used in the assay and K_d the apparent affinity of the receptor. The EC₅₀ is the concentration needed to produce 50% of the maximal effect.

[Cys(ACM)^{2,7}]hCGRP α is basically inactive in the isolated left and right guinea pig atria ($EC_{50} > 710$ nM), it is at least ten-fold more potent ($EC_{50} = 76$ nM) in the rat vas deferens. While this value represented only 2% of the potency of the native peptide, hCGRP α , this finding suggested the existence of differences in the structural requirements of CGRP receptors present in the rat vas deferens as compared to those existing in atrial preparations. It would also appear that the integrity of the N-terminal disulfide bridge is not as critical for the maintenance of the biological activity of CGRP-like molecules in the rat vas deferens. Subsequent studies revealed that the linear analogue was relatively potent on the isolated mouse aorta as inducing hyperthermia in animals (Table 2.1). Thus, the CGRP receptor present in the vas deferens and the mouse aorta seems to behave in a unique manner in regard to the importance of the N-terminal disulfide bridge.

2.2.2 Profile of extended C-terminal fragments as CGRP receptor antagonists in certain preparations

In view of the purported critical importance of amino acid residues between positions 8 and 18 in ensuring appropriate receptor recognition (Lynch and Kaiser, 1988; Tippins *et al.*, 1986; Seifert *et al.*, 1985), we investigated next the binding affinity and biological activity of a variety of C-terminal fragments (Dennis *et al.*, 1990; Dennis *et al.*, 1989; Mimeault *et al.*, 1991).

While short C-terminal fragments failed to demonstrate adequate affinities and biological activities in our assays (in contrast to those of others, Chakder and Rattan, 1990), extended analogs including hCGRP₈₋₃₇, possessed most interesting characteristics (Dennis *et al.*, 1990; Dennis *et al.*, 1989; Mimeault *et al.*, 1991).

As shown in Table 2.2, hCGRP₁₂₋₃₇ retained a relatively high affinity for [¹²⁵I]hCGRP α binding sites present in various tissues. This further supports the concept that the N-terminal portion of the molecule, including the disulfide bridge, is not essential for the maintenance of the affinity of CGRP for its receptors. Of interest, this fragment lacked agonistic properties in a variety of *in vitro* and *in vivo* preparations but acted as a competitive antagonist in certain tissues. For example, while high concentrations of hCGRP₁₂₋₃₇ failed to block the effects induced by hCGRP α in the rat vas deferens and on body temperature, it antagonized the inotropic actions of the native peptide in guinea pig atrial preparations (Table 2.2). Thus, it appears that tissues (such as the vas deferens) that are mostly insensitive to the antagonistic properties of hCGRP₁₂₋₃₇ are better recognized by the agonist [Cys(ACM)^{2,7}]hCGRP α .

Data obtained using the fragment hCGRP₈₋₃₇ also suggest the existence of CGRP receptor subtypes (Dennis *et al.*, 1990; Mimeault *et al.*, 1991). As shown in Table 2.3, the apparent affinity of this fragment for [¹²⁵I]hCGRP α binding sites is extremely high (K_i in the sub-nM range), being at least three to five times higher than that of the native peptide (Dennis *et al.*, 1990). As for hCGRP₁₂₋₃₇, the fragment hCGRP₈₋₃₇ is mostly devoid of agonist activity in the various preparations tested (Dennis *et al.*, 1990). However, it behaves as a potent, fully competitive antagonist in certain assays (Table 2.3)

Table 2.2 Profile of the fragment hCGRP₁₂₋₃₇ in various binding and bioassay preparations.

Binding assays	K_i (nM)	Bioassays	Antagonistic potency (pA ₂)
Rat brain	21.7	Guinea pig left atrium	6.03
Rat spleen	38.0	Guinea pig right atrium	6.09
Guinea pig deferens	---	Rat vas deferens	inactive (up to 10 μ M)
Guinea pig atrium	---	Mouse aorta	inactive (up to 1.0 μ M)
		Hyperthermia (icv)	inactive as blocker (up to 80 μ g)

Note: Values taken from Jolicoeur *et al.* (1992) and Dennis *et al.* (1989) and unpublished results. See legend to Table 2.1 for description of the calculation of K_i . The pA₂ value represents the negative logarithm of the molar concentration of antagonist required to produce a twofold increase in the EC₅₀ (see Table 2.1) of hCGRP α .

(Dennis *et al.*, 1990; Mimeault *et al.*, 1991). For example, hCGRP₈₋₃₇ antagonizes the effects induced by hCGRP α in the guinea pig left and right atria with pA₂ values in the range of 7-7.5 (Mimeault *et al.*, 1991). Similarly, this fragment, at a dose of 10 μ g (icv), completely blocked the hypophagic effect induced by the native peptide (Dennis *et al.*, 1990). This clearly demonstrated the effectiveness, *in vivo*, of hCGRP₈₋₃₇ to antagonize certain actions induced by hCGRP α .

In contrast, hCGRP₈₋₃₇ failed to reverse the lowering in body temperature observed following icv injections of hCGRP α (Table 2.3). Moreover, the potency of this fragment to block the inhibitory effects of hCGRP α in the rat vas deferens and mouse

aorta is at least 10-50 fold lower than that observed in guinea pig atrial preparations (pA_2 values of 6.24 vs. 7.22 to 7.66, respectively; Table 2.3). Chiba *et al.* (1989) were first to report on the antagonistic potential of hCGRP₈₋₃₇ using a biochemical assay in the rat liver. A few other groups also recently reported on the capacity of hCGRP₈₋₃₇ to block some of the effects of hCGRP α in various species (this volume). Thus, the antagonistic potential of hCGRP₈₋₃₇ is not restricted to a given species. Our data clearly demonstrate that hCGRP₈₋₃₇ can reverse the hypophagic effect of hCGRP α (icv) in the rat as well as the inotropic action of the native peptide in the guinea pig atria (Table 2.3) (Dennis *et al.*, 1990). Moreover, we observed that the potency of hCGRP₈₋₃₇ to antagonize the effects of hCGRP α is similar in both rat and guinea pig atria, while being much lower in either the

Table 2.3 Profile of the fragment hCGRP₈₋₃₇ in various binding and bioassay preparations.

Binding assays	K_i (nM)	Bioassays	Antagonistic potency (pA_2)
Rat brain	0.45	Guinea pig left atrium	7.66
Rat spleen	0.58	Guinea pig right atrium	7.22
Guinea pig deferens	0.43	Rat vas deferens	6.24
Guinea pig atrium	0.67	Mouse aorta	inactive (up to 1.0 μ M)
		Food intake (icv)	potent blocker (at 10 μ g)
		Hyperthermia (icv)	inactive as blocker (up to 80 μ g)

Note: Values taken from Jolicoeur *et al.* (1992), Dennis *et al.* (1989,1990), Mimeault *et al.* (1991) and unpublished results. See legend to Table 2.2 for description of K_i and pA_2 values.

rat or guinea pig vas deferens (Fig. 2.1). This provides further evidence that the low antagonistic potential of some C-terminal fragments in the vas deferens is not species-dependent but tissue specific.

We recently completed an investigation aimed at determining which amino acid residues between positions 8 to 12 are most critical to ensure high antagonistic properties (Mimeault *et al.*, 1991). Our results clearly demonstrated that the threonine and histidine

residues in positions 9 and 10, respectively, are of critical importance, while the valine (position 8) and arginine (position 11) residues are not as relevant (Mimeault *et al.*, 1991).

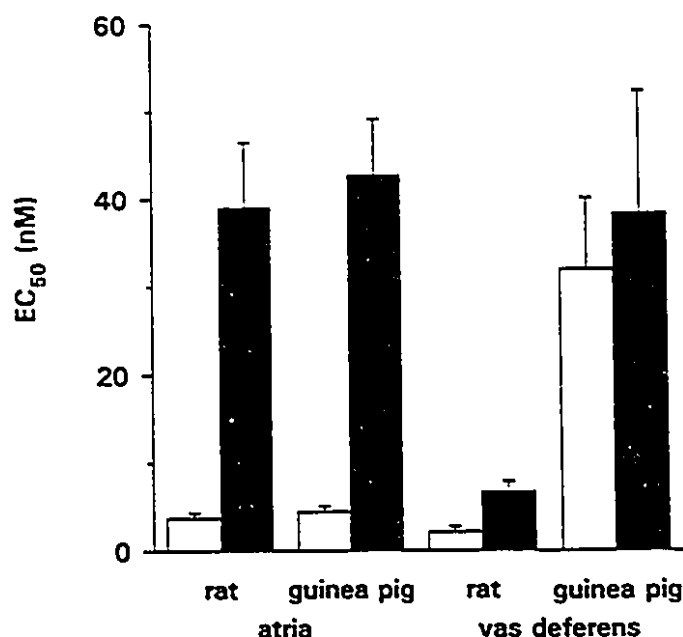


Figure 2.1 Comparative antagonistic activity of the fragment hCGRP₈₋₃₇ in atrial and vas deferens preparations of the rat and guinea pig. The effect of a single concentration (1 μ M) of the antagonist (filled bars) was evaluated for its capacity to block hCGRP α 's action (opened bars) in these four preparations. Data clearly demonstrate that the antagonistic potency of hCGRP₈₋₃₇ is tissue but not species dependent. The blocking activity of the fragment is small in vasa deferentia, while being very important in atrial preparations.

2.2.3 Profile of proposed CGRP receptor subtypes

The differential potencies of the agonist [Cys(ACM)^{2,7}]hCGRP α and the C-terminal fragment antagonists (hCGRP₈₋₃₇ to hCGRP₁₂₋₃₇) in a variety of *in vitro* and *in vivo* bioassays strongly suggest the existence of CGRP receptor subtypes. Early on, we proposed the existence of at least two classes of CGRP receptors, namely the CGRP₁ and CGRP₂ subtypes (Dennis *et al.*, 1990; Dennis *et al.*, 1989). These two subtypes are genuine CGRP receptors for which calcitonin-like peptides (fish or mammalian forms) have little or no affinity. Thus they are distinct from purported calcitonin receptors ("C") and should clearly and unequivocally be dissociated from them. An additional third class of sites has also been reported in the nucleus accumbens, which can recognize hCGRP α , hCGRP₈₋₃₇, [Cys(ACM)^{2,7}]hCGRP α and salmon calcitonin with high affinities (see below) (Sexton *et al.*, 1988; Dennis *et al.*, 1991).

Table 2.4 Profile of proposed CGRP receptor subtypes

	CGRP ₁	CGRP ₂	Other(s)
[Cys(ACM) ^{2,7}]hCGRP α	inactive (up to μ M)	agonist good affinity	lower affinity
hCGRP ₁₂₋₃₇	antagonist	inactive (up to μ M)	not determined
hCGRP ₈₋₃₇	potent antagonist (pA ₂ up to 7.8)	weak antagonist (pA ₂ 6.0) or inactive	lower affinity
Salmon calcitonin	inactive	inactive	high affinity
Prototypic tissues or assay	Atria (guinea pig, rat); Hypophagia (rat)	Vas deferens (rat, guinea pig, mouse aorta); Hyperthermia (rat)	Nucleus accumbens (rat)

Table 2.4 depicts the pharmacologic profile of CGRP₁ and CGRP₂ receptors. The CGRP₁ class is characterized by its lack of affinity for the linear analogue [Cys(ACM)^{2,7}]hCGRP α and its high sensitivity to the blocking activities of C-terminal fragments. In our assays, hCGRP₈₋₃₇ was found to be about 25 to 50 times more potent than hCGRP₁₂₋₃₇. Prototype tissues include the rat and guinea pig atria. The action of hCGRP α (icv) on food intake also appears to be mediated by this receptor subtype (Dennis *et al.*, 1990).

In contrast, the CGRP₂ site is sensitive to the agonist potential of [Cys(ACM)^{2,7}]hCGRP α and more resistant to the blocking activities of hCGRP₈₋₃₇ (Mimeault *et al.*, 1991). Moreover, the fragment hCGRP₁₂₋₃₇ is apparently devoid of significant antagonistic effects on the CGRP₂ site while being active on the CGRP₁ receptors (Table 2.4) (Dennis *et al.*, 1989). Thus, hCGRP₁₂₋₃₇, albeit weaker, might be a more discriminatory probe than hCGRP₈₋₃₇ to distinguish between CGRP₁ and CGRP₂ receptors. Preparations enriched with CGRP₂ sites include the rat and guinea pig vasa deferentia, the mouse aorta, and the neuronal population mediating the hyperthermic effect observed after icv injections of hCGRP α (Dennis *et al.*, 1990). Our group is currently attempting to develop more selective CGRP₁ and CGRP₂ receptor agonists and

antagonists that will allow for a better characterization of their respective functional relevance.

2.2.4 CGRP receptor subtypes in mammalian brain

In view of our interest on the central effects of CGRP-like peptides (Dennis *et al.*, 1990; Dennis *et al.*, 1991), the discrete distribution of CGRP binding sites in mammalian brain as well as the possible existence of CGRP₁ and CGRP₂ subtypes in the CNS were investigated next.

As previously reported by various groups (Henke *et al.*, 1987; Dennis *et al.*, 1991; Seifert *et al.*, 1985; Inagaki *et al.*, 1986; Sexton *et al.*, 1986; Tschopp *et al.*, 1985; Skofitsch and Jacobowitz, 1985b), [¹²⁵I]hCGRP α binding sites are broadly but discretely distributed in the brain. In the rat CNS (Fig. 2.2), the highest density of specific labeling was found in the nucleus accumbens, the amygdaloid formation, the most caudal portion of the striatum (tail of the caudate), and the mammillary body. Moderate levels of binding were detected in the superior colliculus, the auditory temporal cortex, the molecular layer of the cerebellar cortex, the inferior olive and the medial geniculate (Fig. 2.2). Lower, but still significant, labeling was seen in the habenula, the sensory and motor areas of the fifth nerve, the vestibular nucleus, and the area surrounding the central canal (laminae X) of the spinal cord (Fig. 2.2). Other brain regions were globally not enriched with specific [¹²⁵I]hCGRP α binding sites. Similar results were reported in other species, except for

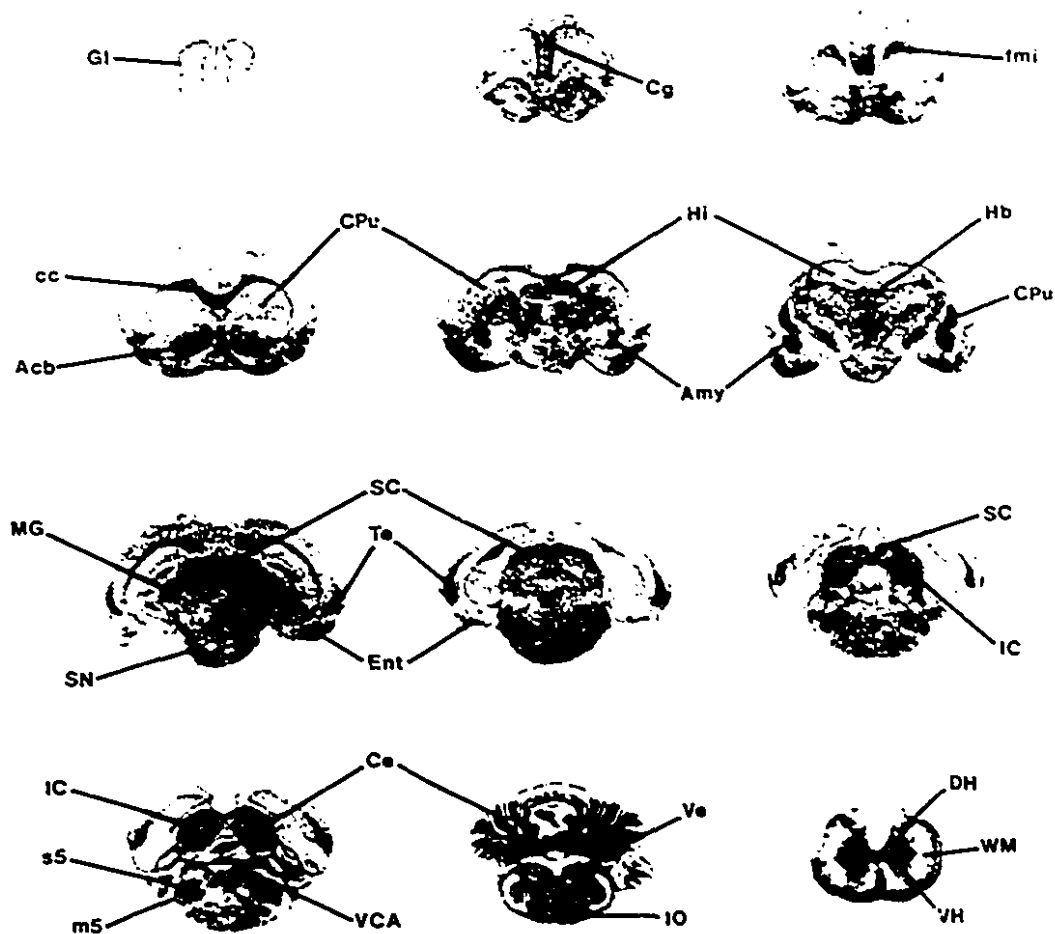


Figure 2.2. Photomicrographs of the autoradiographic distribution of [125 I]hCGRP α binding sites in the rat brain. Sections were incubated in the presence of 20 pM [125 I]hCGRP α . Note the broad but discrete distribution of CGRP binding sites in the rat brain. *Abbreviations:* Acb, nucleus accumbens; Amy, amygdaloid body; cc, corpus callosum; Ce, cerebellum; Cg, cingulate cortex; CPu, caudate-putamen; DH, dorsal horn of the spinal cord; Ent, entorhinal cortex; fmi, forceps minor corpus callosum; Gl, glomerular layer of the olfactory bulb; Hb, habenular nuclei; HI, hippocampus; IC, inferior colliculus; IO, inferior olive; MG, medial geniculate; m5, fifth motor nucleus; SC, superior colliculus; SN, substantia nigra; S5, fifth sensory nucleus; Te, temporal cortex; VCA, ventral cochlear nucleus; Ve, vestibular nucleus; VH, ventral horn of the spinal cord; and WM, white matter.

various nuclei of the basal ganglia, which demonstrated major species differences (Dennis *et al.*, 1991).

In order to investigate the possible existence of CGRP₁ and CGRP₂ sites in the CNS, the first approach consisted in performing *in vitro* receptor autoradiographic studies of [¹²⁵I]hCGRP α binding in presence of either CGRP₁, (CGRP₈₋₃₇), or CGRP₂ ([Cys(ACM)^{2,7}]hCGRP α) preferential ligand. A similar strategy gave positive results for the characterization of the differential distribution of neuropeptide Y receptor subtypes (Dumont *et al.*, 1990) although it was not very successful in the study of opioid receptors (Quirion *et al.*, 1983). We thus investigated the effects of increasing concentrations (10⁻⁹-10⁻⁶ M) of CGRP₈₋₃₇, [Cys(ACM)^{2,7}]hCGRP α , and salmon (sCT) and human (hCT) calcitonins on [¹²⁵I]hCGRP α binding in various brain regions. Prototypical examples of data obtained are shown in Figures 2.3 (at the level of the nucleus accumbens) and 2.4 (at the level of the amygdaloid nuclear group and most caudal portion of the caudate), while quantitative results are given in Figures 2.5a and 2.5b and Table 2.5.

It is clear that this approach failed to provide direct evidence for the existence of CGRP₁ and CGRP₂ receptor subtypes in the rat brain. In all regions studied, CGRP₈₋₃₇ and [Cys(ACM)^{2,7}]hCGRP α demonstrated rather similar affinity profiles with IC₅₀s in the low nM range (Figs. 2.3-2.5, Table 2.5). Thus, other approaches based on the use of more subtype selective competitors or the development of radioligands specifically recognizing a single receptor class (such as [¹²⁵I]hCGRP₁₂₋₃₇) will be required to further investigate the existence of CGRP₁ and CGRP₂ receptors in the CNS. Behavioral data already clearly support this possibility since, for example, CGRP₈₋₃₇ is able to block some, but not all, the effects observed following icv injections of CGRP-like peptides (Dennis *et al.*, 1990; Jolicoeur *et al.*, 1992).

In contrast, the differential competition profiles of sCT in certain brain regions suggested the existence of a unique class of sites possessing strong affinities for both hCGRP α and sCT (Figs. 2.3-2.5, Table 2.5). As shown in Figs. 2.3 and 2.5, sCT (IC₅₀ value of 8.2 nM) competed for [¹²⁵I]hCGRP α binding in the nucleus accumbens, especially in its lateral portion. In contrast, sCT failed to compete for [¹²⁵I]hCGRP α binding in most of the other brain regions studied including the most caudal portion of the caudate nucleus (Fig. 2.4), the amygdaloid formation (Fig. 2.4), and various mesencephalic and brainstem nuclei (Fig. 2.5, Table 2.5). Human calcitonin failed to compete for specific [¹²⁵I]hCGRP α sites in the rat brain (Figs. 2.3-2.5, Table 2.5). These results are in agreement with earlier reports (Sexton *et al.*, 1988; Dennis *et al.*, 1991) and suggest the existence of a unique class of sites, mostly found in the rat nucleus accumbens, which is able to recognize, with high affinity, hCGRP α and salmon (but not human)

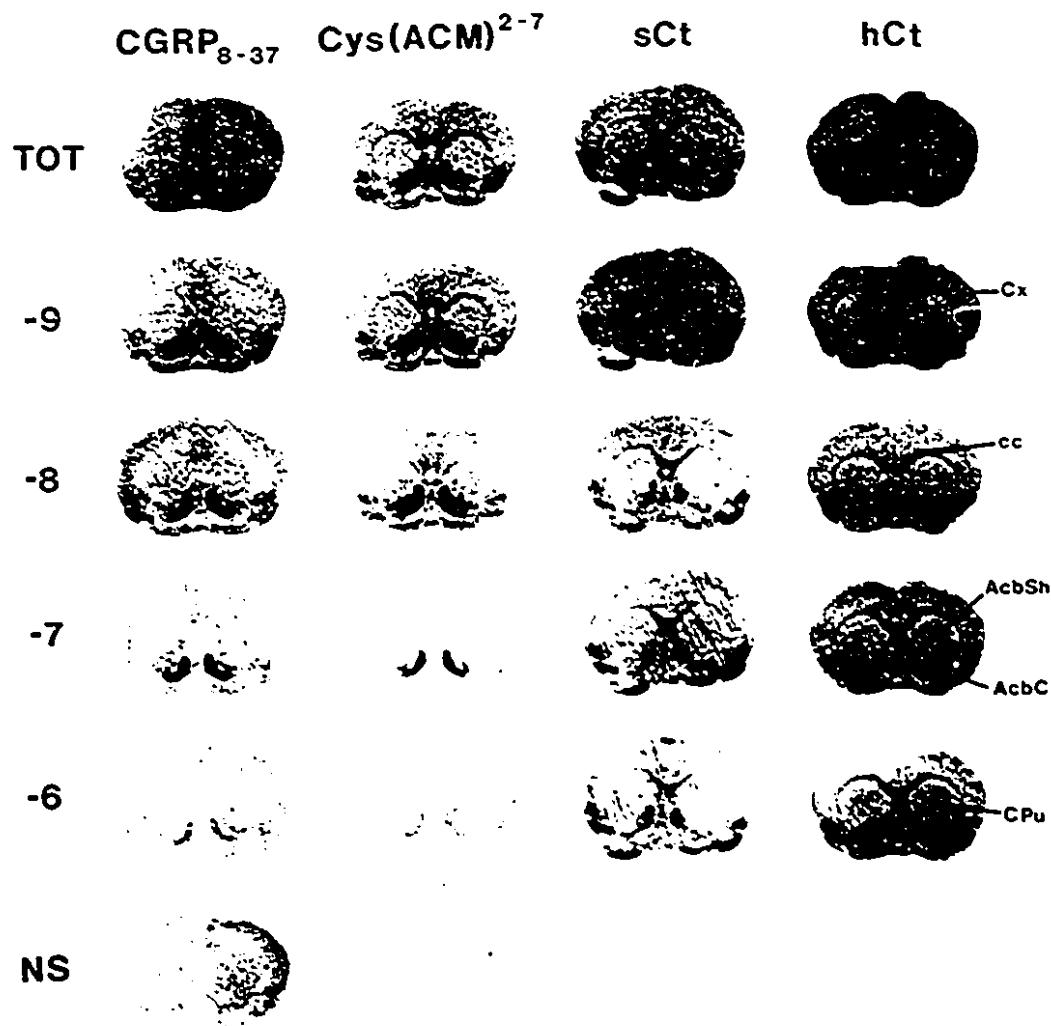


Figure 2.3 Photomicrographs of the effect of increasing concentrations (10^{-9} - 10^{-6} M) of various competitors on [125 I]hCGRP α binding at the level of the nucleus accumbens of the rat brain. hCGRP $_{8-37}$ (first column); [Cys(ACM) $^{2-7}$]hCGRP α (second column); salmon calcitonin, sCT (third column); and human calcitonin, hCT (fourth column). Note that while both CGRP $_{8-37}$ and [Cys(ACM) $^{2-7}$]hCGRP α apparently competed for [125 I]hCGRP α binding with similar potencies in all regions seen at this level, sCT is potent only in the nucleus accumbens. hCT is inactive at all concentrations tested. For abbreviations see legend to Fig. 2.2; also, AcbC, core of the nucleus accumbens; AcbSh, shell of the nucleus accumbens; and Cx, cortex.

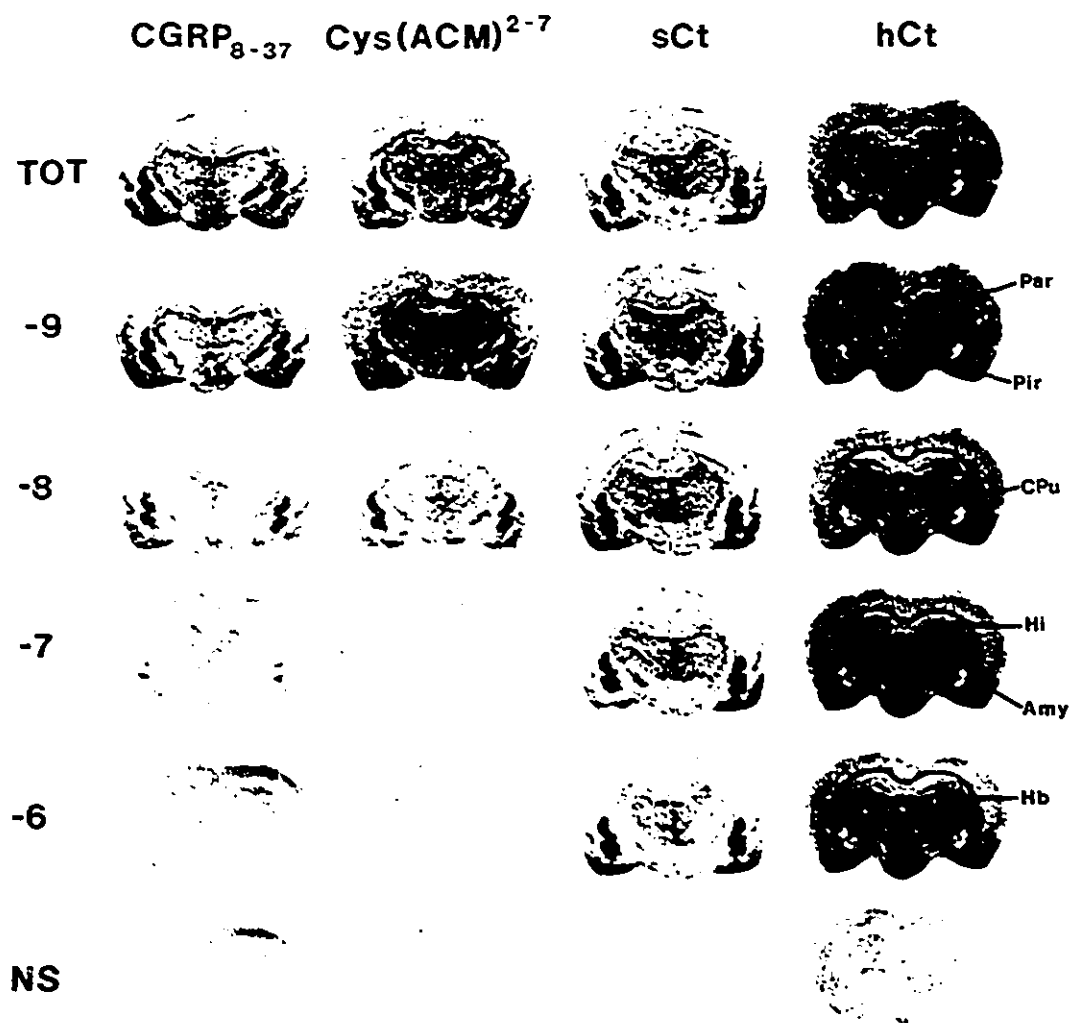


Figure 2.4 Photomicrographs of the effect of increasing concentrations (10^{-9} - 10^{-6} M) of various competitors on [125 I]hCGRP α binding at the level of the amygdaloid nuclei and the most caudal portion of the caudate of the rat brain. hCGRP₈₋₃₇ (first column); [Cys(ACM)^{2,7}]hCGRP α (second column); salmon calcitonin, sCT (third column); and human calcitonin, hCT (fourth column). Note that while both CGRP₈₋₃₇ and [Cys(ACM)^{2,7}]hCGRP α apparently competed for [125 I]hCGRP α binding with similar potencies in all regions seen at this level, sCT and hCT are basically inactive at all concentrations tested. For abbreviations see legend to Fig. 2.2; also, Par, parietal cortex; and Pir, piriform cortex.

Table 2.5 Comparative affinities of various CGRP-related molecules and calcitonins for [125 I]hCGRP α binding in various areas of the rat brain

	Competitor (IC ₅₀ , nM)				
	hCGRP α	hCGRP ₈₋₃₇	[Cys(ACM) ^{2,7}] hCGRP α	sCT	hCT
Glomerular layer of olfactory bulb	0.45	10.9	7.5	>1000	>1000
Accessory olfactory nucleus	0.81	11.8	5.9	>1000	>1000
Frontal cortex	0.71	9.1	5.8	>1000	>1000
Parietal cortex	0.82	8.5	6.2	>1000	>1000
Caudate putamen	0.86	8.4	6.4	>1000	>1000
Caudate putamen (tail)	4.7	5.1	12.1	>1000	>1000
Nucleus accumbens	6.9	21.1	26.7	8.2	>1000
Amygdaloid body	4.1	9.9	17.2	>1000	>1000
Temporal cortex	1.8	5.5	10.0	>1000	>1000
Substantia nigra, pars compacta	1.9	6.1	9.2	>1000	>1000
Medial geniculate	2.2	5.6	9.9	>1000	>1000
Superficial layers of the superior colliculus	1.9	5.6	13.5	>1000	>1000
Pontine nucleus	2.0	5.5	10.7	>1000	>1000
Inferior colliculus	3.2	5.3	10.2	>1000	>1000
Cerebellum	2.8	5.2	11.1	>1000	>1000
Inferior olive	2.7	5.2	9.7	>1000	>1000
Spinal vestibular nucleus	4.2	8.5	—	>1000	>1000

Note: Data are derived from quantitative receptor autoradiography. IC₅₀ represents the concentration of competitors required to compete for 50% of specifically bound [125 I]hCGRP α .

calcitonin. Interestingly, CGRP₈₋₃₇ and [Cys(ACM)^{2,7}]hCGRP α demonstrated somewhat lower, but still significant, affinities for these sites (Tables 2.4 and 2.5). It is unclear, at the present time, whether this rather unique site should be classified as either a CGRP or a sCT receptor binding site. However, since mammalian calcitonins apparently lack affinity for this site (Sexton *et al.*, 1988; Dennis *et al.*, 1991), it would appear that CGRP will act as the endogenous ligand, a salmon calcitonin-like peptide being thus far absent from the mammalian brain. It would be of interest to evaluate whether certain effects observed after intracranial injections of sCT (Pecile *et al.*, 1987) are not in fact due to the selective activation of this unique class of CGRP/sCT receptors.

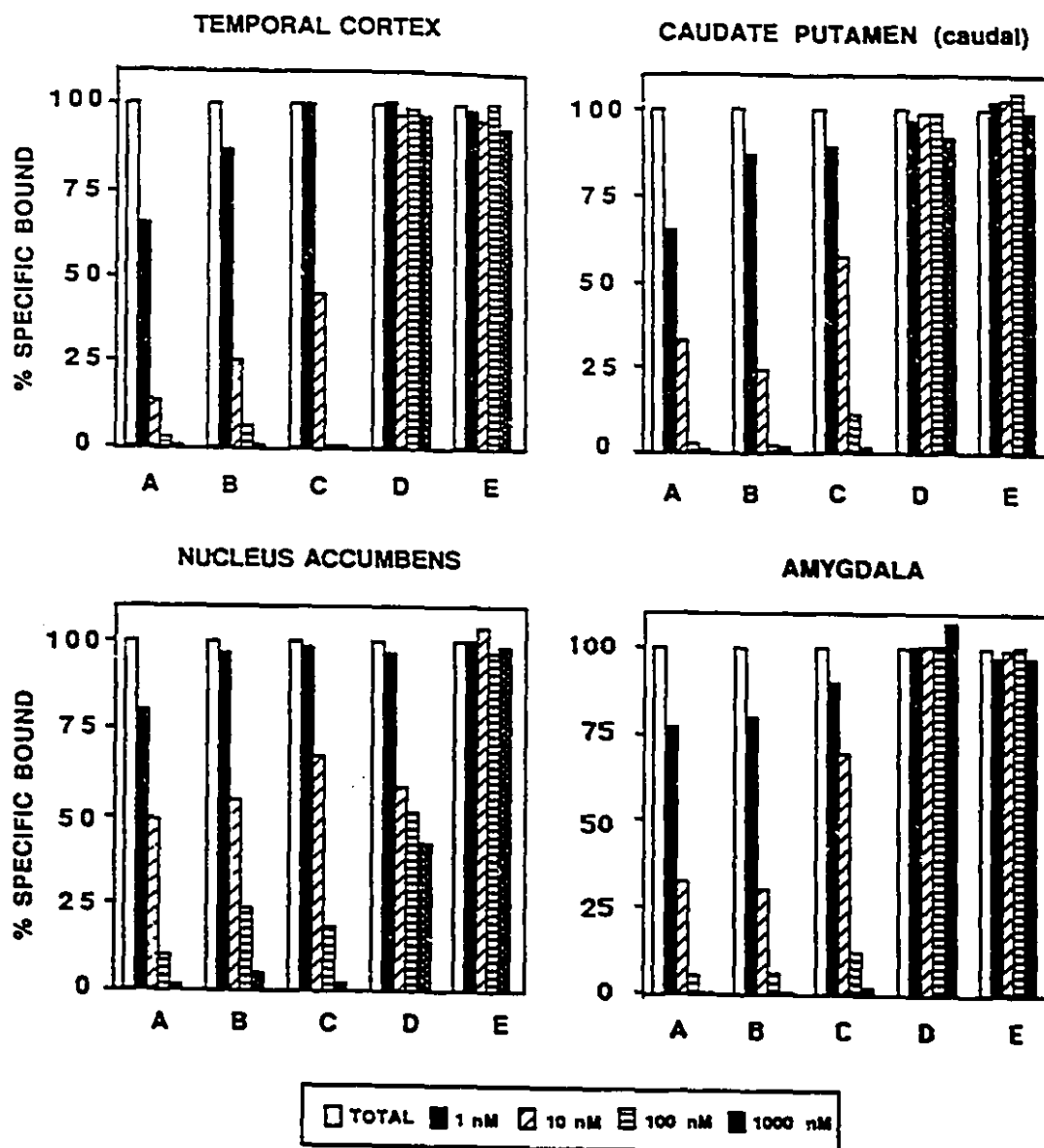


Figure 2.5a. Quantitative analysis of the respective potencies of hCGRP α (A), hCGRP $_{8-37}$ (B), [Cys(ACM) 2,7]hCGRP α (C), salmon calcitonin (D), and human calcitonin (E) against [125]hCGRP α binding in various regions of the rat brain. Data are shown for increasing concentrations (0 or total binding, 1, 10, 100 and 1000 nM) of competitors and are expressed as their potencies to inhibit specifically bound [125]hCGRP α binding. All data are derived from quantitative analysis of film autoradiograms. It is evident that while unlabeled hCGRP α , hCGRP $_{8-37}$, [Cys(ACM) 2,7]hCGRP α are highly potent in all regions, salmon calcitonin is potent only in the nucleus accumbens, and human calcitonin is inactive.

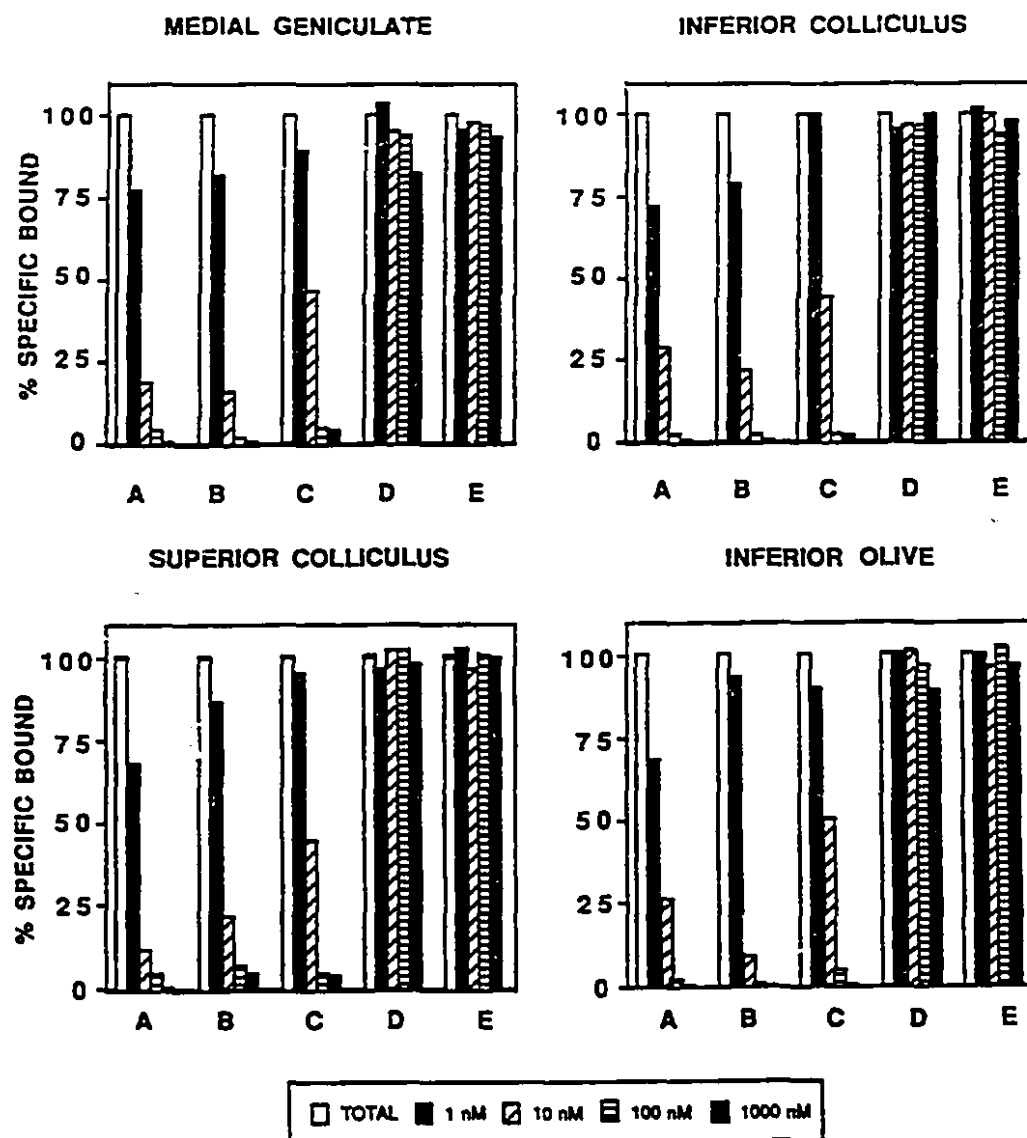


Figure 2.5b. See legend of Fig. 2.5a.

2.3 Conclusions and future directions

In summary, the results discussed here clearly suggest the existence of CGRP receptor subtypes that are differentially expressed in various tissues of the organism. CGRP₁ receptors appear to be especially sensitive to the antagonistic activities of extended C-terminal fragments, whereas CGRP₂ sites possess high affinity for the linear analogue [Cys(ACM)^{2,7}]hCGRP α . A third class of sites can apparently recognize, with high affinity, both hCGRP α and salmon (but not human) calcitonin.

In order to provide additional support for the existence of these various classes of CGRP receptors, the development of highly selective agonists and antagonists is now attempted. Such tools would be extremely valuable to evaluate the respective functional roles of each CGRP receptor subtype. Additionally, it is now important to determine whether these putative receptor subtypes are coupled to different transduction mechanisms. We have recently obtained indirect evidence that suggests that CGRP receptors are possibly coupled to different G-proteins in various tissues (van Rossum *et al.*, 1992). This could provide additional evidence for the existence of CGRP receptor subtypes, although recent studies have revealed that a single receptor class can be coupled to multiple transduction pathways. Naturally, the cloning of the respective CGRP receptor subtypes proposed here will provide definite evidence for their existence.

It would also be of interest to determine whether the CGRP₁ and CGRP₂ receptor subtypes correspond to the previously reported α (I) and β (II)-preferential receptor classes (Dotti-Sigrist *et al.*, 1988; Henke *et al.*, 1987). Consequently, we are currently evaluating the relative affinities and potencies of the α and β forms of both human and rat CGRP in a variety of functional assays. Finally, amylin being a CGRP-related peptide (Cooper *et al.*, 1988), it may be important to determine whether this newly characterized peptide possesses preferential affinity for a given CGRP receptor class. This information should help to better characterize the functional relevance of CGRP and CGRP receptors in the maintenance of body homeostasis.

2.4 Acknowledgments

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CHAPTER 3

Binding profile of a selective calcitonin gene-related peptide (CGRP) receptor antagonist ligand, [¹²⁵I-Tyr]hCGRP₈₋₃₇, in rat brain and peripheral tissues

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3.0 Preface to Chapter 3

As the indirect competitive binding approach presented in the previous chapter failed to provide direct evidence for distinct binding profiles of CGRP₁ and CGRP₂ receptor subtypes in the rat brain, we synthesized a novel radioligand, namely [¹²⁵I-Tyr]hCGRP₈₋₃₇. One advantage of this pharmacological tool is that much lower concentrations (picomolar vs. nanomolar) of the radioligand are required to perform the *in vitro* assays. Moreover, as the unlabeled fragment hCGRP₈₋₃₇ preferentially acts as an antagonist on CGRP₁ binding sites, the radiolabelled counterpart should be useful in comparing agonist vs. antagonist binding properties in addition to possibly preferentially label CGRP₁ binding sites. The present study therefore includes an extensive pharmacological characterization of [¹²⁵I-Tyr]hCGRP₈₋₃₇ binding properties in brain and peripheral tissues.

3.1 Abstract

The calcitonin gene-related peptide (CGRP) C-terminal fragment human CGRP₈₋₃₇ acts as a potent antagonist of various *in vitro* and *in vivo* effects of CGRP. Its iodinated counterpart, [¹²⁵I-Tyr]hCGRP₈₋₃₇, binds with high affinity (K_D values between 7.5×10^{-11} - 2.1×10^{-10} M) to what is apparently a single class of CGRP receptors in brain, atrium and vas deferens membrane preparations. The relative potencies of various CGRP-related fragments and analogues in competing for [¹²⁵I-Tyr]hCGRP₈₋₃₇ binding sites were similar in these three preparations, with hCGRP α being the most potent competitor, followed by unlabeled hCGRP₈₋₃₇, the linear agonist [acetamidomethyl-cysteine^{2,7}]hCGRP α , and finally by rat amylin-amide and salmon calcitonin. Competition profiles suggested the existence of a single affinity site (except in the case of hCGRP α for which competition binding data were best fitted to two apparent affinity constants in all three tissues), whereas rat amylin-amide revealed two affinity constants in the rat brain. Guanylylimidodiphosphate (100 μ M) failed to alter specific [¹²⁵I-Tyr]hCGRP₈₋₃₇ binding in the various tissues studied here. Quantitative receptor autoradiography in the rat brain revealed [¹²⁵I-Tyr]hCGRP₈₋₃₇ binding sites mostly concentrated in the nucleus accumbens (shell), caudate putamen (tail), amygdaloid body, pontine nuclei, cerebellum and inferior olive whereas lower quantities of sites were present in the olfactory tubercle, nucleus accumbens (core), medial geniculate, superior colliculus, temporal cortex, inferior colliculus, lateral lemniscus, vestibular nuclei and principal sensory trigeminal nucleus. [¹²⁵I-Tyr]hCGRP₈₋₃₇ behaves as a high affinity probe for CGRP receptors present in these various tissues; its antagonistic properties being of interest for comparing characteristics of agonist versus antagonist CGRP radioligands.

3.2 Introduction

CGRP is a 37-amino-acid peptide that results from alternative splicing of the calcitonin gene and is widely distributed in both peripheral and central nervous system (Amara *et al.*, 1982; Rosenfeld *et al.*, 1984; for reviews, Ishida-Yamamoto and Tohyama, 1989; Taché *et al.*, 1992; Poyner, 1992). Fragments lacking N-terminal segments such as CGRP₈₋₃₇, CGRP₉₋₃₇ and CGRP₁₂₋₃₇ behave as potent competitive antagonists (Chiba *et al.*, 1989; Dennis *et al.*, 1989; Dennis *et al.*, 1990; Mimeault *et al.*, 1991; Quirion *et al.*, 1992). Shorter C-terminal fragments such as CGRP₁₉₋₃₇, CGRP₂₃₋₃₇ and [Tyr⁰]CGRP₂₈₋₃₇ can also act as competitive antagonists but are markedly less potent with pA₂ values ranging from 4.8 to 7.3 M in various *in vitro* preparations (Chakder and Rattan, 1990; van Valen *et al.*, 1990; Rovero *et al.*, 1992).

The CGRP C-terminal fragment CGRP₈₋₃₇ can antagonize CGRP effects in some *in vitro* and *in vivo* assays (Han *et al.*, 1990; Gardiner *et al.*, 1990; Hughes and Brain, 1991; Wang *et al.*, 1992) though it is inactive in others (Dennis *et al.*, 1990; Evangelista *et al.*, 1992). For example, CGRP₈₋₃₇ was able to potently block the inotropic and chronotropic effects of CGRP in guinea pig atrium (pA₂ ~7.4) but to a lesser extent in the electrically stimulated rat vas deferens (pA₂ ~6.5) (Dennis *et al.*, 1990; Mimeault *et al.*, 1991; Maggi *et al.*, 1991; Quirion *et al.*, 1992). Similar properties were observed with the fragment CGRP₁₂₋₃₇, albeit with lower potencies (Dennis *et al.*, 1989; Quirion *et al.*, 1992). Furthermore, Giuliani *et al.* (1992) have recently reported that CGRP₈₋₃₇, up to 3 µM, did not affect the relaxation produced by either the α or β forms of hCGRP in the guinea pig urinary bladder. These results were taken as suggesting the existence of CGRP receptor subtypes. The CGRP₁ receptor class was thus proposed as the subtype that is highly sensitive to the antagonistic properties of C-terminal fragments (Dennis *et al.*, 1990; Quirion *et al.*, 1992). In addition, the linear analog [Cys(ACM)^{2,7}]hCGRPα behaved as a rather potent agonist in the vas deferens (EC₅₀ 70 nM) in comparison to its effects in the atrial preparations (EC₅₀ >700 nM) (Dennis *et al.*, 1989; Quirion *et al.*, 1992). Stangl *et al.* (1993) also recently reported that this linear analog and hCGRPα produced hCGRP₈₋₃₇-insensitive increases in cAMP formation in the liver. In contrast, [Cys(ACM)^{2,7}]hCGRPα failed to alter cyclase activity in the spleen, a well established effect of the full cyclic CGRP peptide (Stangl *et al.*, 1993). The differential sensitivity of these preparations to the linear agonist as well as the resistance to the antagonistic properties of C-terminal fragments were thus suggested as the key properties of the CGRP₂ receptor subtype (Dennis *et al.*, 1989; Quirion *et al.*, 1992).

Up to now, binding studies of CGRP receptors have been performed almost exclusively using the native peptides in their iodinated forms (h or rat [125 I]CGRP α or β (Henke *et al.*, 1987; Stangl *et al.*, 1991)). However, these radioligands cannot discriminate between putative CGRP receptor subtypes, because they show rather similar affinities for both CGRP $_1$ and CGRP $_2$ receptor subtypes (Quirion *et al.*, 1992). Moreover, the usefulness of antagonists as radioligands is well established in as much as they are less likely to be sensitive to incubation condition variables such as nucleotides and cations. Therefore, we investigated the binding profile of the C-terminal CGRP receptor antagonist hCGRP $_{8-37}$ in its iodinated form, [125 I-Tyr]hCGRP $_{8-37}$, in membrane preparations from the guinea pig atrium (CGRP $_1$ -enriched homogenate), vas deferens (CGRP $_2$ -enriched homogenate) and the rat brain, as well as examining the specific distribution of these sites in the rat brain using *in vitro* autoradiography.

3.3 Materials and Methods

3.3.1 Materials

[Tyr]hCGRP₈₋₃₇, hCGRP α ₁₋₃₇, hCGRP₈₋₃₇, [Cys(ACM)^{2,7}]hCGRP α , rat amylin-NH₂ and sCT were synthesized as previously described in detail (Mimeault *et al.*, 1992). Rat amylin-NH₂ was also purchased from Bachem California (Torrance, California). Sodium iodine (Na-¹²⁵I) (ICN Canada, Mississauga, Ontario), trisma-base, NaCl, bacitracin and leupeptin (Sigma Chemicals Co., St-Louis, MO), bovine serum albumin (Boehringer Mannheim Canada, Laval, Québec), other chemicals of analytical grade (Fisher Scientific, Montréal, Québec).

Male Sprague-Dawley rats (225-250 g) and male Hartley guinea pigs (325-375 g) were obtained from Charles River (St-Constant, Québec). They had free access to food and water and were kept according to the guidelines of the Canadian Council for Animal Care and of McGill University.

3.3.2 Radioligand synthesis

[Tyr]hCGRP₈₋₃₇ was iodinated using a chloramine-T/Na metabisulfite method (modified from Hunter and Greenwood, 1962). Briefly, [Tyr]hCGRP₈₋₃₇ (35 μ g) was added to 0.25 M NaH₂PO₄/Na₂HPO₄, pH 7.5, Na-¹²⁵I (2 mCi) and chloramine-T. After 1 min, the reaction was stopped by adding Na-metabisulfite to the mixture. After pre-purification on an anion exchange sep-pak (Accell QMA cartridge, Millipore) that traps most of free ¹²⁵I-Na, the radiolabelled peptide was further purified by HPLC onto a C-18 reversed-phase column (LKB-Ultropac) and a 10-70% (30 min) of acetonitrile/ 0.06% trifluoroacetic acid. Fractions were collected every 30 sec and the radioactive peak was aliquoted and stored at -20°C until use.

3.3.3 [¹²⁵I-Tyr]hCGRP₈₋₃₇ receptor binding studies

Animals were sacrificed by decapitation. The brains (less cerebellum), atria and *vasa deferentia* were rapidly removed and placed in 15x volume of 25 mM Tris-HCl; 50 mM NaCl; 2 mM MgCl₂; pH 7.4. Tissues were homogenized using Brinkman polytron and centrifuged at 48 000 g for 20 min at 4°C. The pellets were then washed and suspended in the original volume of buffer and re-centrifuged. This step was repeated

twice. For the association experiments, crude membranes [275 µg, 150 µg and 15 µg protein from brain, atrium and vas deferens, respectively, as measured by the method of Lowry *et al.* (1951)] were incubated at room temperature in a final volume of 0.5 ml of 50 mM Tris-HCl, 100 mM NaCl and 4 mM MgCl₂, pH 7.4, 0.2% BSA, 0.02% leupeptin, 0.4 mM bacitracin and ~40 pM [¹²⁵I-Tyr]hCGRP₈₋₃₇ (80 000 CPM; ~2000 Ci/mmol). [¹²⁵I-Tyr]hCGRP₈₋₃₇ binding was determined by incubation for various time intervals (2.5-80 min) in the absence or presence of 1 µM hCGRP₈₋₃₇ for total and nonspecific binding, respectively. The reaction was stopped by rapid filtration under reduced pressure using a Brandel Cell Harvester (Model M24-R, Gaithersburg, MD) through glass fibre filters (#32, Schleicher and Schuell, Keene, NH) pre-soaked in 0.1 % polyethyleneimine. For dissociation experiments, [¹²⁵I-Tyr]hCGRP₈₋₃₇ was incubated for 60 min, at which time an excess of hCGRP₈₋₃₇ (1 µM) was added and residual binding measured at various time points (2.5-50 min). Additionally, direct saturation studies were performed by incubating various concentrations of [¹²⁵I-Tyr]hCGRP₈₋₃₇ (~10-500 pM) for 60 min under the same assay conditions, and competition curves were performed by incubating ~40 pM [¹²⁵I-Tyr]hCGRP₈₋₃₇ with various concentrations (10⁻¹²- 5x 10⁻⁶ M) of hCGRPα, hCGRP₈₋₃₇, [Cys(ACM)^{2,7}]hCGRPα, rat amylin-NH₂ or sCT. Nonspecific binding represented between 15 and 35% of total binding depending on the tissue preparation.

3.3.4 [¹²⁵I-Tyr]hCGRP₈₋₃₇ receptor autoradiography

Brain tissues were snap-frozen in 2-methylbutane (-40°C) and stored at -80°C until use. Serial coronal sections (20 µm) were cut and thaw-mounted onto gelatin coated microscope slides. Tissue sections were then desiccated overnight at 4°C and stored at -80°C until experiments were performed. After a 15-min preincubation at room temperature in 50 mM Tris-HCl buffer, 100 mM NaCl, pH 7.4, the sections were incubated for 60 min in fresh buffer containing 0.2% BSA, 0.02% leupeptin, 0.4 mM bacitracin and ~40 pM [¹²⁵I-Tyr]hCGRP₈₋₃₇. After incubation, sections were washed in ice cold buffer (4 x 1 min) and dipped twice in cold distilled water. Nonspecific binding to adjacent tissue sections was defined by labeling of the ligand in the presence of 1 µM hCGRP₈₋₃₇. It represented ~15% of total binding. Radiolabelled tissue sections were exposed to highly sensitive film (Hyperfilm, Amersham Canada, Ontario) for 3 days. Thereafter, the binding of the various brain regions was quantified using labelled standards (Amersham, Oakville, Ontario) and a computerized image analysis system (MCID System, Image Research Inc., Ste-Catharines, Ontario).

3.3.5 Data analysis

Kinetic data were analyzed according to the procedure of Bennett (1978). The apparent association rate constant (k_{obs}) was calculated as the slope of the plot of $(\ln[B_{eq}/(B_{eq}-B_t)])$ vs. time, where B_{eq} is the amount of specifically bound ligand at equilibrium and B_t is the amount of specifically bound ligand at time t . k_{obs} is equal to $k_f[L] + k_{-f}$ when the binding reaction is performed with no more than 10 % of initial free ligand bound at steady-state, and where k_f corresponds to the association rate constant, $[L]$ to the free ligand concentration and k_{-f} to the dissociation rate constant. k_{-f} was estimated from the slope of the plot of $\ln(B_t/B_0)$ vs. time, where B_0 is the initial amount of specifically bound ligand.

The analysis of both saturation and competition curves from membrane binding assays were performed using the LUNDON-Data Analysis program. ANOVA was used to compare the best goodness of fit between one- and two-sites models. If the ANOVA yielded a probability value greater than 0.05 then the less complex model was accepted as the model that best fitted the data. Values represent the mean $\pm$ S.E.M. of at least three separate experiments, each performed in triplicate.

3.4 Results

3.4.1 Binding characteristics of [125 I-Tyr]hCGRP₈₋₃₇

Ligand association experiments revealed that binding of ~ 40 pM [125 I-Tyr]hCGRP₈₋₃₇ reached equilibrium within 30 min (Fig 3.1) and remained stable for at least 80 min. The data were transformed according to the pseudo first-order rate equation and resolved into a single exponential function from which the association rate constant k_1 was calculated as $1.1 \times 10^{10} \pm 0.3$, $9.2 \times 10^9 \pm 1.0$ and $8.5 \times 10^9 \pm 1.0$ min⁻¹ M⁻¹ in brain, atrium and vas deferens, respectively. Similarly, the rate of dissociation of [125 I-Tyr]hCGRP₈₋₃₇ was resolved into a single exponential function when data were transformed according to a first-order rate reaction from which the dissociation rate constant k_{-1} was calculated as 0.19 ± 0.01 , 0.24 ± 0.04 and 0.17 ± 0.05 min⁻¹ in brain, atrium and vas deferens, respectively.

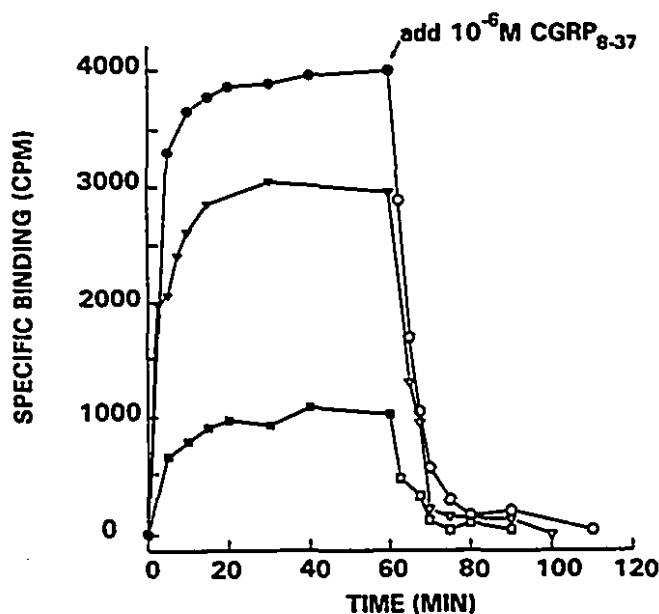


Figure 3.1 Time courses of ligand association (filled symbols) and dissociation (opened symbols) curves of ~ 40 pM [125 I-Tyr]hCGRP₈₋₃₇ binding to brain (●;○), atrium (■;□) and vas deferens (▼;▽). The data are representative of three experiments each performed in triplicate as described in "Material and Methods".

Binding of [125 I-Tyr]hCGRP₈₋₃₇ to rat brain, guinea pig atrium and vas deferens was saturable, was specific and was associated with a single class of sites as revealed by the computer assisted non-linear analysis (see "Material and Methods") and plotted according to the method of Rosenthal (Fig 3.2). Dissociation constants (K_D) values of 1.6×10^{-10} , 2.1×10^{-10} and 7.5×10^{-11} M and B_{max} values of 39, 37 and 443 fmol/mg protein were obtained for the brain, atrium and vas deferens homogenates, respectively.

3.4.2 Comparative affinities of hCGRP α , fragment and analogs for specific [125 I-Tyr]hCGRP₈₋₃₇ binding

As shown in Fig 3.3, the binding profile of [125 I-Tyr]hCGRP₈₋₃₇ was similar in all three membrane preparations. hCGRP α was the most potent in competing against [125 I-Tyr]hCGRP₈₋₃₇ binding, followed by hCGRP₈₋₃₇, [Cys(ACM)^{2,7}]hCGRP α , rat amylin and sCT. All competitors showed one affinity constant for the [125 I-Tyr]hCGRP₈₋₃₇ binding site except for hCGRP α , which revealed two binding affinity constants in all three membrane preparations, and rat amylin-NH₂ in rat brain homogenates (Table 3.1, 3.2). Moreover, the addition to the incubation media of Gpp(NH)p (10^{-8} - 10^{-4} M), a GTP non-hydrolysable analog, had no significant effect on [125 I-Tyr]hCGRP₈₋₃₇ binding (99-102 % of control specific binding) in all three membrane preparations.

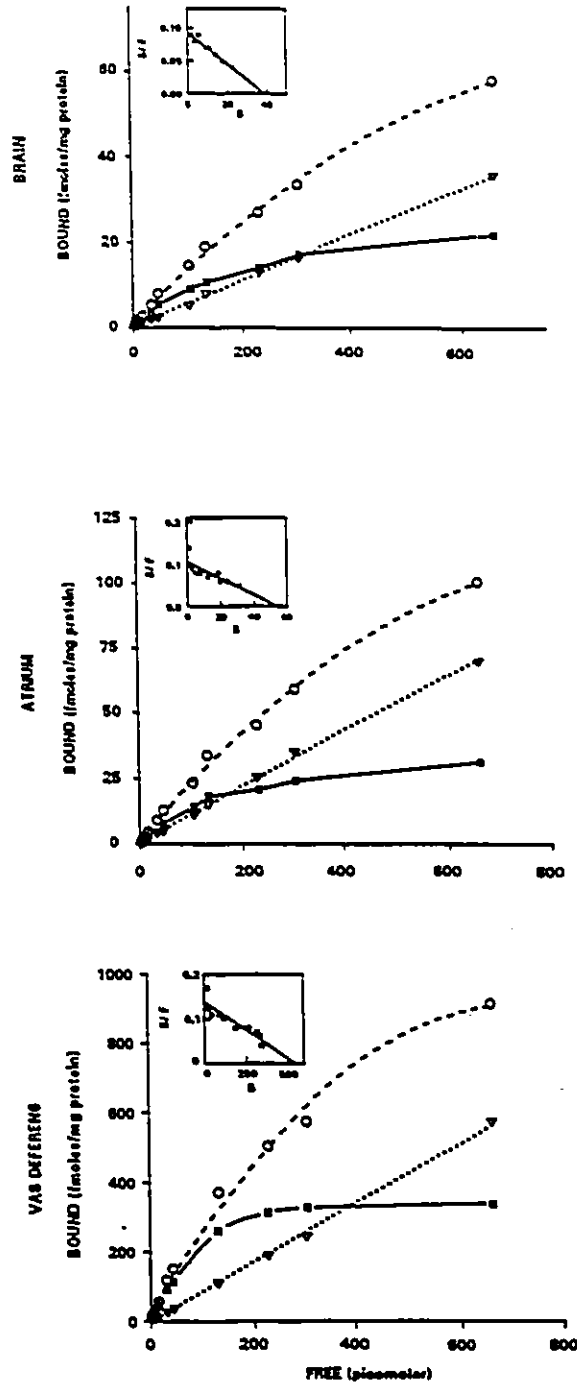


Figure 3.2 Representative saturation curves and respective Rosenthal transformation (*insets*) of $[^{125}\text{I}]\text{-Tyr]hCGRP}_{8-37}$ binding in rat brain (top), guinea pig atrium (middle) and vas deferens (bottom). Total (O), specific (■) and nonspecific (▽) binding. Nonspecific binding was defined in the presence of $1\ \mu\text{M}$ hCGRP $_{8-37}$ and represented 15 to 30% of total binding, depending on tissue and ligand concentrations.

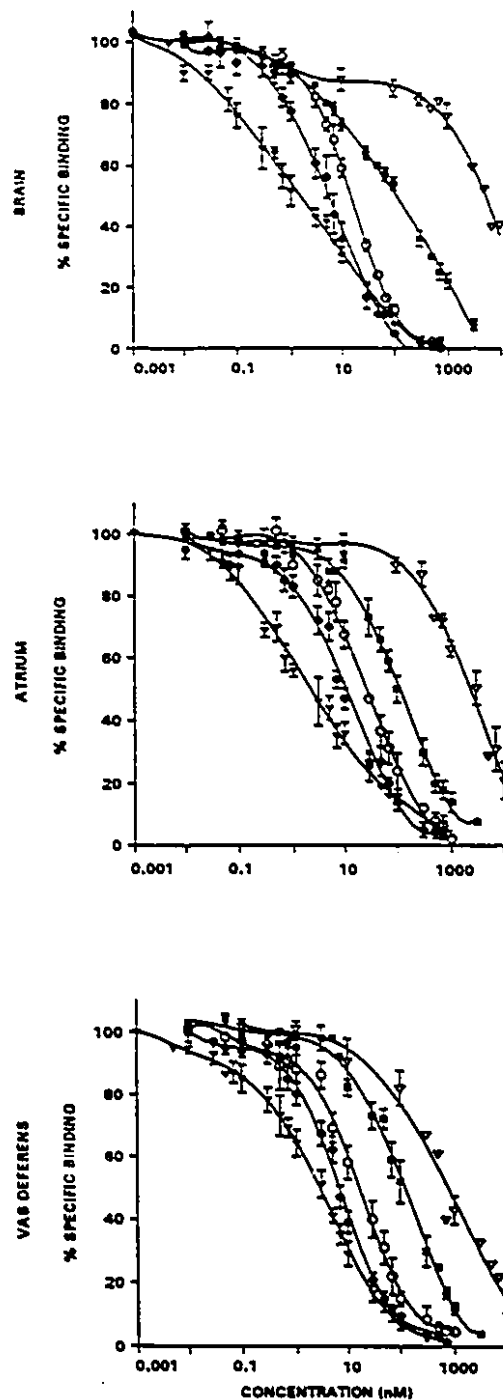


Figure 3.3 Competition binding profiles of hCGRP α , its fragment and analogs for specific $[^{125}\text{I-Tyr}]h\text{CGRP}_{8-37}$ binding in rat brain (top), guinea pig atrium (middle) and vas deferens (bottom). Membranes were incubated with ~ 40 pM $[^{125}\text{I-Tyr}]h\text{CGRP}_{8-37}$ and with increasing concentrations (10^{-12} – 10^{-5} M) of hCGRP α ($\blacktriangledown$), hCGRP $_{8-37}$ ($\bullet$), [Cys(ACM) 2,7]hCGRP α (O), rat amylin-NH $_2$ ($\blacksquare$) and sCT (∇). Data represent mean $\pm$ S.E.M. of at least three separate experiments, each performed in triplicate.

Table 3.1 Comparative affinities of hCGRP α , its fragment and analogs for specific [125 I-Tyr]hCGRP $_{8-37}$ binding sites in the rat brain.

	Rat brain			
	K_H (M) ^a	K_L (M) ^b	R_H (fmol/mg protein) ^c	R_L (fmol/mg protein) ^d
hCGRP α	$3.7 \times 10^{-10} \pm 0.9$	$3.2 \times 10^{-8} \pm 0.8$	12 ± 2	10 ± 2
hCGRP α_{8-37}	---	$3.9 \times 10^{-9} \pm 0.6$	---	27 ± 2
[Cys(ACM) ^{2,7}]hCGRP α	---	$1.2 \times 10^{-8} \pm 0.1$	---	26 ± 2
rat amylin-NH $_2$	$3.2 \times 10^{-9} \pm 0.7$	$3.4 \times 10^{-7} \pm 0.2$	9 ± 1	15 ± 1
sCT	---	$5.6 \times 10^{-6} \pm 0.9$	---	24 ± 3

^a High affinity binding constant

^b Low affinity binding constant

^c Amount of binding sites in high affinity

^d Amount of binding sites in low affinity

Values represent means $\pm$ S.E.M. determined from at least three separate experiments, each performed in triplicate.

Table 3.2 Comparative affinities of hCGRP α , its fragment and analogs for specific [125 I-Tyr]hCGRP $_{8-37}$ binding sites in the guinea pig atrium and vas deferens.

	Atrium (CGRP $_1$)				vas deferens (CGRP $_2$)			
	K $_H$ (M) ^a	K $_L$ (M) ^b	R $_H$ (fmol/mg protein) ^c	R $_L$ (fmol/mg protein) ^d	K $_H$ (M) ^a	K $_L$ (M) ^b	R $_H$ (fmol/mg protein) ^c	R $_L$ (fmol/mg protein) ^d
hCGRP α	4.0 x10 $^{-10}$ $\pm$ 0.4	5.6 x10 $^{-8}$ $\pm$ 1.8	21 $\pm$ 1	14 $\pm$ 0.1	2.5 x10 $^{-10}$ $\pm$ 0.6	9.4x10 $^{-9}$ $\pm$ 4	193 $\pm$ 41	247 $\pm$ 29
hCGRP α_{8-37}	---	1.1 x10 $^{-8}$ $\pm$ 0.2	---	35 $\pm$ 1	---	5.0x10 $^{-9}$ $\pm$ 0.6	---	415 $\pm$ 48
[Cys(ACM) 2,7]hCGRP α	---	2.4 x10 $^{-8}$ $\pm$ 0.5	---	33 $\pm$ 2	---	1.4x10 $^{-8}$ $\pm$ 0.2	---	403 $\pm$ 48
rat amylin-NH $_2$	---	1.1 x10 $^{-7}$ $\pm$ 0.2	---	31 $\pm$ 3	---	9.3x10 $^{-8}$ $\pm$ 1.8	---	280 $\pm$ 29
sCT	---	2.0 x10 $^{-6}$ $\pm$ 0.5	---	34 $\pm$ 0.1	---	1.2x10 $^{-6}$ $\pm$ 0.5	---	408 $\pm$ 65

^a High affinity binding constant

^b Low affinity binding constant

^c Amount of binding sites in high affinity

^d Amount of binding sites in low affinity

Values represent means $\pm$ S.E.M. determined from at least three separate experiments, each performed in triplicate.

3.4.3 Autoradiographic distribution of [125 I-Tyr]hCGRP₈₋₃₇ binding sites in rat brain

The distribution of [125 I-Tyr]hCGRP₈₋₃₇ binding sites was investigated using *in vitro* receptor autoradiography on coronal rat brain sections (Fig 3.4). Quantification of the data in various brain regions is shown in Fig 3.5. The highest densities (>2.5 fmol/mg tissue, wet wt.) of [125 I-Tyr]hCGRP₈₋₃₇ binding were present in the shell of the nucleus accumbens, caudate putamen (tail), amygdaloid body, pontine nuclei, cerebellum and inferior olive. Moderate densities (between 1.5-2.5 fmol/mg tissue, wet wt.) were observed in the piriform cortex, olfactory tubercle, nucleus accumbens core, habenular, medial geniculate, superior colliculus, temporal cortex, inferior colliculus, lateral lemniscus, vestibular nuclei, principal sensory trigeminal nucleus and spinal trigeminal nucleus. Low densities (<1.5 fmol/mg tissue, wet wt.) of [125 I-Tyr]hCGRP₈₋₃₇ labeling were located at the level of the frontal cortex, parietal cortex, caudate putamen, hilus of the dentate gyrus, red nucleus, substantia nigra, central gray, motor trigeminal nucleus and reticulotegmental nucleus of the pons. Nonspecific binding corresponded to ~15 % of total binding in the various brain areas except for nucleus accumbens, where nonspecific represented 28 % of total binding (Fig 3.4).

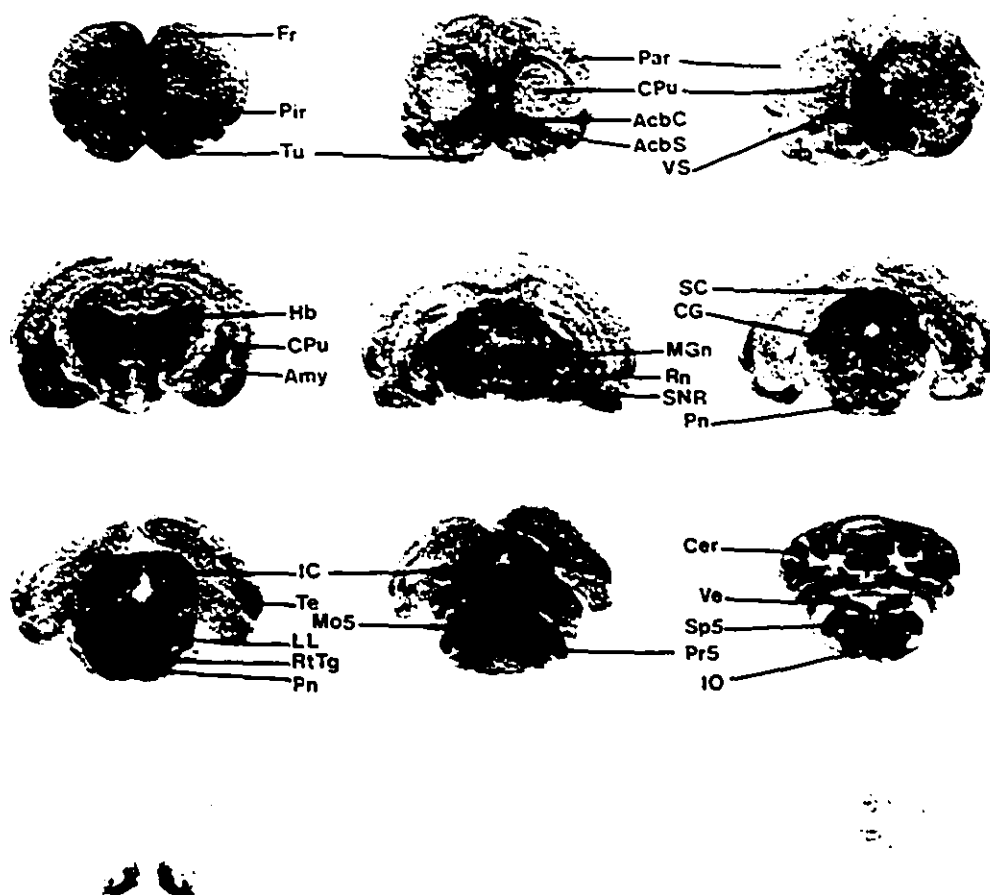


Figure 3.4 Photomicrographs of the autoradiographic distribution of [125 I-Tyr]hCGRP₈₋₃₇ (~40 pM) binding sites in coronal sections of the rat brain. Nonspecific binding was defined in the presence of 1 μ M hCGRP₈₋₃₇ on adjacent coronal sections and represented 15 to 28% of total binding (bottom row). *Abbreviations:* AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; Amy, amygdaloid body; CPu, caudate putamen; CG, central gray; Cer, cerebellum; Fr, frontal cortex; Hb, habenular nucleus; IC, inferior colliculus; IO, inferior olive; LL, lateral lemniscus; MGn, medial geniculate; Mo5, motor trigeminal nucleus; Par, parietal cortex; Pir, piriform cortex; Pn, pontine nuclei; Pr5, principal sensory trigeminal nucleus; Rn, red nucleus; RtTg, reticulotegmental nucleus of the pons; SC, superior colliculus; SNR, substantia nigra; Sp5, spinal trigeminal nucleus; Te, temporal cortex; Tu, olfactory tubercle; Ve, vestibular nuclei; VS, ventral striatum.

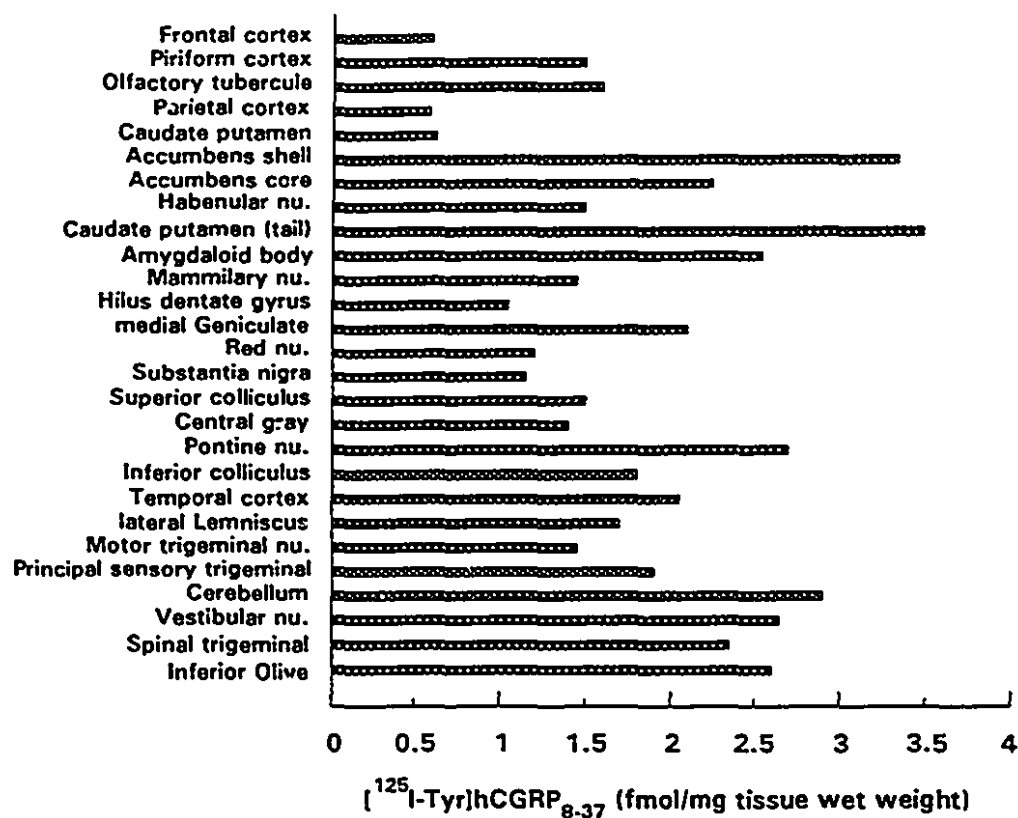


Figure 3.5 Quantitative autoradiographic analysis of specific [¹²⁵I-Tyr]hCGRP₈₋₃₇ (~40 pM) binding in selected areas of the rat brain. Values represent the mean of at least three different brains. Nonspecific binding was defined in the presence of 1 μM hCGRP₈₋₃₇ on adjacent coronal sections and represented 15 to 28% of total [¹²⁵I-Tyr]hCGRP₈₋₃₇ binding.

3.5 Discussion

The binding of [125 I-Tyr]hCGRP₈₋₃₇ to the guinea pig atrium (CGRP₁-enriched tissue), vas deferens (CGRP₂-enriched tissue) and the rat brain was saturable, and was best fitted to a single class of high affinity binding sites. The relative potencies of hCGRP α , hCGRP₈₋₃₇, [Cys(ACM)^{2,7}]hCGRP α , rat amylin-NH₂ and sCT to compete for [125 I-Tyr]hCGRP₈₋₃₇ binding were similar in all three membrane preparations. Furthermore, [125 I-Tyr]hCGRP₈₋₃₇ binding sites were widely distributed in the rat brain, with the highest densities of sites being observed in the nucleus accumbens, caudate putamen (tail), amygdaloid body, pontine nuclei, cerebellum and inferior olive. Thus, [125 I-Tyr]hCGRP₈₋₃₇ represents a new high affinity probe for the study of CGRP receptors in various tissues.

Saturation and competition experiments of [125 I-Tyr]hCGRP₈₋₃₇ binding by unlabelled hCGRP₈₋₃₇ revealed the existence of a single affinity site in all three tissue preparations. [125 I-Tyr]hCGRP₈₋₃₇ binding was also found to be insensitive to the presence of up to 100 μ M Gpp(NH)p in the incubation medium. This is in marked contrast to the well established sensitivity of the agonist radioligand, [125 I]hCGRP α , to GTP and its metabolically stable analogues (Umeda and Arisawa, 1989; Roa and Changeux, 1991; Chatterjee and Fisher, 1991; van Rossum *et al.*, 1993; see Poyner, 1992, for recent review). Thus in accordance with previous binding studies with antagonist radioligands of other receptor classes (Liang, 1989; Jackson *et al.*, 1992; Sinkins and Wells, 1993; Hunter *et al.*, 1993), [125 I-Tyr]hCGRP₈₋₃₇ binding was not affected by the GTP analogues, probably because of its ability to recognize the uncoupled state of the CGRP receptors and its relative insensitivity to the existence of multiple affinity states of the CGRP receptor complex. In this regard, it is of interest that the agonist hCGRP α revealed the existence of two affinity constants in competing for specific [125 I-Tyr]hCGRP₈₋₃₇ binding in all three membrane preparations of the present study. Similar results were obtained using [125 I]hCGRP α as agonist radioligand (Dennis *et al.*, 1990; van Rossum *et al.*, 1993). These two affinity constants may correspond either to two affinity states of a single receptor protein or to two distinct receptor classes. The former hypothesis appears more likely on the basis of recent results demonstrating the existence of multiple receptor affinity states sensitive to the addition of guanine nucleotides in these tissues (van Rossum *et al.*, 1993).

The highest quantity of [125 I-Tyr]hCGRP₈₋₃₇ binding sites was detected in the vas deferens (~450 fmol/mg protein), whereas lesser amounts were found in both atria and brain (~30 fmol/mg protein). A comparison of maximal binding capacities of the agonist

ligand, [125 I]hCGRP α (Dennis *et al.*, 1990; van Rossum *et al.*, 1993), reveals significantly lower values when [125 I-Tyr]hCGRP $_{8-37}$ is used as radioligand. This was somewhat unexpected; previous reports have generally shown higher apparent $B_{\max}$ values when antagonist vs. agonist ligands were compared, because the latter would preferentially label only the high affinity state of the receptor (Jarvis *et al.*, 1991; Jackson *et al.*, 1992). The significantly lower maximal amounts of binding sites observed here using [125 I-Tyr]hCGRP $_{8-37}$ may thus reflect the relative selectivity of this radioligand for the purported CGRP $_1$ receptor subtype as suggested by the results of functional *in vitro* and *in vivo* bioassays using the unlabelled fragment (Dennis *et al.*, 1990; Jolicœur *et al.*, 1992; Giuliani *et al.*, 1992; Quirion *et al.*, 1992; Stangl *et al.*, 1993).

Rat amylin-NH $_2$ is a 37 amino acid peptide showing ~50% amino acid sequence homology with CGRP (Nishi *et al.*, 1990). This peptide was able to compete for specific [125 I-Tyr]hCGRP $_{8-37}$ binding with rather low affinity (~100 nM) except in the rat brain, in which rat amylin-NH $_2$ revealed both high- and low-affinity binding constants for [125 I-Tyr]hCGRP $_{8-37}$ sites. Recently, high-affinity [125 I]amylin binding sites were reported in rat brain (Beaumont *et al.*, 1993). A few recent reports have shown that hCGRP $_{8-37}$ can potentially antagonize the hypotensive and tachycardiac effects induced by either amylin or CGRP (Gardiner *et al.*, 1991), their inhibitory activity on glycogen turnover in the soleus muscle (Deems *et al.*, 1991) and their positive inotropic actions in the isolated guinea pig atrium (Giuliani *et al.*, 1992). However, only relatively high concentrations (10-100 μ M) of this antagonist were able to block the action of amylin in liver (Morishita *et al.*, 1990) and skeletal muscle (Wang *et al.*, 1991) preparations. Furthermore, hCGRP $_{8-37}$ failed to antagonize amylin's actions on the isolated guinea pig urinary bladder and the rat vas deferens, two CGRP $_2$ receptor-enriched tissues (Giuliani *et al.*, 1992). It thus remains to be determined whether amylin acts mostly via CGRP receptors and/or through genuine amylin receptors such as those recently reported to be present in the lung (Bhogal *et al.*, 1992) and the brain (Beaumont *et al.*, 1993). The complex competition profile of amylin for [125 I-Tyr]hCGRP $_{8-37}$ binding in the rat brain revealed in the present study suggests that rat amylin-NH $_2$ can either differentiate two affinity states of the CGRP receptors or recognize two different populations of receptors that cannot be distinguished by [125 I-Tyr]hCGRP $_{8-37}$.

[125 I]hCGRP α /sCT-sensitive binding sites were reported to be present in various areas of the brain, especially in the nucleus accumbens (Sexton *et al.*, 1988; Dennis *et al.*, 1991; Quirion *et al.*, 1992). Because this peptide was only able to compete for [125 I-Tyr]hCGRP $_{8-37}$ binding with low affinity, it is unlikely that this new radioligand recognizes a significant proportion of the sCT receptors. It also further demonstrates the dissociation

between CGRP and CT receptor subtypes in most tissue preparations.

The quantitative autoradiographic distribution of [125 I-Tyr]hCGRP₈₋₃₇ sites is similar to that reported using the agonist radioligand [125 I]hCGRP α (Tschopp *et al.*, 1985; Skofitsch and Jacobowitz, 1985; Inagaki *et al.*, 1986; Sexton *et al.*, 1986; Quirion *et al.*, 1992) with [125 I-Tyr]hCGRP₈₋₃₇ binding concentrated in mesolimbic areas such as the nucleus accumbens and the amygdaloid body, in addition to medial geniculate, inferior olive, cerebellum and various brainstem nuclei. This distinctive distribution pattern observed throughout the brain suggests a role for CGRP and related peptides in the modulation of the activities of various limbic structures as well as of sensory and motor nuclei. Already, CGRP has been reported to modulate various centrally mediated behaviours, including nociception (Pecile *et al.*, 1987; Welch *et al.*, 1988; Jolicoeur *et al.*, 1992), motor activity (Krahn *et al.*, 1984; Jolicoeur *et al.*, 1992), body temperature (Dennis *et al.*, 1990; Jolicoeur *et al.*, 1992) and food intake (Krahn *et al.*, 1984; Dennis *et al.*, 1990; Jolicoeur *et al.*, 1992) as well as the release of neurotransmitters such as noradrenaline (Fisher *et al.*, 1983) and dopamine (Drumheller *et al.*, 1992); this is consistent with the high concentrations of [125 I-Tyr]hCGRP₈₋₃₇ sites in the nucleus accumbens and amygdaloid body. The fact that the distribution pattern of [125 I-Tyr]hCGRP₈₋₃₇ binding sites is similar to that of [125 I]hCGRP α in the rat brain suggests that hCGRP₈₋₃₇, on the basis of its antagonistic properties, should be useful in investigating the physiological role(s) of CGRP in the central nervous system.

In summary, [125 I-Tyr]hCGRP₈₋₃₇ represents a novel antagonist radioligand with high affinity for CGRP receptors in brain and peripheral tissues. Its unique features are related to its high affinity, low levels of nonspecific labeling, apparent insensitivity to GTP nucleotides and specific distribution pattern in the CNS. It should thus be a useful pharmacological tool to further characterize CGRP receptors present in a variety of tissues.

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CHAPTER 4

Autoradiographic distribution and receptor binding profile of [125I]Bolton Hunter-rat amylin binding sites in the rat brain

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4.0 Preface to Chapter 4

Islet amyloid polypeptide or amylin is a recently isolated peptide demonstrating about 50% sequence homology with CGRP. This peptide is currently generating much interest because of its potential role in the pathology in non-insulin dependent diabetes. A variety of biological effects have been reported for amylin both on peripheral tissues as well as in the brain. Until very recently, the existence of specific receptor sites for amylin was unknown. Moreover, as several biological actions of amylin are shared with those of CGRP, it has been proposed that amylin mostly acted through CGRP receptors to induce its effects. However, other data suggested the possible existence of unique population of amylin receptors. Therefore, we were especially interested in investigating the affinity of amylin for [125 I]hCGRP α binding sites present in brain and various peripheral tissues. Furthermore, we studied the possible existence of a unique class of receptor sites for amylin in rat brain. These data suggest the genuine existence of amylin receptor sites in the rat.

4.1 Abstract

Amylin is a recently isolated peptide from amyloid plaques in non-insulin dependent diabetic patients and showed high sequence homology with calcitonin gene-related peptide. We investigated the distribution and the binding profile of [125 I]Bolton Hunter-rat amylin ([125 I]BH-rat amylin) binding sites in the rat brain, as well as the affinity of rat amylin for [125 I]hCGRP α binding sites in the brain, atrium (CGRP $_1$ receptor-enriched tissue) and vas deferens (CGRP $_2$ receptor-enriched tissue). High amounts of high affinity [125 I]BH-rat amylin binding sites were observed in the nucleus accumbens, various hypothalamic nuclei, amygdaloid body, dorsal raphe, tegmental and parabrachial nuclei, and the locus coeruleus. Interestingly, both rat amylin and salmon calcitonin revealed low nanomolar affinities (2-19 nM) for [125 I]BH-rat amylin binding sites in the various brain areas whereas human calcitonin gene-related peptide- α (hCGRP α) showed lower affinities ranging between 13 to 150 nM. Moreover, the affinity of rat amylin was much lower than that of hCGRP α for [125 I]hCGRP α binding in the brain, atrium and vas deferens, except for very few areas such as the nucleus accumbens and ventral striatum. Similarly, rat amylin was much weaker (100- to 400- fold) than hCGRP α to induce a biological effect in the atrium and vas deferens. These results thus suggest the existence of unique [125 I]BH-rat amylin binding sites in the rat brain as well as limited cross-reactivity between rat amylin and [125 I]hCGRP α receptors present in the brain, atrium and vas deferens.

4.2 Introduction

Islet amyloid polypeptide or amylin was first isolated from amyloid deposits from human insulinoma (Westermarck *et al.* 1986; Westermarck *et al.* 1987) and from the pancreases of type 2 diabetic patients (Cooper *et al.* 1987; for review, Nishi *et al.* 1990; Betsholtz *et al.* 1993). This peptide subsequently revealed strong amyloidogenic properties (Westermarck *et al.* 1990). Amylin shows ~ 50% amino acid sequence homology with CGRP and likely shares a common ancestral gene with the CGRP/calcitonin family (Nishi *et al.* 1990). Amylin mRNA is found in high concentrations in the pancreas whereas lower levels are detected in the stomach, gastrointestinal tract, lung and dorsal root ganglia (Ferrier *et al.* 1989). Similarly, by using a specific radioimmunoassay, amylin-like immunoreactivity was detected in the pancreas, stomach and various parts of the gastrointestinal tract but failed at first to be detected in the central nervous system (Asai *et al.* 1990). Subsequently, however, Chance *et al.* (1991) detected picomolar amounts of the peptide in the hypothalamus whereas the content of amylin in other brain areas was below detectable levels. With respect to amylin receptors, Bhogal *et al.* (1992) reported the presence of specific [¹²⁵I]amylin binding sites in lung membrane preparations from various species as well as in the stomach, spleen, liver and various regions of the brain. However, no specific binding was detected in the pancreas, soleus muscle or kidney.

In the periphery, both amylin and CGRP are potent inhibitors of glucose uptake and glycogen synthesis in skeletal muscle *in vitro* (Leighton and Cooper, 1988; Deems *et al.* 1991b). Amylin also shares the hypocalcaemic (Datta *et al.* 1989; MacIntyre, 1989) and anorexic (Chance *et al.* 1991; Chance *et al.* 1992) properties of both calcitonin (Freed *et al.* 1979; Yamamoto *et al.* 1982; Fischer and Born, 1985) and CGRP (Krahn *et al.* 1984; Roos *et al.* 1986). On the basis of the similarities between the biological actions of amylin and CGRP, several reports have focused on the possible existence of a common receptor for these two peptides in a variety of preparations including the liver (Morishita *et al.* 1990; Galeazza *et al.* 1991; Zhu *et al.* 1991; Chantry *et al.* 1991), skeletal muscle (Galeazza *et al.* 1991; Chantry *et al.* 1991), central nervous system (Galeazza *et al.* 1991; Zhu *et al.* 1991) and L6 myocytes (Zhu *et al.* 1991; Poyner *et al.* 1992). However, in

most preparations amylin was found to be 30 to 100 fold less effective than CGRP in competing for specific [125 I]CGRP binding. Furthermore, the actions of amylin could not be explained fully by the activation of CGRP receptors in L6 myocytes (Poyner *et al.* 1992; Kreutter *et al.* 1993). It thus remains to be determined if amylin acts exclusively via CGRP receptors and/or through a unique amylin receptor class as reported in lung membranes (Bhagal *et al.* 1992) and possibly the nucleus accumbens (Beaumont *et al.* 1993), to produce its biological effects.

The major objective of the present study was thus to investigate the existence of unique amylin receptors in the brain. We therefore examined the existence and distribution of binding sites for [125 I]BH-rat amylin as well as its selectivity profile in the rat brain. Furthermore, the potential cross-reactivity between CGRP and amylin sites was examined in rat brain sections. Finally, the affinity and biological activity of amylin were investigated in the electrically stimulated atrium and vas deferens. These two tissues were chosen on the basis of their enrichment with CGRP₁ and CGRP₂ receptor subtypes, respectively (Dennis *et al.* 1989; Dennis *et al.* 1990; Dennis *et al.* 1991; Giuliani *et al.* 1992). The CGRP₁ subtype is defined by its preferential antagonism by C-terminal fragments such as CGRP₈₋₃₇ and CGRP₉₋₃₇ whereas the linear fragment [Cys(ACM)^{2,7}]hCGRP α has preferential agonistic effects on the CGRP₂ receptor subtype (Mimeault *et al.* 1991; for review, Quirion *et al.* 1992; Stangl *et al.* 1993).

4.3 Materials and Methods

4.3.1 Materials

Rat amylin-NH₂, hCGRP α and [Tyr⁰]hCGRP α were synthesized in our laboratories as previously described in detail (Mimeault *et al.* 1992). Rat amylin-NH₂ also was purchased from Bachem California (Torrance, California). [¹²⁵I]BH-rat amylin (2000 Ci/mmol) and (2-[¹²⁵I]iodohistidyl¹⁰)-hCGRP α (2000 Ci/mmol) were purchased from Amersham Canada (Oakville, Ontario, Canada). Na-iodine (Na-¹²⁵I) was obtained from ICN Canada (Mississauga, Ontario, Canada). Trisma-base, NaCl, bacitracin, leupeptin and chymostatin were supplied by Sigma Chemicals Co. (St-Louis, MO) and BSA by Boehringer Mannheim Canada (Laval, Québec, Canada). Glass fibre filters #32 were purchased from Schleicher and Schuell (Keene, NH). Other chemicals were of analytical grade and were obtained from Fisher Scientific (Montréal, Québec, Canada).

Male Sprague-Dawley rats (225-250 g) and male Hartley guinea pigs (325-375 g) were obtained from Charles River (St-Constant, Québec, Canada). They had free access to food and water and were kept according to the guidelines of the Canadian Council for Animal Care and McGill University.

4.3.2 Iodination of hCGRP α

[Tyr⁰]hCGRP α was iodinated by using a modified chloramine-T/Na metabisulfite method (Hunter and Greenwood, 1962). Briefly, [Tyr⁰]hCGRP α was added to phosphate buffer 0.25 M, pH 7.5, Na-¹²⁵I and chloramine-T. After 1 min, the reaction was stopped by adding Na-metabisulfite to the mixture. After pre-purification on an anion exchange sep-pak (Accell QMA cartridge, Millipore), the radiolabeled peptide was further purified by high-performance liquid chromatography by using a C-18 reversed-phase column (LKB-Ultropac) and a 10 to 70% (30 min) acetonitrile-0.06% trifluoroacetic acid gradient. Thirty-second fractions were collected and the radioactive peak was aliquoted and stored at -20°C until use.

4.3.3 [¹²⁵I]BH-rat amylin and [¹²⁵I]hCGRP α receptor autoradiography

Brain tissues were snap-frozen in 2-methylbutane (-40°C) and stored at -80°C until use. Serial coronal sections (20 μ m) were cut and thaw-mounted onto gelatin coated

microscope slides. Tissue sections were then desiccated overnight at 4°C and stored at -80°C until experiments were performed.

Binding profile of [¹²⁵I]BH-rat amylin in rat brain was obtained by pre-incubating adjacent coronal rat brain sections for 15 to 20 min in 50 mM Tris-HCl buffer, 100 mM NaCl and 4 mM MgCl₂, pH 7.4, followed by a 60-min incubation in fresh buffer containing 35 pM [¹²⁵I]BH-rat amylin containing 0.2% BSA, 0.4 mM bacitracin, 4 µg/ml of leupeptin and 2 µg/ml of chymostatin and increasing concentrations (1-1000 nM) of either rat amylin-NH₂, sCT or hCGRPα. The sections were then washed in ice cold buffer (4 x 2 min) and dipped twice in cold distilled water. Nonspecific binding was evaluated on adjacent sections incubated with 1 µM of rat amylin-NH₂ and corresponded to ~40% of total binding. Radiolabeled tissue sections were exposed to highly sensitive film (Hyperfilm, Amersham Canada, Ontario, Canada) for 18 days. Thereafter, specific binding in various brain regions was quantified by using commercially available standards and computerized image analysis system (MCID System, Image Research Inc., Ste-Catharines, Ontario, Canada). A similar protocol was used for [¹²⁵I]hCGRPα binding except for the following: after a 15 min pre-incubation, coronal rat brain sections were incubated with either 25 pM [¹²⁵I]hCGRPα or ~25 pM [¹²⁵I-Tyr⁰]hCGRPα for 90 min. Nonspecific binding to adjacent tissue sections was defined in the presence of 1 µM hCGRPα and represented less than 10% of total [¹²⁵I]hCGRPα binding and ~25% of [¹²⁵I-Tyr⁰]hCGRPα labeling. The radiolabeled tissue sections were exposed to Hyperfilm for 5 days and quantified as above.

4.3.4 [¹²⁵I]hCGRPα membrane binding

Animals were sacrificed by decapitation. Atria and *vasa deferentia* were removed rapidly and placed in 15x volume of 25 mM Tris-HCl, 50 mM NaCl and 2 mM MgCl₂ at pH 7.4. Tissues were homogenized by using Brinkman polytron and centrifuged at 48 000 x g for 20 min at 4°C. Pellets were washed and suspended in the original volume of buffer and re-centrifuged. This step was repeated twice. The crude membranes [150 µg and 15 µg of protein from atria and *vasa deferentia*, respectively, as measured by the method of Lowry *et al.* (1951)] were incubated for 1 hour at room temperature in a final volume of 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 4 mM MgCl₂, 0.2% BSA, 0.4 mM bacitracin, 40 pM [¹²⁵I]hCGRPα and increasing concentrations of either hCGRPα or rat amylin-NH₂ (10⁻¹² to 10⁻⁶ M). Incubations were terminated by rapid filtration under reduced pressure by using a Brandel Cell Harvester (Model M24-R, Gaithersburg, MD) through glass fibre filters pre-soaked in 0.1 % polyethyleneimine. Nonspecific

binding was defined in the presence of an excess of hCGRP α (1 μ M) and represented between ~30% of total binding depending upon the tissue preparation.

4.3.5 *In vitro* bioassays

Animals were sacrificed by decapitation. Guinea pig left atrium and rat vas deferens (*pars prostatica*) were removed quickly and placed in oxygenated Krebs-Ringer's solution. Tissues were then prepared for isometric tension recording, as described previously (Dennis *et al.* 1989). In brief, a resting tension of 1 g was applied to all tissue preparations and an equilibration period of 1.5 h was allowed before electrical stimulation was applied. The atria and *vasa deferentia* were driven electrically at maximal voltage and frequencies of 3 Hz (0.3 ms) and 0.12 Hz (0.5 ms), respectively. Concentration-response curves of hCGRP α or rat amylin-NH₂ were determined by increasing bath concentrations applied in a cumulative manner by using previously reported methods that have revealed the absence of tachyphylaxis in these tissues (Dennis *et al.* 1989). Concentrations up to 5 μ M of rat amylin-NH₂ were tested in the guinea pig atria, followed by the addition (without wash) of 0.1 μ M hCGRP α to evaluate the maintenance of the maximal response of this tissue and the full effectiveness of hCGRP α under these conditions.

4.3.6 Data analysis

IC₅₀ values (i.e. concentration of the peptide required to compete for 50% of specific binding of the radioligand) of the various peptides were calculated in autoradiographic binding studies by using nonlinear regression by GraphPAD InPlot which quantitate goodness of fit by the method of Marquardt (1963) with modification of Tabata and Ito (1975). Values are expressed as mean $\pm$ S.E.M. from at least 3 brains. The analysis of the competition curves obtained from membrane binding assays was performed using the LUNDON-2 Competition Data Analysis program which is based on a modified version of the Cheng-Prusoff method. Analysis of variance was used to compare the goodness of fit between the one- and two-site models. If the analysis of variance yielded a probability value greater than 0.05, the less complex model was accepted as the model which best fitted the data. Values represent the mean $\pm$ S.E.M. of at least three separate experiments each performed in triplicate. Finally, EC₅₀ values from *in vitro* bioassays were computed using nonlinear regression by GraphPAD InPlot (see above). Data are expressed as mean $\pm$ S.E.M. of 4 to 10 individual preparations. Student's *t* test was performed to compare peptide affinities and activities in the various preparations.

4.4 Results

4.4.1 Autoradiographic distribution and binding profile of [125 I]BH-rat amylin binding sites in rat brain

The distribution of [125 I]BH-rat amylin binding sites in coronal rat brain sections is shown in Figure 4.1 and quantified in Table 4.1. Highest amounts of specific [125 I]BH-rat amylin labeling (> 7 fmol/mg of tissue, wet weight) were observed in the nucleus accumbens, fundus striati, central and medial amygdaloid nuclei, amygdalostriatal transition zone, dorsal raphe and locus coeruleus. Moderate levels of specific binding (between 5-7 fmol/mg of tissue, wet weight) were quantified in the medial preoptic area and dorsomedial and arcuate hypothalamic nuclei. Low levels of binding (< 5 fmol/mg of tissue, wet weight) were detected in the ventromedial hypothalamic nucleus, the medial tuberal nucleus, the laterodorsal tegmental and the lateral parabrachial nuclei. Significant amounts of specific [125 I]BH-rat amylin binding failed to be detected in most cortical areas, hippocampus and cerebellum (Figure 4.1).

Residual [125 I]BH-rat amylin binding in the presence of increasing concentrations (1-1000 nM) of either rat amylin-NH₂, sCT or hCGRP α is shown in Figure 4.2. The apparent affinity of rat amylin-NH₂ and sCT for [125 I]BH-rat amylin was similar in all brain regions whereas hCGRP α demonstrated 5- to 35- fold lower affinity (Table 4.1). In brain areas also known to be enriched in [125 I]hCGRP α binding such as the nucleus accumbens and the amygdaloid body (Dennis *et al.* 1991; Quirion *et al.* 1992), hCGRP α competed with greater affinity for [125 I]BH-rat amylin sites than in areas not containing significant amounts of [125 I]hCGRP α binding such as in various hypothalamic nuclei (Table 4.1).

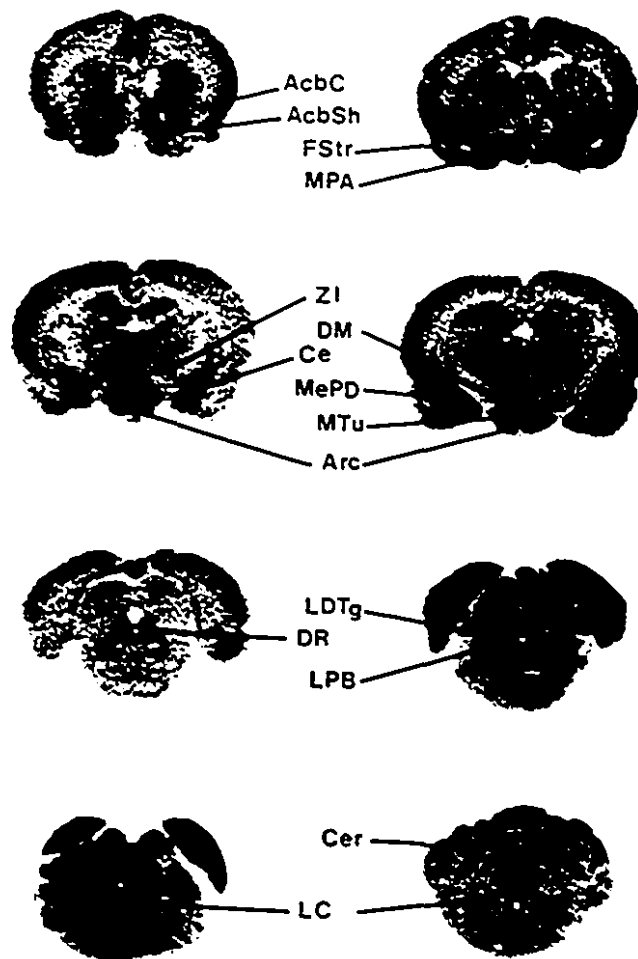


Figure 4.1 Photomicrographs of the autoradiographic distribution of $[^{125}\text{I}]\text{BH-rat amylin}$ binding sites in coronal sections of the rat brain. Sections were incubated with 35 pM $[^{125}\text{I}]\text{BH-rat amylin}$ and nonspecific binding was determined in the presence of 1 μM amylin on adjacent sections. *Abbreviations:* AcbC, nucleus accumbens, core; AcbSh, nucleus accumbens, shell; Arc, arcuate hypothalamic nucleus; Ce, central amygdala nucleus; Cer, cerebellum; DM, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; FStr, fundus striati; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LPB, lateral parabrachial nucleus; MePD, medial amygdaloid nucleus, posterodorsal; MPA, medial preoptic area; MTu, medial tuberal nucleus; ZI, zona incerta.

4.4.2 Competition of $[^{125}\text{I}]\text{hCGRP}\alpha$ binding by rat amylin- NH_2 in various areas of the rat brain

The quantitative analysis of residual $[^{125}\text{I}]\text{hCGRP}\alpha$ binding seen in the presence of increasing concentrations of rat amylin- NH_2 (1-1000 nM) is shown in Figure 4.4 and Table 4.2. Interestingly, rat amylin- NH_2 was highly potent in competing for specific

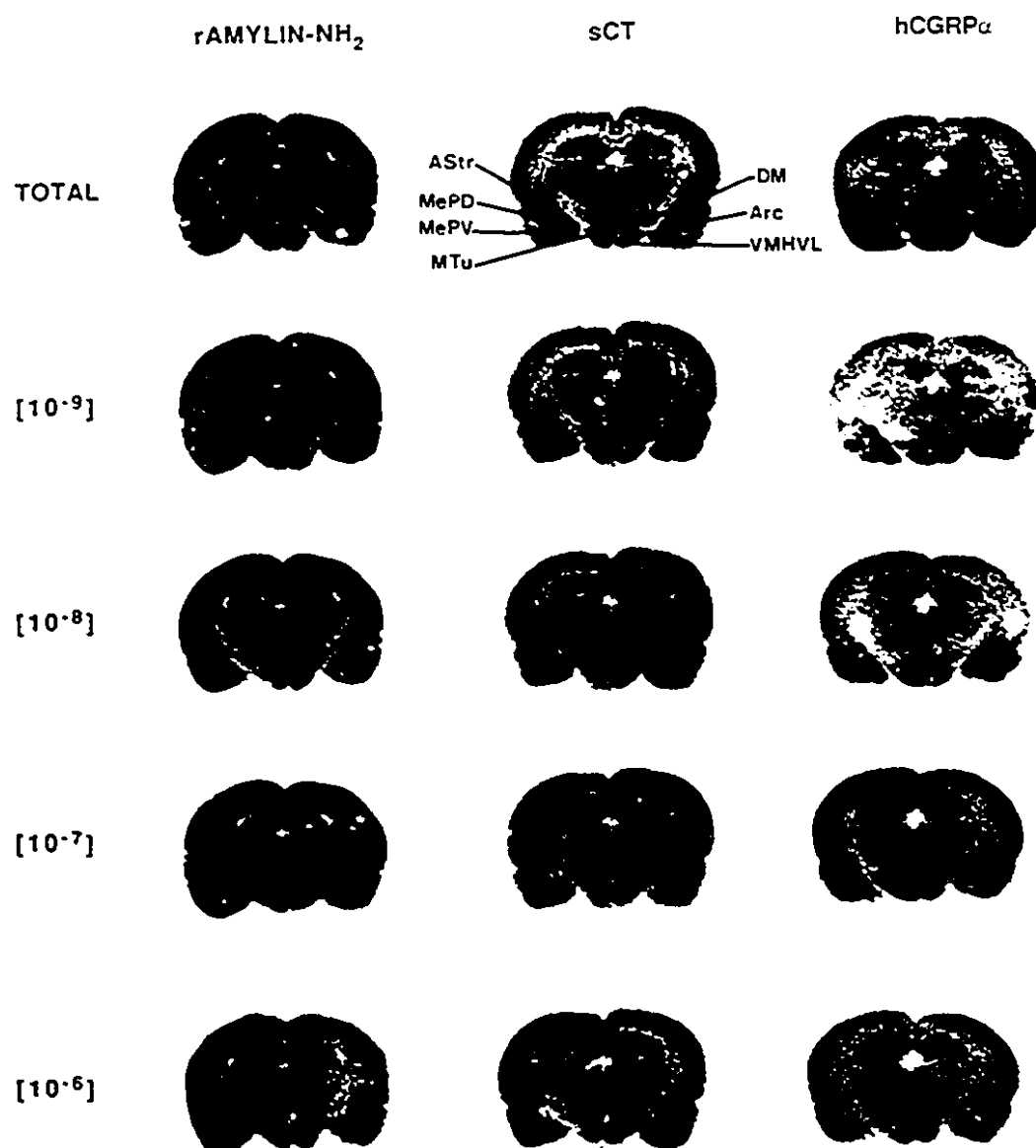


Figure 4.2 Photomicrographs of the competition profiles of rat amylin-NH₂, sCT and hCGRPα for specific [¹²⁵I]BH-rat amylin-NH₂ in rat brain. Nonspecific binding was defined as [¹²⁵I]BH-rat amylin-NH₂ binding in the presence of 1 μM of rat amylin-NH₂. *Abbreviations:* Arc, arcuate hypothalamic nucleus; AStr, amygdalostratial transition zone; DM, dorsomedial hypothalamic nucleus; MePD, medial amygdaloid nucleus, posterodorsal; MePV, medial amygdaloid nucleus, posteroventral; MTu, medial tuberal nucleus; VMHVL, ventromedial hypothalamic nucleus, ventrolateral.

Table 4.1 Quantitative autoradiographic analysis and comparative affinities of amylin and related peptides for specific [125 I]BH-rat amylin binding in selected rat brain areas.

	Total	IC ₅₀ (nM)*		
	(fmol/mg tissue, wet weight)	rat amylin	sCT	hCGRP α
Accumbens n. (shell)	9.1 $\pm$ 0.5	18.7 $\pm$ 1.2	16.7 $\pm$ 1.1	130 $\pm$ 12
Accumbens n. (core)	7.4 $\pm$ 0.5	7.8 $\pm$ 1.4	9.2 $\pm$ 1.0	25 $\pm$ 1
Olf. tubercle	7.9 $\pm$ 0.6	1.5 $\pm$ 1.0	4.7 $\pm$ 1.2	17 $\pm$ 2
Fundus striati	8.5 $\pm$ 1.2	14.5 $\pm$ 1.2	8.7 $\pm$ 1.1	100 $\pm$ 13
Medial preoptic area	6.1 $\pm$ 1.0	13.2 $\pm$ 1.3	5.3 $\pm$ 1.4	77 $\pm$ 3
Central amygdala n.	7.0 $\pm$ 0.6	3.2 $\pm$ 1.0	4.5 $\pm$ 1.8	29 $\pm$ 1
Dorsomedial hypoth. n.	6.2 $\pm$ 0.4	3.6 $\pm$ 1.2	5.1 $\pm$ 1.1	150 $\pm$ 12
Arcuate hypoth. n.	5.0 $\pm$ 0.2	2.3 $\pm$ 1.2	4.2 $\pm$ 1.4	118 $\pm$ 12
Ventromedial hypoth. n., ventrolateral	4.8 $\pm$ 0.4	2.9 $\pm$ 1.2	3.3 $\pm$ 1.4	53 $\pm$ 2
Medial tuberal n.	4.5 $\pm$ 0.8	3.8 $\pm$ 1.3	4.3 $\pm$ 1.4	24 $\pm$ 1
Amygdalostratial transition zone	8.3 $\pm$ 0.5	3.6 $\pm$ 1.1	4.7 $\pm$ 1.8	14 $\pm$ 1
Medial amygdaloid n., posterodorsal	7.1 $\pm$ 0.5	2.9 $\pm$ 1.1	3.1 $\pm$ 1.3	14 $\pm$ 1
Medial amygdaloid n., posteroventral	6.9 $\pm$ 1.2	2.2 $\pm$ 1.2	3.2 $\pm$ 1.6	13 $\pm$ 1
Dorsal raphe n.	7.3 $\pm$ 1.1	5.8 $\pm$ 2.6	3.5 $\pm$ 1.2	38 $\pm$ 2
Laterodorsal tegmental n.	3.8 $\pm$ 0.2	3.2 $\pm$ 1.2	4.6 $\pm$ 1.1	24 $\pm$ 1
Lateral parabrachial n.	4.4 $\pm$ 0.7	4.2 $\pm$ 1.0	3.7 $\pm$ 1.2	75 $\pm$ 12
Locus coeruleus	7.3 $\pm$ 1.9	9.1 $\pm$ 1.0	2.8 $\pm$ 1.2	100 $\pm$ 13

*IC₅₀, the concentration of peptide required to compete for 50% of specific [125 I]BH-rat amylin binding. Adjacent rat brain coronal sections were incubated with either 35 pM [125 I]BH-rat amylin alone or with increasing concentrations (1-1000 nM) of the respective competitors. Nonspecific was defined as [125 I]BH-rat amylin binding in the presence of 1 μ M of rat amylin-NH₂. Data represent mean $\pm$ S.E.M. of at least three separate brains.

[¹²⁵I]hCGRPα binding in two regions only (Figures 4.3 and 4.4), namely the nucleus accumbens and the ventral striatum which are also enriched in [¹²⁵I]BH-rat amylin sites (Figure 4.1). All other brain areas examined were much less sensitive to rat amylin-NH₂ with IC₅₀ values significantly above 100 nM (Figure 4.4, Table 4.2).

4.4.3 Comparative affinities of rat amylin-NH₂ and hCGRPα for [¹²⁵I]hCGRPα binding in CGRP₁ and CGRP₂ enriched membrane homogenates

The comparative affinities of rat amylin-NH₂ and hCGRPα for specific [¹²⁵I]hCGRPα binding in membrane homogenates enriched with the CGRP₁ (atrium) and CGRP₂ (vas deferens) receptor subtypes was investigated next. Rat amylin-NH₂ was able to compete, in a concentration-dependent manner, for [¹²⁵I]hCGRPα binding in these two tissues. Rat amylin-NH₂ showed ~200-fold lower affinity than previously reported hCGRPα affinity values for specific [¹²⁵I]hCGRPα binding in atrium (K_H: 2.7 × 10⁻¹⁰) and vas deferens (K_H: 2.1 × 10⁻¹⁰) (van Rossum *et al.* 1993). However, rat amylin-NH₂ appeared to compete more potently (P<0.05) for the [¹²⁵I]hCGRPα binding in a CGRP₂ (K_D 45 ± 1 nM) over a CGRP₁ (K_D 61 ± 4 nM) enriched membrane preparations.

4.4.4 *In vitro* biological activity in CGRP₁ and CGRP₂ preparations

To further determine if rat amylin-NH₂ shows preferential affinity for a CGRP receptor subtype in functional assays, the comparative effects of hCGRPα, hCGRPβ and rat amylin-NH₂ on the electrically stimulated guinea pig left atrium (prototypical CGRP₁ assay) and rat vas deferens (prototypical CGRP₂ assay) were investigated. These three peptides induced a concentration-dependent positive inotropic effect in the guinea pig atria (Table 4.3). However, rat amylin-NH₂ was markedly less potent than hCGRPα showing ~400-fold lower affinity and reaching only 60% of the maximal effect induced by hCGRPα. hCGRPβ revealed similar maximal effect but with ~20-fold lower affinity than hCGRPα. In the rat vas deferens, hCGRPα, hCGRPβ and rat amylin-NH₂ produced a concentration-dependent inhibition of the electrically evoked twitch response (Table 4.3). In this preparation, rat amylin-NH₂ was ~100-fold less potent than hCGRPα. The hCGRPβ also showed lower affinity (6-fold) than hCGRPα in the rat vas deferens. All peptides including rat amylin-NH₂, were able to fully inhibit the electrically evoked twitch response in the rat vas deferens, suggesting preferential affinity of rat amylin-NH₂ for the CGRP₂ vs. CGRP₁ receptor subtype, albeit with much lower potencies than CGRP and related homologue.

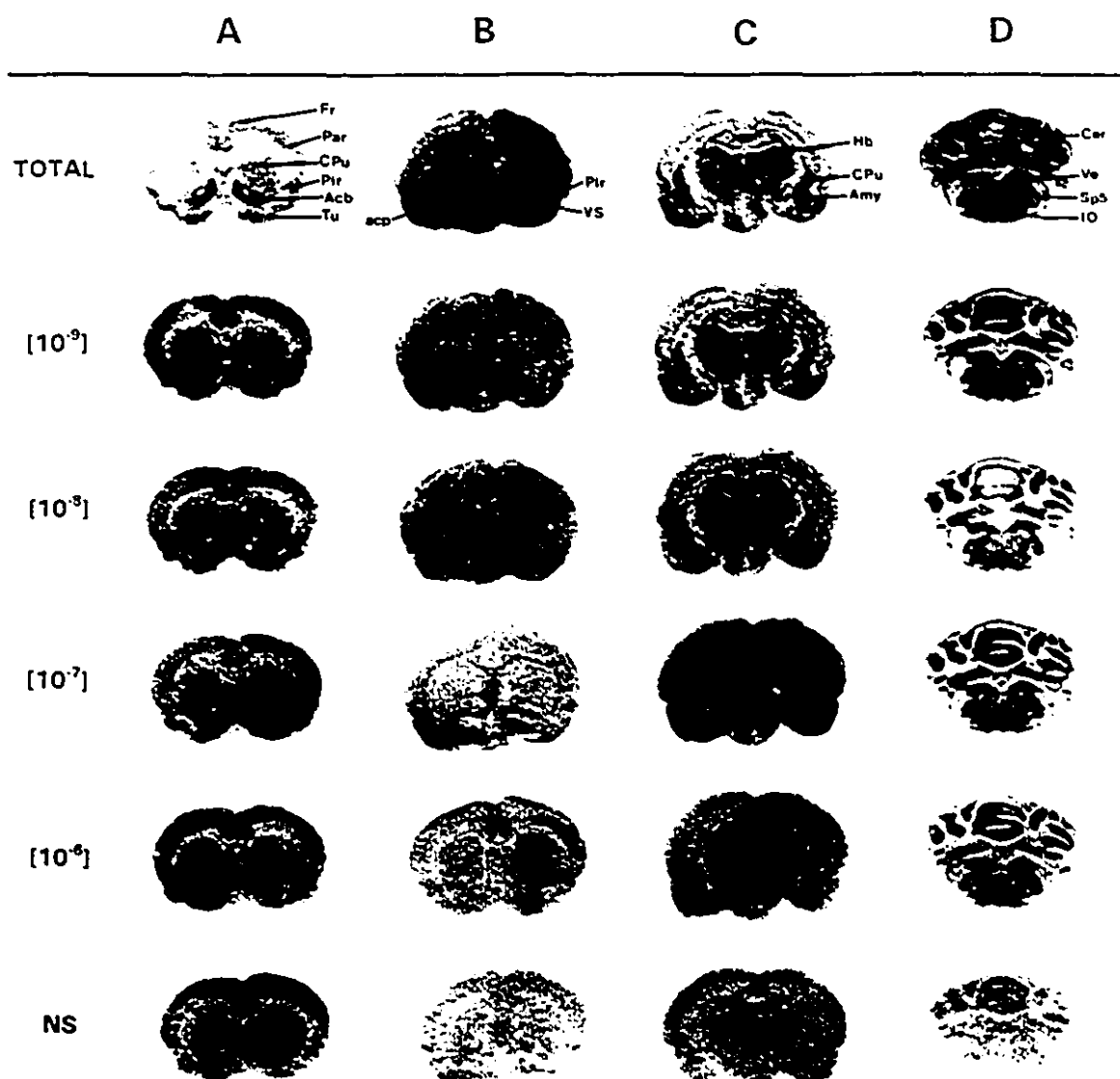


Figure 4.3 Photomicrographs of the autoradiographic distribution of $[^{125}\text{I}]\text{hCGRP}\alpha$ binding sites in the presence of increasing concentrations (1-1000 nM) of rat amylin- NH_2 at four different levels in the rat brain. Nonspecific binding was determined by the addition of 1 μM hCGRP α . *Abbreviations:* Fr, Frontal cortex; Par, Parietal cortex; CPu, Caudate Putamen; Pir, Piriform cortex; Acb, nucleus Accumbens; Tu, Olfactory tubercle; VS, Ventral striatum; acp, anterior commissure posterior; Amy, Amygdala nuclei; Cer, Cerebellum; Ve, Vestibular nuclei; Sp5, Spinal trigeminal nucleus; IO, Inferior olive.

Table 4.2 Affinities of rat amylin-NH₂ for [¹²⁵I]hCGRP α binding in various areas of the rat brain.

Brain areas	IC ₅₀ (nM)*
Frontal Cortex	163 $\pm$ 25
Parietal Cortex	110 $\pm$ 45
Piriform Cortex	128 $\pm$ 41
Olfactory Tubercle	126 $\pm$ 34
Tenia Tecta	238 $\pm$ 30
Accumbens nucleus (Shell)	50 $\pm$ 13
Caudate-Putamen	183 $\pm$ 40
Ventral Striatum	18 $\pm$ 10
Amygdaloid nuclei	233 $\pm$ 37
Caudate-Putamen (Tail)	228 $\pm$ 35
Temporal Cortex	275 $\pm$ 62
Medial Geniculate	184 $\pm$ 60
Superior Colliculus	150 $\pm$ 32
Substantia Nigra	235 $\pm$ 64
Pontine nucleus	166 $\pm$ 57
Inferior Colliculus (External Cortex)	168 $\pm$ 54
Cerebellum	167 $\pm$ 24
Vestibular nuclei	170 $\pm$ 30
Motor Trigeminal nucleus	195 $\pm$ 5
Spinal Trigeminal nucleus	330 $\pm$ 79
Inferior Olive	193 $\pm$ 24

* IC₅₀ represents the concentration of rat amylin-NH₂ required to compete for 50% of specifically bound [¹²⁵I]hCGRP α . Data are expressed in nanomolar and represent means $\pm$ S.E.M. of at least four different brains. Nonspecific binding was determined in the presence of 1 μ M unlabeled hCGRP α and corresponded to ~25% of total binding.

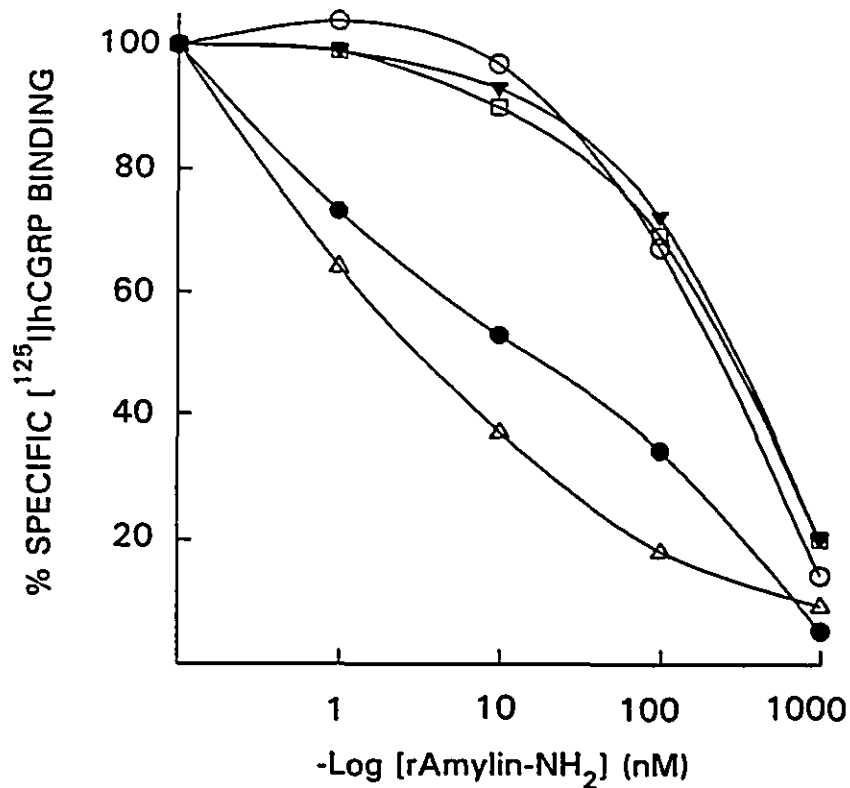


Figure 4.4 Competition profiles of rat amylin-NH₂ for [¹²⁵I]hCGRPα binding in various regions of the brain: ●, nucleus accumbens; Δ, ventral striatum; □, caudate putamen (tail); ▼, amygdaloid body and ○, cerebellum. Adjacent coronal brain sections were incubated with 25 pM [¹²⁵I]hCGRPα and increasing concentrations of rat amylin-NH₂ (1-1000 nM). Nonspecific binding was evaluated in the presence of 1 μM hCGRPα and corresponded to ~10% of total binding.

Table 4.3 Comparative potency of hCGRP α , hCGRP β and rat amylin-NH $_2$ on CGRP $_1$ and CGRP $_2$ representative *in vitro* bioassays.

	Guinea pig atrium (CGRP $_1$)			Rat vas deferens(CGRP $_2$)		
	EC $_{50}$ (nM)*	RP(%)	E $_{max}$ (%)	EC $_{50}$ (nM)*	RP(%)	E $_{max}$ (%)
hCGRPα	6.7 $\pm$ 0.4	100	100	1.9 $\pm$ 0.3	100	100
hCGRPβ	152 $\pm$ 14	4.4	100	11 $\pm$ 3	17	100
rat amylin-NH$_2$	2550 $\pm$ 790	0.3	59 $\pm$ 2	180 $\pm$ 55	1.1	100

* EC $_{50}$, the concentration required to produce 50% of the maximal response and is expressed as mean $\pm$ S.E.M. of data obtained from 4 to 10 individual preparations. RP, the calculated potency compared to hCGRP α . E $_{max}$ corresponds to the percentage of the maximal response observed with hCGRP α .

4.5 Discussion

The present study demonstrates the existence of specific, high affinity, saturable [^{125}I]BH-rat amylin binding sites in few areas of the rat brain including the nucleus accumbens, some hypothalamic nuclei, amygdaloid body, dorsal raphe, tegmental and parabrachial nuclei and the locus coeruleus. Both rat amylin-NH₂ and sCT revealed nanomolar affinities for these specific [^{125}I]BH-rat amylin binding sites whereas hCGRP α showed 5 to 35 times lower affinity depending upon the region examined. Furthermore, rat amylin-NH₂ competed with rather low affinity for [^{125}I]hCGRP α binding sites in the brain, atrium (CGRP₁ enriched tissue) and vas deferens (CGRP₂ enriched tissue). Exceptions were observed in the nucleus accumbens and ventral striatum; two regions in which rat amylin-NH₂ demonstrated nanomolar affinities for specific [^{125}I]hCGRP α binding. In addition, rat amylin-NH₂ was clearly less potent than hCGRP α to induce a positive inotropic effect in the isolated guinea pig atria and to inhibit the *in vitro* contraction of the electrically stimulated rat vas deferens, two prototypical CGRP bioassays (Dennis *et al.* 1989; Quirion *et al.* 1992).

By using *in vitro* receptor autoradiography, we were able to detect the existence of specific [^{125}I]BH-rat amylin binding sites in various nuclei of the basal ganglia, diencephalon and brainstem. As shown in Figure 4.1, levels of specific [^{125}I]BH-rat amylin binding varied between the various zones of the basal ganglia with the highest levels in the shell of the nucleus accumbens, followed by fundus striati, olfactory tubercle and core of the nucleus accumbens. Interestingly, in the caudate putamen, only the most ventral part showed specific [^{125}I]BH-rat amylin labeling (Figure 4.1). Despite multiple shared features between the caudate putamen, accumbens and olfactory tubercle, significant differences have been reported as to their respective connectivity, morphology and chemical neuroanatomy (Groenewegen and Russchen, 1984; Zaborszky *et al.* 1985; Voorn *et al.* 1989; Meredith *et al.* 1992; for review, Zahm and Brog, 1992). The unique distribution of [^{125}I]BH-rat amylin binding in these structures further supports the high differentiation of the basal ganglia and may be relevant to the role of this peptide in the brain.

High amounts of [^{125}I]BH-rat amylin binding were also observed in several nuclei of the hypothalamus with the highest levels present in the medial preoptic and dorsomedial nuclei. Bidirectional projections have been demonstrated between the medial preoptic nucleus and diverse areas of the brainstem including the parabrachial and laterodorsal tegmental nuclei and the locus coeruleus (Swanson, 1987). Similar pathways also exist

between the dorsomedial nucleus and certain brainstem nuclei believed to play a role in the relay of taste and/or visceral sensory information (Swanson, 1987) and hence may suggest a role for amylin in these behaviours. A lower level of [125 I]BH-rat amylin binding was detected in the ventrolateral part of the ventromedial nucleus of the hypothalamus. In contrast, its dorsomedial part was devoid of [125 I]BH-rat amylin labeling supporting functional differences between these two areas of the ventromedial nucleus (Swanson, 1987). Furthermore, massive inputs from the amygdaloid body to the ventromedial nucleus have been identified with the posterior part of the medial nucleus of the amygdala particularly innervating the ventrolateral portion of the nucleus (Swanson, 1987). These projections are in a position to relay olfactory information to the ventromedial nucleus. On the other hand, this nucleus has a widespread network of efferents including some to the amygdala and various brainstem nuclei. The pathway from the ventromedial nucleus to the central nucleus of the amygdala may be of particular importance as the latter projects massively to parasympathetic sensory and motor nuclei of the brainstem (Swanson, 1987). Indeed, the amygdaloid complex can be seen as a major interface between hypothalamic and brainstem areas involved in the regulation of endocrine and autonomic functions (Price *et al.* 1987). The presence of [125 I]BH-rat amylin in the ventromedial nucleus of the hypothalamus may thus be relevant in that regard.

The dorsal raphe and locus coeruleus are highly enriched with specific [125 I]BH-rat amylin binding sites. The dorsal raphe projects throughout the brain and particularly to the medial preoptic nucleus, caudate putamen, locus coeruleus and the parabrachial nuclei (Tork, 1985). Reciprocally, afferents to the dorsal raphe arise from the locus coeruleus, laterodorsal tegmental nucleus and the parabrachial nuclei (Tork, 1985). Additionally, afferents and efferents of the locus coeruleus include reciprocal projections to the dorsomedial nucleus of the hypothalamus, central nucleus of the amygdala as well as to the dorsal raphe (Loughlin and Fallon, 1985). The purported roles of the locus coeruleus are diverse and include the modulation of pain, various limbic activities and neuroendocrine systems (Loughlin and Fallon, 1985). The enrichment of [125 I]BH-rat amylin sites in these areas are likely of functional significance.

Taken together, the discrete distribution of [125 I]BH-rat amylin binding throughout the rat brain thus suggests that this peptide might play a role in the modulation of food and water intakes, the control of body temperature as well as in various sensory, neuroendocrine and autonomic functions. Indeed, amylin already was reported to have anorexic and adipic effects upon its injection into the hypothalamus (Chance *et al.* 1991; Chance *et al.* 1992) as well as to alter the metabolism of dopamine and serotonin in the hypothalamus and the striatum (Chance *et al.* 1991). Systemic injection of amylin also

was shown to induce both hyperglycaemia and anorexia (Chance *et al.* 1993) suggesting that this peptide could be involved as a link between the peripheral and central modulation of appetite.

[¹²⁵I]BH-rat amylin binding was found to be highly specific in a variety of brain areas and was competed in a concentration-dependent manner with high affinity by both rat amylin-NH₂ and sCT, whereas hCGRPα had lower affinity in most regions. Few reports have described the characterization of putative [¹²⁵I]amylin binding sites (Sheriff *et al.* 1992; Bhogal *et al.* 1992; Beaumont *et al.* 1993), and the recent solubilisation and partial purification of a [¹²⁵I]amylin receptor binding protein from rat lung membranes further support the existence of a distinct class of amylin receptors (Bhogal *et al.* 1993). *In vitro* [¹²⁵I]amylin binding using brain membrane preparations revealed the existence of specific labeling in the rat cortex, hypothalamus, brainstem and cerebellum (Bhogal *et al.* 1992). Moreover, the presence of high affinity [¹²⁵I]BH-amylin binding sites in the nucleus accumbens (IC₅₀: 42 pM) also was reported recently (Beaumont *et al.* 1993). The apparent discrepancies between the reported amylin affinity values in rat brain might be related to the use of different assay conditions including ligand concentrations, buffer composition as well as the competition profiles being performed in membrane homogenates vs. brain sections. In contrast to specific amylin binding sites, amylin peptide mRNA could not be detected in nervous tissues except in the dorsal root ganglia (Ferrier *et al.* 1989). Similarly, amylin failed at first to be detected in the brain (Asai *et al.* 1990) or could only be detected in the hypothalamus (Chance *et al.* 1991) by using a highly specific radioimmunoassay. Whereas these findings may be related to the relative sensitivity of these methods, it is noteworthy that both [¹²⁵I]BH-amylin and [¹²⁵I]sCT binding sites (Skofitsch and Jacobowitz, 1992) are similarly distributed in the brain. Therefore, the recently isolated sCT-like molecule from the rat brain (Sexton and Hilton, 1992) might be an endogenous ligand for the amylin binding sites characterized here. However, it appears that rat amylin failed to compete for the [¹²⁵I]sCT binding sites recently cloned from the rat brain (Lin *et al.* 1991; Sexton *et al.* 1993; Albrandt *et al.* 1993) and showed over 1000-fold lower affinity than sCT, for [¹²⁵I]sCT binding in rat skeletal muscle (Kenney *et al.* 1993). Further studies will be required to clarify the precise nature of the endogenous ligand acting on the [¹²⁵I]BH-amylin binding sites characterized in the present study.

Rat amylin-NH₂, unlike hCGRPα (Quirion *et al.* 1992), demonstrated rather high affinity for [¹²⁵I]CGRP binding only in the shell of the nucleus accumbens and the ventral striatum, whereas lower affinities were observed in most other areas. Similarly, the existence of a unique population of CGRP/sCT sensitive sites was reported previously in the nucleus accumbens, fundus striati, lateral border of the lateral bed nucleus of the stria

terminalis and part of the central amygdaloid nucleus of the rat brain (Sexton *et al.* 1988; Dennis *et al.* 1991; Quirion *et al.* 1992). These data support the existence of an atypical class of CGRP receptors in the nucleus accumbens and the ventral striatum as both amylin and sCT demonstrated high affinity for these sites, whereas comparable competition profiles failed to be observed in various other brain regions (Quirion *et al.* 1992). The cloning of this atypical site is awaited to confirm the present data mostly derived from pharmacological studies.

Rat amylin-NH₂ showed a very low potency (micromolar concentrations) to induce a positive inotropic effect in the guinea pig atrium while being relatively more active (~15 times) to inhibit the electrically stimulated contraction of the rat vas deferens, two prototypical CGRP bioassays (Quirion *et al.* 1992). The greater potency of amylin in the rat vas deferens suggests a preferential affinity of amylin for the CGRP₂ vs. CGRP₁ receptor subtype. Moreover, the capacity of the C-terminal fragment hCGRP₈₋₃₇ to act as a potent antagonist is a key feature of the CGRP₁ receptor class (Dennis *et al.* 1990; Quirion *et al.* 1992). The blocking activity of this CGRP fragment for various effects induced by amylin has been reported. For example, CGRP₈₋₃₇ acted as a potent blocker of the hypotensive and tachycardiac effects of both amylin and CGRP *in vivo* (Gardiner *et al.* 1991) and their inhibitory action on glycogen turnover in the soleus muscle (Deems *et al.* 1991a). However, only very high concentrations (10-100 µM) of the CGRP fragment were able to antagonize the action of amylin in the liver (Morishita *et al.* 1990) and skeletal muscle (Wang *et al.* 1991). Additionally, CGRP₈₋₃₇ was unable to antagonize the action of amylin in both the isolated guinea pig urinary bladder and the rat vas deferens (Giuliani *et al.* 1992). Taken together, these findings suggest that amylin, whereas mostly acting on its own class of high affinity receptors, can also activate at higher concentrations CGRP receptors showing a preferential affinity for the putative CGRP₂ vs. CGRP₁ receptor subtypes.

In summary, the present data provide further evidence for the existence of [¹²⁵I]BH-rat amylin binding sites in the rat brain. Both rat amylin-NH₂ and sCT competed with high affinity for [¹²⁵I]BH-rat amylin binding in various brain regions. The cross-reactivity between amylin and CGRP receptors in brain and peripheral tissues appears to be rather limited (except for the atypical amylin/CGRP/sCT sites present in the nucleus accumbens) further supporting the presence of preferential amylin sites in the brain.

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CHAPTER 5

Comparative affinities of human adrenomedullin for [¹²⁵I]hCGRP α and [¹²⁵I]BH-rat amylin specific binding sites in the rat brain

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5.0 Preface to Chapter 5

Adrenomedullin is the most recent isolated peptide belonging to the CGRP/calcitonin family of peptides. It revealed approximately 25% sequence homology with CGRP. Thus far, adrenomedullin has essentially been studied for its peripheral vasodilating properties. Only one study has reported adrenomedullin-mediated CNS effects. Similar to amylin, very little is currently known about receptor sites mediating the effects of adrenomedullin. We have thus studied the affinity of this newly isolated peptide homologue for the various putative CGRP receptor subtypes in rat brain. We have also evaluated the potential affinity of adrenomedullin for amylin binding sites present in the rat brain. These findings further distinguish the characteristics of brain amylin and CGRP binding sites.

5.1 Abstract

Adrenomedullin (ADM) is a recently identified peptide that shows some homology (~25%) with calcitonin gene-related peptide (CGRP) and is now considered to be a new member of this family. As it shares biological effects with CGRP, we evaluated the possible affinity of human adrenomedullin (hADM) for [125 I]hCGRP α binding sites in the rat brain. Moreover, we evaluated the potential existence of cross-reactivity for [125 I]BH-rat amylin binding sites, another member of this peptide family. In all brain areas investigated, hADM only competed with rather low affinities for both [125 I]hCGRP α and [125 I]BH-rat amylin binding sites with IC₅₀ values generally in the high nanomolar-low micromolar range; the lowest affinity being observed for [125 I]BH-rat amylin binding sites. Interestingly, the lowest affinities of hADM against both radioligands were detected in the nucleus accumbens and ventral striatum. These areas are known to be enriched with atypical CGRP/salmon calcitonin/amylin-sensitive sites. It would thus appear that hADM is unlikely to bind to this atypical site. Moreover, hADM demonstrated limited affinity for either [125 I]hCGRP α or [125 I]BH-rat amylin binding sites in the rat brain. This suggests that the potential biological effects of ADM in the brain could be mediated through a different class of receptors with higher affinity for this newly isolated peptide.

5.2 Introduction

Adrenomedullin (ADM) is a peptide discovered very recently by monitoring the elevation of activity of platelet cAMP in human pheochromocytoma (Kitamura *et al.* 1993a) . The human form of this peptide consists of 52 amino acids, has one intramolecular disulfide bond and shows homology (~25%) with the calcitonin gene-related peptide or CGRP. ADM mRNA and the corresponding peptide, as measured by RNA blot analysis and radioimmunoassay respectively, were detected in a variety of human and porcine tissues including the adrenal medulla, ventricle, lung and kidney (Kitamura *et al.* 1993b; Ichiki *et al.* 1994; Kitamura *et al.* 1994). Only low amounts (0.31 fmol/mg tissue wet weight) of immunoreactive ADM were detected in human brain cortex (Ichiki *et al.* 1994).

Intravenous injections of ADM elicit a potent and long lasting reduction in blood pressure in rats , mainly by inducing a vasodilatation (Ishiyama *et al.* 1993). Interestingly, the CGRP receptor antagonist, hCGRP₈₋₃₇ (Chiba *et al.* 1989; Dennis *et al.* 1990), was shown to inhibit in a dose-dependent manner, the ADM-induced increases in cAMP formation in vascular smooth muscle cells (Eguchi *et al.* 1994). In contrast to its vasodepressor effects upon peripheral injection, ADM was shown to increase blood pressure when injected directly into the rat brain (Takahashi *et al.* 1994). Similar opposing effects have been reported for CGRP (Fisher *et al.* 1983).

In spite of the blocking effect of CGRP₈₋₃₇, it remains to be determined if ADM mostly acts through CGRP receptors, or via a unique ADM receptor to induce its biological effects. We thus investigated the comparative affinity of hADM for either [¹²⁵I]hCGRP α or [¹²⁵I]BH-rat amylin receptor binding sites known to be present and differentially distributed in the rat brain (Dennis *et al.* 1991; Beaumont *et al.* 1993; van Rossum *et al.* 1994). Amylin is a well known member of this peptide family showing rather high homology (~50%) with CGRP (Westermarck *et al.* 1986; Westermarck *et al.* 1987).

5.3 Materials and Methods

5.3.1 Materials

Male Sprague-Dawley rats (225-250 g) were obtained from Charles River (St-Constant, Québec, Canada). They had free access to food and water and were kept according to the guidelines of the Canadian Council for Animal Care and McGill University.

Rat amylin was purchased from Bachem California (Torrance, California). hCGRP α was synthesized in our laboratories as previously described in detail (Mimeault *et al.* 1992) and human adrenomedullin (hADM) was obtained from Phoenix Pharmaceutical Inc. (2-[125 I]iodohistidyl 10)-hCGRP α (2000 Ci/mmol), [125 I]BH-rat amylin (2000 Ci/mmol), Hyperfilm and 3H microscale standards were purchased from Amersham Canada (Oakville, Ontario). Trisma-base, NaCl, bacitracin, leupeptin and chymostatin were supplied by Sigma Chemicals Co. (St-Louis, MO) and bovine serum albumin (BSA) by Boehringer Mannheim Canada (Laval, Québec, Canada). Other chemicals were of analytical grade and were obtained from Fisher Scientific (Montréal, Québec, Canada).

5.3.2 [125 I]hCGRP α and [125 I]BH-rat amylin in vitro receptor autoradiography

Brain tissues were snap-frozen in 2-methylbutane (-40°C) and stored at -80°C until use. Serial coronal sections (20 μm) were cut and thaw-mounted onto gelatin coated microscope slides. Tissue sections were then desiccated overnight at 4°C and stored at -80°C until experiments were performed.

The competition profile of ADM for [125 I]hCGRP α binding sites in the rat brain was determined by pre-incubating adjacent coronal rat brain sections for 15-20 min in 50 mM Tris-HCl buffer, 100 mM NaCl, 4 mM MgCl_2 , pH 7.4, followed by a 60 min incubation in fresh buffer containing 25 pM [125 I]hCGRP α , 0.2% BSA, 0.4 mM bacitracin, 4 $\mu\text{g/ml}$ leupeptin and 2 $\mu\text{g/ml}$ chymostatin and increasing concentrations (10^{-9} - 10^{-6} M) of ADM. The sections were then washed in ice cold buffer (4 x 2 min) and dipped twice in cold distilled water. Nonspecific binding to adjacent tissue sections was

determined in the presence of 1 μ M hCGRP α and represented 10-15 % of total [125 I]hCGRP α binding (see Dennis *et al.*, 1991 for details). Radiolabelled tissue sections were exposed alongside with standards to highly sensitive film for 5 days. Thereafter, specific binding in various brain regions was quantified using commercially available standards and computerized image analysis system (MCID System, Image Research Inc., Ste-Catharines, Ontario). A similar protocol was used for [125 I]BH-rat amylin binding except for the following: after a 15 min pre-incubation, coronal rat brain sections were incubated in fresh buffer with 35 pM [125 I]BH-rat amylin (see van Rossum *et al.* , 1994 for details). Nonspecific binding was evaluated on adjacent sections incubated with 1 μ M rat amylin and corresponded to ~30% of total binding. The radiolabelled tissue sections were exposed to Hyperfilm for 7 days and quantified as above. The comparative affinities of hADM for [125 I]hCGRP α and [125 I]BH-rat amylin binding sites are derived from competition curves and expressed as IC₅₀ values.

5.4 Results

Using *in vitro* receptor autoradiography, high densities of binding sites for [125 I]hCGRP α were found to be especially concentrated in the shell of the nucleus accumbens, the tail of the caudate putamen and the amygdaloid body (Figure 5.1, total, left side). On the other hand, [125 I]BH-rat amylin binding sites were observed in the nucleus accumbens (core and shell), various hypothalamic nuclei including the dorsomedial and arcuate nuclei and in the amygdala (Figure 5.1, total, right side). The residual binding of either [125 I]hCGRP α or [125 I]BH-rat amylin in the presence of increasing concentrations of hADM (10^{-9} - 10^{-6} M) is also shown in Figure 5.1; quantitative data being summarized in Table 5.1. Overall, hADM demonstrated relatively higher affinities for specific [125 I]hCGRP α binding sites (130-880 nM) compared to specific [125 I]BH-rat amylin binding sites (330-18 000 nM) in all brain areas examined (Table 5.1). Interestingly, the lowest affinities of ADM were observed in the nucleus accumbens and ventral striatum for both radioligands; differences between these two regions and all others studied here being especially marked for [125 I]BH-rat amylin labeled sites (Table 5.1).

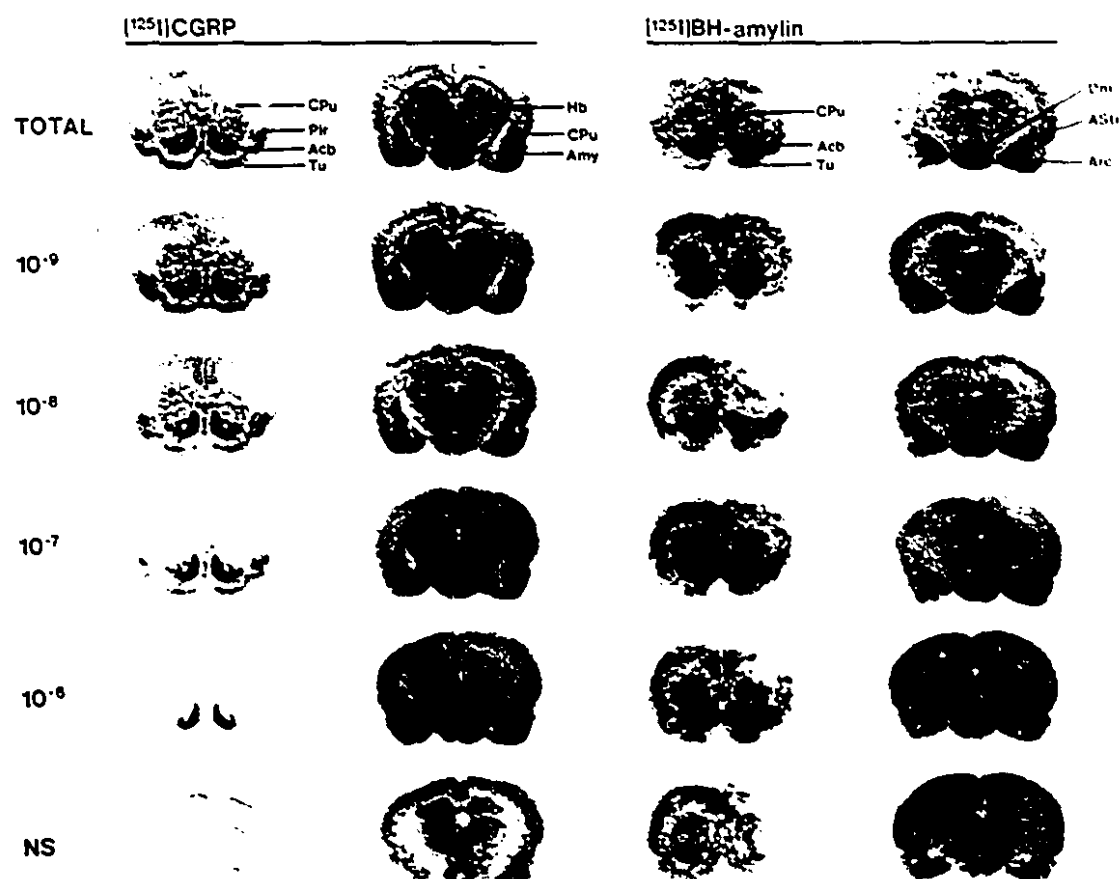


Figure 5.1 Photomicrographs of the autoradiographic distribution of $[^{125}\text{I}]\text{hCGRP}\alpha$ and $[^{125}\text{I}]\text{BH-rat amylin}$ binding sites in the presence of increasing concentrations (10^{-9} - 10^{-6} M) of hADM at two different levels of the rat brain, namely the nucleus accumbens and the dorsal hippocampus. Nonspecific binding (NS) was determined in the presence of $1\ \mu\text{M}$ hCGRP α or rat amylin, respectively and represented between 10 to 30% of total binding. *Abbreviations:* Acb, nucleus accumbens; Amy, amygdaloid body; Arc, arcuate hypothalamic nucleus; AStr, amygdalostratial transition zone; CPu, caudate putamen; DM, dorsomedial hypothalamic nucleus; Hb, habenular nucleus; Pir, piriform cortex and Tu, olfactory tubercle.

Table 5.1 Comparative affinities of human adrenomedullin (hADM) for specific [125 I]hCGRP α and [125 I]BH-amylin binding in various areas of the rat brain.

Brain areas	IC ₅₀ (nM) hADM*	
	[125 I]hCGRP α binding	[125 I]BH-amylin binding
Olfactory tubercle	130 $\pm$ 20	ND
Accumbens nucleus (shell)	880 $\pm$ 150	18 000 $\pm$ 10 000
Ventral striatum	500 $\pm$ 66	13 000 $\pm$ 400
Amygdaloid nuclei	330 $\pm$ 52	710 $\pm$ 160
Caudate-putamen (tail)	350 $\pm$ 14	ND
Temporal cortex	240 $\pm$ 35	ND
Dorsomedial hypothalamic nucleus	ND	890 $\pm$ 160
Arcuate hypothalamic nucleus	ND	565 $\pm$ 95
Superior colliculus	140 $\pm$ 11	ND
Pontine nucleus	170 $\pm$ 14	ND
Dorsal raphe nucleus	ND	565 $\pm$ 76
Laterodorsal tegmental nucleus	ND	560 $\pm$ 32
Lateral parabrachial nucleus	ND	330 $\pm$ 40
Locus coeruleus	ND	1600 $\pm$ 560
Cerebellum	160 $\pm$ 40	ND
Inferior olive	360 $\pm$ 73	ND

* IC₅₀ represents the concentration of hADM required to compete for 50% of either [125 I]hCGRP α or [125 I]BH-amylin specific binding. Data are expressed in nanomolar (nM) and represent means $\pm$ S.E.M. of three to four different brains.

5.5 Discussion

The present study reveals the limited affinity of hADM for both [125 I]hCGRP α and [125 I]BH-rat amylin binding sites in the rat brain. However, hADM showed a substantially higher affinity (high nanomolar range) for [125 I]hCGRP α sites compared to [125 I]BH-rat amylin (low micromolar range) sites in all brain areas investigated.

Very little is known thus far concerning the potential role(s) of ADM in the brain. Only one study reported the presence of a low amount of ADM-like immunoreactivity in the human brain cortex, as measured by radioimmunoassay (Ichiki *et al.* 1994). More recently, Takahashi *et al.* (1994) showed that intracerebroventricular injections of ADM (1-3 nmol/kg) in the rat brain produced an increase in blood pressure suggesting the existence of specific sites of action for ADM in the brain. However, it was not clear from this study if these sites represented unique ADM receptors or if ADM acted through CGRP receptors to produce this hypertensive effect especially as CGRP was shown to induce similar effects upon intracerebroventricular injection (Fisher *et al.* 1983).

In the present study, hADM revealed over 100 fold lower affinity (IC_{50} : 130-880 nM) for [125 I]hCGRP α binding sites compare to hCGRP α itself (IC_{50} : 0.4-6.9 nM; Quirion *et al.* 1992). Similarly, hADM showed over 200 fold lower affinity (IC_{50} : 330-18 000 nM) than rat amylin (IC_{50} : 1.5-18 nM; van Rossum *et al.* 1994) for [125 I]BH-rat amylin binding sites. In view of these limited affinities of hADM for both CGRP and amylin receptor sites in the rat brain, it would appear that the potent biological effects of ADM might be mediated by a unique class of ADM-preferring receptors such as those reported in vascular smooth muscle cells (Eguchi *et al.* 1994). However, future experiments using radiolabeled adrenomedullin as ligand will be required to fully establish the existence of this unique receptor class in rat brain. Furthermore, the rather low affinity of hADM for CGRP and amylin binding sites in rat brain is unlikely to be due to limited interspecies crossreactivity as hADM has been reported to be potent in eliciting a variety of biological responses in several species including rats and cats (Ishiyama *et al.* 1993; Ishizaka *et al.* 1994; Lipton *et al.* 1994; Takahashi *et al.* 1994).

Our laboratory (Dennis *et al.* 1991; Quirion *et al.* 1992) as well as others (Sexton *et al.* 1988) have reported the existence of a unique population of CGRP/salmon

calcitonin-sensitive binding sites in the rat nucleus accumbens. Moreover, rat amylin revealed its highest affinity for [125 I]hCGRP α binding sites in this area over all other brain areas investigated (van Rossum *et al.* 1994). In contrast, hADM showed its lowest affinity for both CGRP and amylin labeled sites in the nucleus accumbens. This demonstrates further the uniqueness of the nucleus accumbens CGRP/salmon calcitonin/amylin sites for which hADM is unlikely to be an endogenous ligand.

5.6 Acknowledgments

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CHAPTER 6

Effect of guanine nucleotides and temperature on calcitonin gene-related peptide receptor binding sites in brain and peripheral tissues

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6.0 Preface to Chapter 6

As no highly selective CGRP analogs are thus far available to uniquely label and study putative CGRP receptor subtypes, we undertook the investigation of the characterization of potential transduction mechanisms associated with the CGRP receptors. Different transduction mechanisms coupled to the CGRP receptors could provide some evidence, albeit indirect, for the existence of multiple CGRP receptor subtypes. CGRP receptors apparently belong to the G protein-coupled receptor superfamily. We have thus studied the effects of a non-hydrolyzable GTP nucleotide analogue on the binding affinity of CGRP. We compared these effects in several peripheral and central tissues known to be enriched with the various putative CGRP receptor subtypes, such as the atrium and vas deferens.

6.1 Abstract

Recent data have suggested the existence of at least two major classes of calcitonin gene-related peptide (CGRP) receptors in brain and peripheral tissues [Henke *et al.*, Brain Res. 410 (1987) 404-408; Dennis *et al.*, J.Pharmacol.Exp.Ther., 251 (1989) 718-725; *ibid*, 254 (1990) 123-128; Quirion *et al.*, Ann.NY Acad.Sci., 657 (1992) 88-105]. However, little is currently known on the structure characteristics of CGRP receptors as its cloning has as yet to be reported. In the present study, the sensitivity of [¹²⁵I]humanCGRP α binding to guanine nucleotides and temperature was investigated in guinea pig atria (prototypical CGRP₁ tissue) guinea pig vas deferens (prototypical CGRP₂ tissue) and in the rat brain and cerebellum (mixed assay). Binding isotherms of [¹²⁵I]hCGRP α in those four preparations were curvilinear and best fitted to a two-site model under most assay conditions. The high affinity binding component was highly temperature-sensitive and accounted, under experimental conditions, for up to 18% of the total population of receptors. Moreover, these high affinity sites were also highly sensitive to guanine nucleotides (Gpp(NH)p, 100 μ M) in all preparations although to a different extent depending upon assay temperatures. Taken together, this suggests that the different CGRP receptor subtypes present in these tissues all belong to a G protein-coupled receptor family.

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide that arises from the alternate processing of the RNA transcript of the calcitonin gene (Amara *et al.* 1982; Rosenfeld *et al.* 1984). Two forms (α and β) of CGRP like-immunoreactive peptides as well as specific CGRP receptor sites are widely distributed throughout the central (CNS) and peripheral (PNS) nervous systems (Skofitsch and Jacobowitz, 1985; Tschopp *et al.* 1985; Kruger *et al.* 1988; Quirion *et al.* 1992; for review, Ishida-Yamamoto and Tohyama, 1989). Based on the differential potencies of CGRP analogues in various *in vitro* and *in vivo* bioassays, we proposed the existence of at least two classes of CGRP receptors, the CGRP₁ and CGRP₂ subtypes (Dennis *et al.* 1989; Dennis *et al.* 1990). The CGRP₁ receptor is highly sensitive to the antagonistic properties of C-terminal fragments hCGRP₈₋₃₇ to hCGRP₁₂₋₃₇ and is present in peripheral tissues such as the guinea pig and rat atria, whereas CGRP₂ sites demonstrate preferential affinity for the linear CGRP agonist, [Cys(ACM)^{2,7}]hCGRP α with the rat and guinea pig *vasa deferentia* representing prototypical tissues (Mimeault *et al.* 1991; for review, Quirion *et al.* 1992). More recently, Maggi and co-workers (Evangelista *et al.* 1992; Giuliani *et al.* 1992) reported that the effects of CGRP on the rat stomach and the guinea pig urinary bladder are also resistant to the antagonistic properties of the fragment hCGRP₈₋₃₇, suggesting the existence of non-CGRP₁ receptors, possibly CGRP₂, in these two preparations. In addition, a population of salmon calcitonin-sensitive CGRP binding sites has been demonstrated in few selected areas of the rat brain including the nucleus accumbens and the central amygdaloid nucleus (Henke *et al.* 1985; Sexton *et al.* 1988; Dennis *et al.* 1991; Quirion *et al.* 1992). Taken together, these data clearly demonstrate the existence of multiple CGRP receptor subtypes in brain and peripheral tissues.

The activation of CGRP receptors of unidentified subtypes was reported to induce increases in intracellular cAMP content and/or to activate adenylate cyclase in a variety of tissues including the rat and guinea pig heart (Ishikawa *et al.* 1988; Wang and Fiscus, 1989), rat liver (Yamaguchi *et al.* 1988), guinea pig pancreas (Zhou *et al.* 1986), chick skeletal muscle (Laufer and Changeux, 1989) as well as in the neuroblastoma cell line SK-N-MC (van Valen *et al.* 1990) and astrocytic culture (Lazar *et al.* 1991). Moreover,

guanine nucleotide-sensitive [125 I]CGRP binding sites were reported to be present in the rat cerebellum (Chatterjee and Fisher, 1991), chick skeletal muscle (Takamori and Yoshikawa, 1989; Roa and Changeux, 1991), guinea pig lung (Umeda and Arisawa, 1989) and neonatal rat cardiac myocytes (Chatterjee *et al.* 1991). Hence, in these tissues, CGRP receptors might belong to a family of G protein-coupled receptors.

In contrast, the activation of CGRP receptors present in guinea pig small intestinal myenteric neurons (Palmer *et al.* 1987) and in rat spinal cord (Goltzman and Mitchell, 1985) did not appear to directly involve cAMP production. Furthermore, the biological effects induced by CGRP are believed to be at least partly mediated through increases in both cGMP and cAMP formation in the rat aorta (Wang *et al.* 1991; Fiscus *et al.* 1991), activation of K^+ _{ATP} channels in rabbit mesenteric arteries (Nelson *et al.* 1990), increases in Ca^{++} inward current and K^+ permeability in the guinea pig atria (Ohmura *et al.* 1990) and activation of muscarinic-gated K^+ current in rat atrial cells (Kim, 1991). It remains to be established if the mobilisation of these different mechanisms by CGRP is mediated through G protein-coupled receptors.

To further investigate if the various putative CGRP receptor subtypes characterized in our earlier studies (Dennis *et al.* 1989; Dennis *et al.* 1990; Mimeault *et al.* 1991, Quirion *et al.* 1992) all belong to a G protein-coupled receptor family, we evaluated the effects of a non-hydrolysable analog of GTP, Gpp(NH)p, on [125 I]hCGRP α binding parameters in various CNS and peripheral tissues including the guinea pig atria and vas deferens, and the rat brain (minus cerebellum) and the cerebellum itself as it is most enriched with CGRP receptors (Henke *et al.* 1987; Chatterjee and Fisher, 1991). The first two tissues are believed to be enriched with the CGRP₁ and CGRP₂ receptor subtypes respectively (Dennis *et al.* 1989; Dennis *et al.* 1990; Quirion *et al.* 1992) while the precise nature of the CGRP receptor subtype(s) present in different brain areas remains to be established although recent evidence suggests coupling to G protein in rat cerebellum (Chatterjee and Fisher, 1991).

6.3 Materials and Methods

6.3.1 Materials

hCGRP α was synthesized in our laboratories as previously described (Mimeault *et al.* 1992). (2-[125 I] iodohistidyl 10)-hCGRP α (specific activity ~ 2000 Ci/mmol) was purchased from Amersham Canada (Oakville, Ontario, Canada). Gpp(NH)p and bovine serum albumin (BSA) was supplied by Boehringer Mannheim Canada, Laval, Québec. GTP γ S, GTP, GDP, GMP, ATP γ S, bacitracin and polypep were obtained from Sigma Chemical Co., St-Louis, MO. All the other chemicals were of analytical grade and were obtained from Fisher Scientific, Montréal, Québec, Canada.

6.3.2 Membrane Preparation

Male Sprague-Dawley rats (225-250 g) and male Hartley guinea pigs (325-375 g) obtained from Charles River (St-Constant, Québec) were sacrificed by decapitation. The guinea pig atria (as prototypical CGRP $_1$ assay, Quirion *et al.* 1992) and vas deferens (as prototypical CGRP $_2$ assay, Quirion *et al.* 1992) and the rat brain (minus cerebellum) and rat cerebellum were isolated and prepared as described by Sexton *et al.* (1986). Briefly, tissues were homogenized in 20x vol. of ice-cold 25 mM Tris-HCl, 50 mM NaCl, pH 7.4 using Brinkmann polytron (setting 6, 30 sec) and centrifuged at 48 000 g for 20 min at 4°C. The pellets were then washed and resuspended in the original volume of buffer three successive times. Final pellets were resuspended in 50 mM Tris-HCl containing 100 mM NaCl (pH 7.4) and subsequently used in assays on the very same day.

6.3.3 Receptor binding assays

Either guinea pig atria, guinea pig vas deferens, rat brain (minus cerebellum) or rat cerebellum corresponding to 150 μ g, 15 μ g, 250 μ g and 300 μ g of protein, respectively was incubated for 2 hours at 4°C, 1 hour at 20°C (room temperature) or 30 min. at 37°C, in a final volume of 0.5 ml consisting of 50 mM Tris-HCl, 100 mM NaCl, 0.2% BSA, 0.5% polypep, 0.4 mM bacitracin, 5 mM MgCl $_2$, 40 pM [125 I]hCGRP α and increasing concentrations of hCGRP α (10^{-12} - 10^{-6} M), in the presence or absence of various concentrations of cyclic nucleotides (10^{-8} - 10^{-4} M). At the end of the incubation, bound [125 I]hCGRP α was separated from free ligand by rapid filtration under reduced pressure

using a Brandel Cell Harvester (Model M24-R, Gaithersburg, MD) through glass-fibre filters (#32, Schleicher and Schuell, Keene, NH) presoaked in 0.1% polyethyleneimine solution. Tubes were rinsed with 3x 5 ml of ice-cold buffer. The radioactivity trapped on the filters was measured using a gamma counter (1282 Compugamma, LKB, Rockville, MD). Non-specific [125 I]hCGRP α binding was defined by the addition of 1 μ M unlabelled hCGRP α to the various incubation media and represented between 15–45 % of total binding depending upon the tissue preparation. Proteins were measured by the method of Lowry *et al.* (1951) using BSA as standard.

6.3.4 Data Analysis

The analysis of the competition curves was performed using the LUNDON-2 Competition Data Analysis program which is based on a modified version of the Cheng-Prusoff method. The *F*-test was used to compare the goodness of fit between the one and two-site models. If the *F*-test indicated that the probability value was greater than 0.05, the less complex model was accepted as the model which better fitted the data. The sum of squared error (SSE) values for the two consecutive models along with their corresponding degree of freedom are used to determine the *F* value. Significance between values was determined by analysis of variance and Scheffé's *post hoc* analysis for multiple comparisons. Data represent the mean $\pm$ S.E.M. of at least three separate experiments, each performed in triplicate.

6.4 Results

6.4.1 [125 I]hCGRP α binding characteristics in rat cerebellum

As shown in Figure 6.1a, the effect of Gpp(NH)p on [125 I]hCGRP α binding in the cerebellum is temperature dependent. At 4°C, in the absence of the GTP analog, [125 I]hCGRP α binding best fitted a one-site model (Table 6.1). However, by increasing the temperature to 20°C or 37°C, a higher affinity site (K_H) could be detected and accounted for about 10% of the total population of receptor (Table 6.1). In the presence of Gpp(NH)p, the one-site model best fitted [125 I]hCGRP α competition curves at all temperatures indicating a shift of K_H to a lower affinity site (K_L) at both 20°C and 37°C (Table 6.1; Fig 6.1a). However, in one experiment at 20°C, receptors under R_H could still be detected and corresponded to 4% of R_T (vs. 10% in control) with affinity constant values of 3.3×10^{-11} M and 2.1×10^{-9} M for K_H and K_L respectively. Similarly, in two replicate experiments at 37°C, the two-site model best fitted the data obtained ($P < 0.05$) in the presence of Gpp(NH)p with affinity constants of 2.1×10^{-10} M and 4.1×10^{-8} M respectively.

6.4.2 [125 I]hCGRP α binding characteristics in rat brain

As in the cerebellum, the effect of Gpp(NH)p on [125 I]hCGRP α binding in rat brain (minus cerebellum) is temperature dependent (Figure 6.1b). At 4°C, Gpp(NH)p failed to alter [125 I]hCGRP α binding: both competition curves best fitted the one-site model (Table 6.1). At higher temperatures (20°C and 37°C), a significant proportion of R_H could be detected (up to 15% of R_T , Table 6.1). In the presence of Gpp(NH)p, both K_H and K_L could still be detected at 20°C, CGRP competition curves best fitted the two-site model. At 37°C, in the presence of Gpp(NH)p, a single affinity site could be detected in two separate experiments (Table 6.1). However, in two other experiments, the CGRP competition curves in the presence of the GTP analog still best fitted the two-site model ($P < 0.05$), with affinity constants of 4.4×10^{-11} M and 1.6×10^{-9} M respectively and decreased R_H from 7% to 4% (not significant, NS).

We next examined the effects of Gpp(NH)p on prototypical CGRP $_1$ (guinea pig atria) and CGRP $_2$ (guinea pig vas deferens) receptor subtypes to evaluate the relevance of data observed in CNS preparations and to better characterize CGRP receptor subtypes.

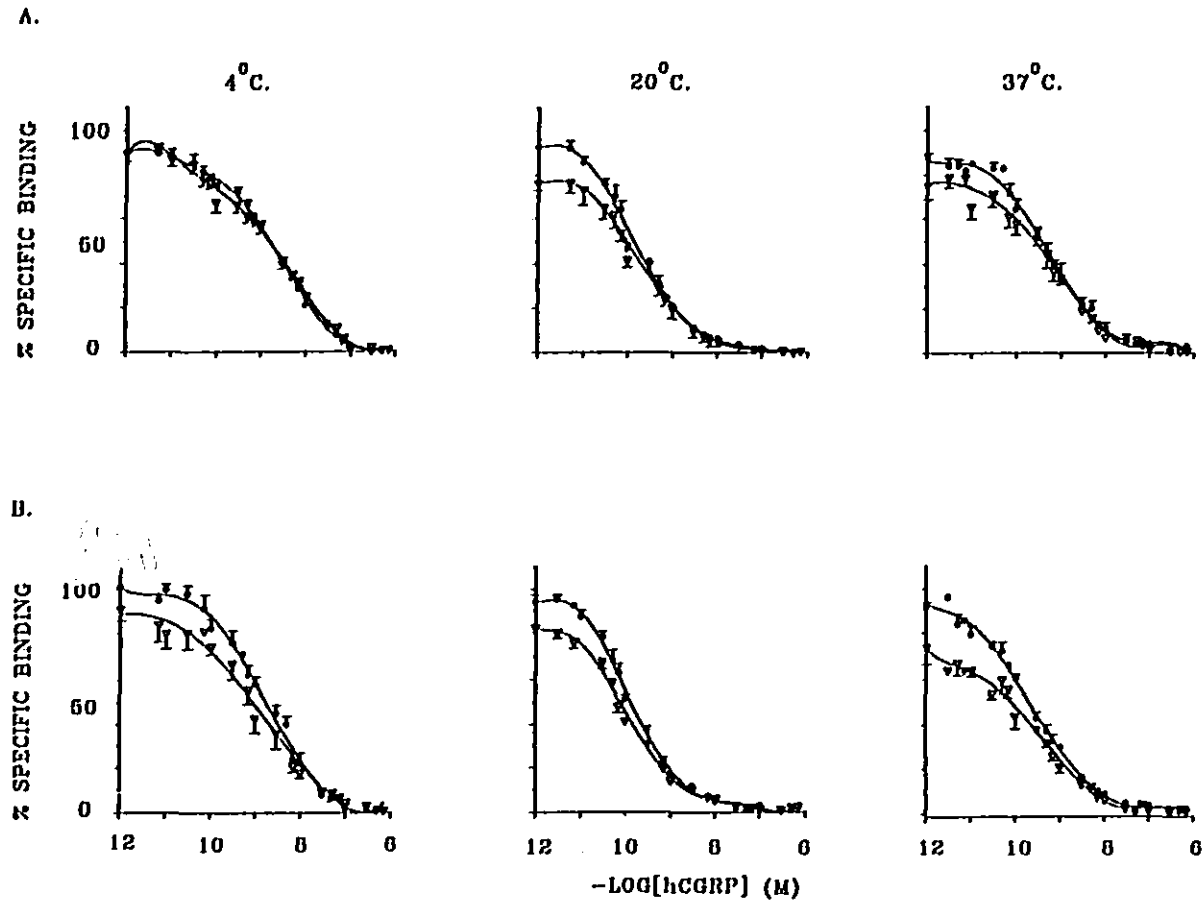


Figure 6.1 [125 I]hCGRP α binding profile in the rat cerebellum (A) and brain (minus cerebellum; B) in the presence or absence of non-hydrolysable GTP analogue, Gpp(NH)p. Membranes were incubated with 40 pM [125 I]hCGRP α in the presence (∇) or absence ($\bullet$) of 100 μ M Gpp(NH)p at 4°C, 20°C or 37°C. Gpp(NH)p curves are expressed as percentage of the specific binding observed under control conditions. Non-specific binding was determined in presence of 1 μ M unlabeled hCGRP α and represented ~30% of total binding. Data represent means $\pm$ S.E.M. of at least 3 different experiments, each performed in triplicate.

Table 6.1 [125 I]hCGRP α binding characteristics in rat central nervous system in the presence or absence of a non-hydrolysable GTP analogue, Gpp(NH)p.

	Control						+Gpp(NH)p			
	Temp (°C)	<i>n</i>	K_H (M)	K_L (M)	R_T (fmol/mg protein)	% R_H	K_H (M)	K_L (M)	R_T (fmol/mg protein)	% R_H
Rat cerebellum	4	4	--	$3.1 \times 10^{-9} \pm 0.7$	131 ± 15	ND	--	$3.9 \times 10^{-9} \pm 0.6$	156 ± 17	ND
	20	3	$3.7 \times 10^{-11} \pm 1$	$1.8 \times 10^{-9} \pm 0.2$	56 ± 0.5	9.5 ± 0.5^A	--	$2.6 \times 10^{-10} \pm 0.5$	22 ± 3	ND ^C
	37	4	$1.7 \times 10^{-10} \pm 0.4$	$1.5 \times 10^{-8} \pm 1.1$	69 ± 18	9 ± 2^A	--	$9.1 \times 10^{-10} \pm 0.8$	24 ± 3	ND ^C
Rat brain	4	4	--	$1.4 \times 10^{-9} \pm 0.3$	73 ± 12	ND	--	$1.8 \times 10^{-9} \pm 0.4$	91 ± 11	ND
	20	4	$6.1 \times 10^{-11} \pm 0.7$	$2.9 \times 10^{-9} \pm 1.1$	54 ± 7	$18 \pm 3^{A,B}$	$4.5 \times 10^{-11} \pm 0.6$	$4.0 \times 10^{-9} \pm 2.4$	52 ± 9	$14 \pm 2^{A,B}$
	37	4	$8.2 \times 10^{-11} \pm 3$	$2.6 \times 10^{-9} \pm 0.8$	36 ± 5	11 ± 4^A	--	$3.5 \times 10^{-10} \pm 0.6$	13 ± 2	ND ^C

^A Best goodness fit of the two-site model with $P < 0.01$

^B Best goodness fit of the two-site model with $P < 0.05$

^C See "Results" for further detailed values

n represents number of replicates, each performed in triplicate; K_H and K_L refer to high and low affinity binding components, respectively; R_T refers to total number of binding sites while % R_H relates to their proportion under high affinity. ND: Not Detected.

6.4.3 [125 I]hCGRP α binding characteristics in guinea pig atria (CGRP $_1$ tissue)

As in the two CNS preparations, a single class of [125 I]hCGRP α binding site was detected at 4 $^{\circ}$ C in both control and Gpp(NH)p membrane treated preparations (Table 6.2, Fig 6.2a). Increasing the temperature to 20 $^{\circ}$ C revealed the existence of a very small population of R $_H$ (2% of R $_T$) while failed to be detected in the presence of Gpp(NH)p (Table 6.2). The assay was not carried at 37 $^{\circ}$ C due to low specific binding.

6.4.4 [125 I]hCGRP α binding characteristics in guinea pig vas deferens (CGRP $_2$ tissue)

As observed in the other three membrane preparations (Fig 6.1a, 6.1b, 6.2a), [125 I]hCGRP α binding characteristics varied with temperature in the guinea pig vas deferens (Figure 6.2b). In the presence or absence of Gpp(NH)p, data best fitted the one-site model at 4 $^{\circ}$ C ($P > 0.05$, Table 6.2). At higher temperatures, the presence of a high affinity site was detected and accounted for about 4% of R $_T$ (Table 6.2). Treatments with Gpp(NH)p shifted [125 I]hCGRP α binding to a single population of sites as results best fitted the one-site model at 20 $^{\circ}$ C and 37 $^{\circ}$ C. However, in two separate experiments at 37 $^{\circ}$ C, data best fitted the two-site model ($P < 0.01$) even in the presence of Gpp(NH)p, with affinity constant values of 2.8×10^{-10} M and 4×10^{-8} M for the K $_H$ and K $_L$ respectively and with 1% of R $_T$ under K $_H$ vs. 3% in non-treated membrane preparations (NS).

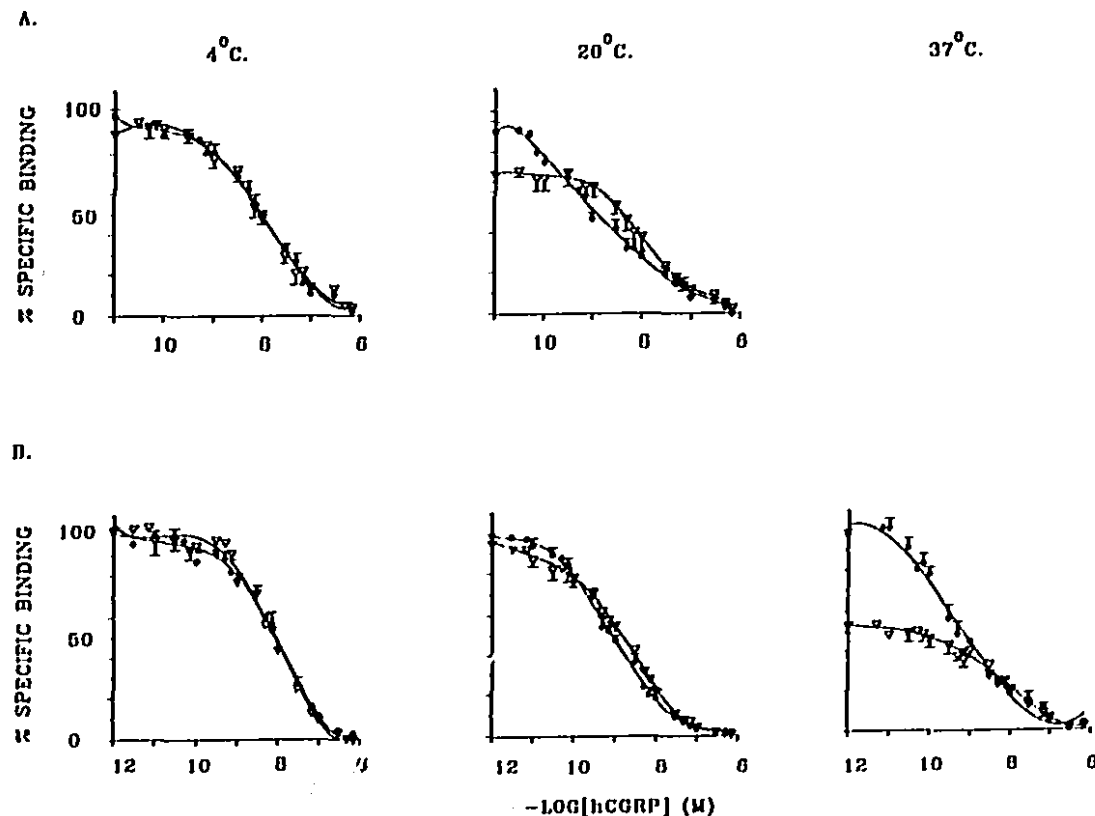


Figure 6.2 [125 I]hCGRP α binding profile in prototypical CGRP₁ (guinea pig atria, A) and CGRP₂ (guinea pig vas deferens, B) in the presence or absence of a non-hydrolysable GTP analogue, Gpp(NH)p. Membranes were incubated with 40 pM [125 I]hCGRP α in the presence (▽) or absence (●) of 100 μ M Gpp(NH)p at 4°C, 20°C or 37°C. Gpp(NH)p curves are expressed as percentage of the specific binding observed under control conditions. Non-specific binding was determined in the presence of 1 μ M unlabeled hCGRP α and represented between 15 to 35% of total binding. Data represent means $\pm$ S.E.M. of at least 3 different experiments performed in triplicate. No data were obtained at 37°C in the guinea pig atria as specific binding was highly unstable likely because of proteolytic degradation of the radioligand.

Table 6.2 [125 I]hCGRP α binding characteristics in prototypical CGRP $_1$ and CGRP $_2$ assays in the presence or absence of a non-hydrolysable GTP analogue, Gpp(NH)p.

	Control						+ Gpp(NH)p			
	Temp (°C)	n	K _H (M)	K _L (M)	R _T (pmol/mg protein)	%R _H	K _H (M)	K _L (M)	R _T (pmol/mg protein)	%R _H
Guinea pig atria (CGRP $_1$)	4	4	--	8.8x10 ⁻⁹ ±1.3	1.1±0.5	ND	--	1.1x10 ⁻⁸ ±0.7	1.3±0.5	ND
	20	3	2.7x10 ⁻¹⁰ ±0.9	2.1x10 ⁻⁸ ±0.4	0.7±0.05	2±1 ^A	--	9.0x10 ⁻⁹ ±1.1	0.7±0.2	ND
Guinea pig vas deferens (CGRP $_2$)	4	4	--	9.4x10 ⁻⁹ ±1.8	17±1	ND	--	7.4x10 ⁻⁹ ±0.7	20±0.7	ND
	20	4	2.1x10 ⁻¹⁰ ±0.5	7.2x10 ⁻⁹ ±2.8	9±3	5±2.4 ^A	--	2.9x10 ⁻⁹ ±0.2	6.3±0.6	ND
	37	4	2.5x10 ⁻¹⁰ ±0.9	9.2x10 ⁻⁸ ±2.9	10±2	3±1.5 ^A	--	4.3x10 ⁻⁹ ±0.7	1.7±0.5	ND ^C

^A Best goodness fit of the two-site model with P < 0.01

^C See "Results" for further detailed values

n represents number of replicates, each performed in triplicate; K_H and K_L refer to high and low affinity binding components, respectively; R_T refers to total number of binding sites while %R_H relates to their proportion under high affinity. ND: Not Detected.

6.4.5 Nucleotide specificity

The effect of various nucleotides and nucleotide analogues on [125 I]hCGRP α binding in guinea pig vas deferens is shown in Figure 6.3. GTP γ S was the most effective nucleotide to inhibit [125 I]hCGRP α specific binding with maximal effect (~45%) at 1 μ M. Gpp(NH)p, GTP and GDP had similar effects in decreasing [125 I]hCGRP α specific binding at higher concentrations (10–100 μ M). GMP and ATP γ S had very little effect at concentrations up to 100 μ M.

6.4.6 Temperature and Gpp(NH)p-dependent changes in R_T

In all tissues studied, the addition of Gpp(NH)p (100 μ M) to membrane preparations and the increment of the assay temperature tend to decrease R_T . For example, significant decreases were observed in rat cerebellum ($P < 0.05$ at 4 $^{\circ}$ C vs. 20 $^{\circ}$ C and 37 $^{\circ}$ C) and rat brain (minus cerebellum) ($P < 0.05$ at 4 $^{\circ}$ C vs. 37 $^{\circ}$ C). Significant decreases were also detected in the presence of Gpp(NH)p in the rat cerebellum at 20 $^{\circ}$ C ($P < 0.05$), the rat brain (minus cerebellum) at 37 $^{\circ}$ C ($P < 0.05$) as well as in the guinea pig vas deferens at 37 $^{\circ}$ C ($P < 0.05$).

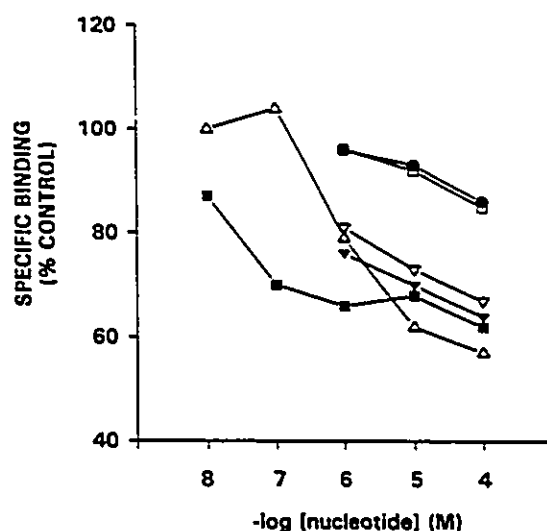


Figure 6.3 Effect of various cyclic nucleotides on specific [125 I]hCGRP α binding in guinea pig vasa deferentia. Membranes were incubated with 40 pM [125 I]hCGRP α for 30 min at 37 $^{\circ}$ C, in the presence of increasing concentrations of GTP γ S (■), Gpp(NH)p (Δ), GTP (▼), GDP (▽), GMP (●) and ATP γ S (□). Data are expressed as percentage of the specific binding observed under control conditions and represent the means of three separate experiments.

6.5 Discussion

We (Dennis *et al.* 1989; Dennis *et al.* 1990; Mimeault *et al.* 1991; Quirion *et al.* 1992) and others (Henke *et al.* 1985; Henke *et al.* 1987; Sexton *et al.* 1988; Giuliani *et al.* 1992) have reported the existence of multiple CGRP receptor subtypes in brain and peripheral tissues. In the present study, we focused on the sensitivity of [125 I]hCGRP α binding to guanine nucleotide analog such as Gpp(NH)p, and temperature in various preparations. The results suggest that CGRP receptors present in all these tissues belong to a G protein-coupled receptor family. High affinity [125 I]hCGRP α binding sites were detected, in a temperature-dependent manner, in the four tissues studied, namely the brain (minus cerebellum), cerebellum, atria (prototypical CGRP $_1$ assay) and vas deferens (prototypical CGRP $_2$ assay). At low temperature (4°C), high affinity sites could not be detected in any preparations. By raising the incubation temperature to 20°C or 37°C, the presence of a high affinity population of sites became apparent which corresponded to between 2 and 18% of the total receptor population. Addition of the guanine nucleotide analog reduced or induced the disappearance of this high affinity [125 I]hCGRP α binding component. This suggests that Gpp(NH)p induced a shift to a lower affinity receptor state, as expected for G protein-coupled receptors.

A variety of dissociation constant values has been previously reported for [125 I]CGRP binding in rat cerebellum (Sexton *et al.* 1986; Inagaki *et al.* 1986; Yoshizaki *et al.* 1987; Chatterjee and Fisher, 1991), brain (Yoshizaki *et al.* 1987; Henke *et al.* 1987) and atria (Yoshizaki *et al.* 1987). Three different laboratories reported the existence of a single high affinity site in the rat cerebellum with K_D values ranging from 1.1×10^{-10} M to 3.9×10^{-9} M (Sexton *et al.* 1986; Inagaki *et al.* 1986; Chatterjee and Fisher, 1991). On the other hand, Yoshizaki *et al.* (1987) described the existence of two [125 I]CGRP binding components with constant values of 1.5×10^{-11} M and 6.1×10^{-8} M respectively. These apparent discrepancies could be related to the use of different assay conditions. For example, Sexton *et al.* (1986) used a 1 hour incubation period at 20°C ($K_D = 3.9 \times 10^{-9}$ M) in comparison to 30 minutes at 37°C for Chatterjee *et al.* (1991) ($K_D = 2.2 \times 10^{-10}$ M). In the present study, we obtained values of 3.7×10^{-11} M and 1.8×10^{-9} M at 20°C and of 1.7×10^{-10} M and 1.5×10^{-8} M at 37°C for the two affinity components present in cerebellar membrane preparations. Interestingly, K_D values reported by the other laboratories (Sexton *et al.* 1986; Chatterjee and Fisher, 1991) are very close to those obtained here for the low and high affinity constant values at 20°C and 37°C, respectively.

The binding of ligands to their receptors is well known to be a temperature-dependent process (for example, Weiland *et al.* 1980). Binding and thermodynamic properties at various temperatures have been extensively studied for β -adrenergic agonists and antagonists in a variety of preparations (Pike and Lefkowitz, 1978; Weiland *et al.* 1980; Contreras *et al.* 1986). One characteristic of adrenergic agonist binding to their receptors relates to their higher affinity at lower temperatures (Insel and Sanda, 1979; Contreras *et al.* 1986). In contrast, opiate and adenosine related agonists demonstrate lower affinities for their respective receptors at low temperatures (Murphy and Snyder, 1982; Lohse *et al.* 1984; Simantov *et al.* 1976). The major factor leading to these differences is thought to result from an enthalpy-driven interaction in the case of adrenergic agonists in contrast to an entropy-driven interaction for opiates and adenosine (Weiland *et al.* 1980; Lohse *et al.* 1984). The affinity of [125 I]hCGRP α for its receptors in the cerebellum and vas deferens (CGRP $_2$ tissue) is inversely related to temperature (Tables 6.1 and 6.2) suggesting by analogy, an enthalpy-driven interaction between CGRP and its receptors in these tissues. On the other hand, [125 I]hCGRP binding affinities were apparently unaffected by temperature in the other two preparations used here, namely the rat brain (minus cerebellum) and guinea pig atria (CGRP $_1$ tissue). Further experiments will be required to address the nature of these differences and to determine if they can be related to different proportion of CGRP $_1$ and CGRP $_2$ receptor subtypes in these various preparations.

The formation of a high affinity state complex of the β -adrenergic receptors decreased in parallel with temperature. This likely results from a decrease in the availability of the guanine nucleotide-binding protein (G protein) or to its reduced mobility in the cytoplasmic membrane at lower temperatures (Contreras *et al.* 1986). Similarly, the proportion of brain adenosine receptors present in the high affinity state is largely decreased at 0°C compared to 37°C (35% vs. 80% respectively) (Lohse *et al.* 1984). In agreement with these reports, high affinity [125 I]hCGRP α binding components could not be detected at 4°C in any of the tissue preparations used in the present study but accounted for between 2 and 18% of the total population of receptors at 20°C (without further increase at 37°C).

GTP and GTP analogues prevent the formation of high affinity Ligand (L)-Receptor (R)-G protein (G) complex favouring a lower affinity state (LR) by activating the G protein which rapidly dissociates from the ternary complex (LR*G) (DeLean *et al.* 1980; for review, Birnbaumer *et al.* 1990). [125 I]hCGRP α binding is shown here to be sensitive to various GTP analogues, especially GTP γ S and Gpp(NH)p. The similar potency of GDP and GTP to affect [125 I]hCGRP α receptor affinities in the guinea pig vas

deferens was previously reported in a variety of other preparations (Iyengar *et al.* 1980; Rojas and Birnbaumer, 1985; Mattera *et al.* 1985). It is not obvious why GDP can shift [¹²⁵I]hCGRP α binding to a similar lower affinity state as GTP. One possibility could be that at least two pathways could lead to the formation of low affinity binding site (R_L): one being the GTP-dependent activation of the G protein and the other being a direct dissociation of GDP from R_L (Birnbaumer *et al.* 1990).

Multiple affinity components, sensitive to Gpp(NH)p were detected for [¹²⁵I]hCGRP α binding in the four preparations used in the present study. In most cases, addition of the nucleotide analog fully shifted all [¹²⁵I]hCGRP α binding sites to a lower affinity (K_L) suggesting that CGRP receptors are coupled to G proteins. However, at both 20°C and 37°C in the CNS and the vas deferens, a small percentage of high affinity site, with K_H values very similar to control, could still be detected even in the presence of Gpp(NH)p. In agreement with this observation, Chatterjee *et al.* (Chatterjee *et al.*, 1991; Chatterjee and Fisher, 1991) recently reported that CGRP receptors in the rat cerebellum and cardiac myocytes may exist under multiple affinity states even before complexing with their respective G proteins. These authors observed that the dissociation of [¹²⁵I]hCGRP α from its cerebellar receptors revealed a multi-exponential dissociation curve which could be described by fast and slow dissociating components, either in the presence or absence of GTP γ S (Chatterjee and Fisher, 1991). Based on these and our results, one possible scheme for CGRP receptor isomerisation and subsequent coupling to a G protein could be as follows:



in which the receptor undergoes a conformational change before forming a high affinity ternary complex (LR*G) by associating with a G protein. According to our results, the additional intermediary (LR*) could be of similar affinity as LR*G. This receptor isomerisation sequence would explain the small percentage of [¹²⁵I]hCGRP α binding still observed in the K_H state in the presence of the GTP analog under certain experimental conditions. In fact, the percentage of CGRP receptors in high affinity would be expected to be lower since in the presence of Gpp(NH)p only LR* is detected vs. LR* + LR*G under control assay conditions. The LR* intermediary is usually difficult to detect and quantify because of its very small amounts seen at equilibrium (1-4% of the total [¹²⁵I]hCGRP α receptor population).

It is also of interest to note that the formation of high affinity receptor complexes without coupling to a G protein has been previously reported (Lynch *et al.* 1988; Hausdorff *et al.* 1990). For example, Lynch *et al.* (1988) reported that following the uncoupling of the G protein from the α_1 -adrenergic receptor by treatments with NaHPO₄

or CaCl_2 , the high affinity binding component of α_1 -agonists was still observed. Similarly, a mutation of the β_2 -adrenergic receptor was recently shown to impair the activation of adenylyl cyclase by isoproterenol without affecting the high affinity binding component, suggesting the existence of an additional intermediary between LR^*G and the moieties which is able to stimulate adenylyl cyclase (Hausdorff *et al.* 1990). It is also likely that more than one CGRP receptor class are present in most tissues; one subtype being highly sensitive to GTP while the other is not. Addition of $\text{Gpp}(\text{NH})\text{p}$ would thus shift one receptor subtype to a lower affinity state while leaving the other into its higher affinity state. This possibility appears unlikely on the basis of the comparative sensitivity of K_H to variations in temperature.

The proportion of K_H and K_L is known to vary among tissues and receptor classes. For example, while β -adrenergic receptors were reported to exist mostly (80%) under a high affinity state in frog erythrocyte (Kent *et al.* 1980), rat brain muscarinic receptors represented between 19 and 49% of the total population regardless of the agonist tested (Birdsall *et al.* 1978). For $[^{125}\text{I}]\text{hCGRP}\alpha$ sites, this percentage appears to vary between 2 and 18% depending upon the preparation studied here. This is a rather low proportion as compared to other G protein coupled receptors (Birdsall *et al.* 1978; Kent *et al.* 1980). Lin *et al.* (1991) have recently reported the cloning of the calcitonin receptor. Interestingly, the amino acid sequence of this receptor showed very little homology with other previously cloned, well known G protein-coupled receptors (except for the parathyroid hormone and secretin receptors which shared about 50% sequence homology (Juppner *et al.* 1991; Ishihara *et al.* 1991)). These authors suggested that the calcitonin, parathyroid hormone and secretin receptors belong to a new super-family of G protein-coupled receptors. One essential feature of this family resides in the short cytoplasmic loop between hydrophobic domains V and VI which is thought to be involved in the coupling to the G protein of the $\text{G}_{\text{S}\alpha}$ type. This region is not similar to the corresponding regions of other adenylate cyclase-coupled receptors (Lin *et al.*, 1991). The cloning of CGRP receptors has yet to be reported but it is likely to belong to this new family of G protein-coupled receptor on the basis of the common calcitonin-CGRP precursor, peptide sequence homologies and sharing of some biological activities and possibly receptors (Amara *et al.* 1982; Goltzman and Mitchell, 1985; Dennis *et al.* 1991). The differences between this newly identified family of G protein-coupled receptors and the others might also account for the lower proportion of receptors in K_H related to a different type of interaction between the G protein and the receptor complex.

In brief, the present findings suggest that CGRP receptor binding sites present in the brain, atria and vas deferens belong to a family of G protein-coupled receptors which exist under multiple affinity states depending upon their micro-environment.

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CHAPTER 7

Profile of CGRP-, amylin- and salmon calcitonin-induced depression of locomotion following intracerebroventricular injection

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As presented in Chapters 2 and 4, the nucleus accumbens is enriched in binding sites for both CGRP and amylin. Other laboratories have reported that this nucleus is also enriched with salmon calcitonin binding sites. The pharmacological characteristics of the CGRP binding sites in the nucleus accumbens is atypical as [^{125}I]hCGRP α binding in this tissue is highly sensitive to both amylin and salmon calcitonin in comparison to most other areas of the brain. Furthermore, these CGRP/salmon calcitonin/amylin-sensitive binding sites do not share the pharmacological characteristics of either the CGRP $_1$ or CGRP $_2$ receptor subtypes. As the respective binding affinity of CGRP, salmon calcitonin and amylin is very similar in the accumbens, the primary goal of the present study was to develop an assay to differentiate between the possible functional relevance of these binding sites. We first evaluated the effects of these peptide homologues on amphetamine-induced locomotion since the nucleus accumbens plays a critical role in the amphetamine-induced hyperactivity. Second, in an attempt to differentiate their actions as well as to delineate possible mechanisms of action, we studied the effects of a GABA antagonist on each peptide-induced decreased locomotor activity.

7.1 Abstract

Calcitonin gene-related peptide (CGRP)-, amylin- and calcitonin-like immunoreactivity, as well as their respective receptors, are widely distributed in the brain. Few brain areas, including the nucleus accumbens, are enriched with binding sites for all three peptide homologues. Specific neurobehavioral profiles are induced following the central administration of either CGRP, amylin or salmon calcitonin. In the present study, we investigated the effects of each of these peptides on amphetamine-induced and spontaneous locomotor behaviors. Intracerebroventricular injections of each peptide decreased, in a dose-dependent manner, the amphetamine-induced locomotion. Salmon calcitonin demonstrated the highest potency followed by amylin and hCGRP α . A similar profile of potency was observed on spontaneous locomotion. Interestingly, peripheral injection of bicuculline methiodide (0.5 mg/kg), a GABA $_A$ receptor antagonist, selectively blocked the decrease in spontaneous locomotion induced by hCGRP α whereas it had no effects on amylin and salmon calcitonin-induced decrease in spontaneous locomotor activity. The present findings thus suggest the existence of functionally distinct receptors for CGRP vs. those of amylin and salmon calcitonin to modulate locomotor activity in the rat brain.

CGRP results from the alternative splicing of the calcitonin gene and is widely distributed in both peripheral and central nervous systems (for review, Ishida-Yamamoto and Tohyama, 1989). CGRP receptors are broadly but discretely localized in the CNS (Tschopp *et al.* 1985; Henke *et al.* 1985; Kruger *et al.* 1988). Moreover, CGRP has been shown to induce a variety of effects following intracerebroventricular (i.c.v.) injections such as hyperthermia (Jolicoeur *et al.* 1992), catalepsy (Jolicoeur *et al.* 1992; Clementi *et al.* 1992), anorexia (Krahn *et al.* 1984; Jolicoeur *et al.* 1992) as well as increase in mean arterial pressure and heart rate (Fisher *et al.* 1983).

In addition to CGRP, the presence of calcitonin-like immunoreactivity and binding sites have been described in brain (Cooper *et al.* 1980; Fischer *et al.* 1981; Rizzo and Goltzman, 1981; for review, Skofitsch and Jacobowitz, 1992). The binding sites for calcitonin were detected most often using [¹²⁵I]salmon calcitonin (sCT) as [¹²⁵I]human calcitonin revealed a more limited distribution of binding sites in the rat brain likely because of its rather low affinity (Skofitsch and Jacobowitz, 1992). The partial isolation of sCT-like peptide(s) from the mammalian brain supports the existence of endogenous ligand(s) for these binding sites (Fischer *et al.* 1983; Sexton and Hilton, 1992). The central administration of sCT has been reported to induce anorexia (Freed *et al.* 1979; Yamamoto *et al.* 1982; De Beaurepaire and Freed, 1987a), hyperthermia (Sellami and De Beaurepaire, 1993), analgesia (Sellami and De Beaurepaire, 1993) and to decrease locomotor activity (Twery *et al.* 1983). On the other hand, the central administration of human calcitonin failed to produce significant anorexic effects in the rat (Yamamoto *et al.* 1982). This unique neurobehavioral profile suggests a distinct role for sCT-like peptide in the rat brain.

CGRP shares ~50% sequence homology with amylin, a recently isolated peptide from human insulinoma and from pancreases of type 2 diabetic patients (Westermarck *et al.* 1986; Cooper *et al.* 1987; Nishi *et al.* 1990). Thus far, only low amounts of amylin-like immunoreactivity have been detected in the rat hypothalamus (Chance *et al.* 1991). On the other hand, recent studies have reported the existence and distribution of specific amylin binding sites in the rat brain. The localization of amylin sites is different from that of CGRP sites, but rather similar to the one reported for sCT sites (Goltzman and Mitchell, 1985; Beaumont *et al.* 1993; van Rossum *et al.* 1994). Direct administration of amylin in the brain has been shown to produce anorexia (Chance *et al.* 1991), adipisia

(Balasubramaniam *et al.* 1991; Chance *et al.* 1992a) and to induce amnesia (Flood and Morley, 1992).

These three structurally related peptides thus seem to share some central effects like anorexia, albeit with different potencies (Krahn *et al.* 1984; Chance *et al.* 1991). Similarly, few brain areas, including the nucleus accumbens and the ventral striatum, are enriched with specific receptor sites for all three peptides (Henke *et al.* 1985; Kruger *et al.* 1988; Skofitsch and Jacobowitz, 1992; van Rossum *et al.* 1994). However, limited cross-reactivity appears to exist between each of these three receptor sites in most brain areas. Only a few areas such as the nucleus accumbens have revealed atypical binding properties with sCT and amylin having rather high affinity for [¹²⁵I]hCGRP α sites (Sexton *et al.* 1988; Dennis *et al.* 1991; van Rossum *et al.* 1994). We were therefore interested to evaluate the potential functional significance of hCGRP α , sCT and amylin and their receptors in the nucleus accumbens. Activation of dopamine neurons in the mesoaccumbens pathway is critical for the initiation of locomotion (Kelly *et al.* 1975; Makanjuola *et al.* 1980; Clarke *et al.* 1988). At first, we determined the effects of each peptide on amphetamine-induced locomotion as it is well accepted that the nucleus accumbens plays a critical role in amphetamine-induced hyperactivity (Pijnenburg *et al.* 1976; Clarke *et al.* 1988; for review, Seiden *et al.* 1993), as well as in spontaneous locomotion. Several other neurotransmitters including acetylcholine, glutamate, GABA and enkephalins are also involved in locomotor behaviors (for recent review, Angulo and McEwen, 1994). Amongst these, the existence of strong interaction between dopamine and GABA in modulating locomotion within limbic areas is well established (Austin and Kalivas, 1989; Kalivas *et al.* 1991; Wong *et al.* 1991). CGRP and sCT-like peptides have been reported to not directly modulate mesolimbic dopaminergic transmission (De Beaupaire and Freed, 1987b; Masuo *et al.* 1991; Clementi *et al.* 1992; Drumheller *et al.* 1992). We have therefore explored the possible involvement of GABAergic pathways in the mediation of the effects of hCGRP α , amylin and sCT on locomotor behaviors.

7.3 Materials and Methods

7.3.1. Animals and Materials

Male Sprague-Dawley rats (250-275 g) were purchased from Charles River (St-Constant, Québec, Canada) and kept according to the Guidelines of the Canadian Council for Animal Care and McGill University. Food and water were available *ad libitum*. Animals tested for spontaneous motor activity were kept in an inverse light-dark cycle room for a minimum of two weeks prior to experiments.

D-amphetamine sulfate was obtained by the Health Protection Branch of Health and Welfare Canada. (-)-Bicuculline methiodide was purchased from Research Biochemicals International (MA). hCGRP α was synthesized in our laboratory as previously described in details elsewhere (Mimeault *et al.* 1992), whereas sCT and rat amylin were purchased from Bachem California (Torrance, CA). All drugs were dissolved in sterile 0.9% saline solution (Abbott Laboratories, Montréal, Canada) and prepared immediately before the experiments.

7.3.2 Surgery

For cannulae implantation, anesthesia was induced using a mixture of ketamine (80 mg/kg)-xylazine (12 mg/kg) given intraperitoneally. Animals were placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA). The skull was exposed by a sagittal incision of 1.5 cm, cleaned and dried. A stainless-steel guide cannula (23 gauge) was stereotaxically implanted at 2 mm above the left lateral ventricle (anteroposterior, -0.4; and midlateral, +1.5 mm relative to bregma). The cannula was then fixed to the skull with dental acrylic cement (Durelon, Espe-Premier, Ontario, Canada) and a protection cap was inserted into the cannulae. The animals were allowed to recover from surgery for at least 5 days before experiments.

7.3.3 Measurements of locomotion

Locomotion was measured in activity chambers equipped with 2 photocell detectors. Rats were first habituated to the activity chambers for a minimum of 30 min prior to treatments. Individual animals were then taken, injected subcutaneously with either d-amphetamine sulfate (2 mg/kg), (-)-bicuculline methiodide (0.5 mg/kg) or saline

and immediately returned to their respective activity chambers. Fifteen min after, intracerebroventricular injections of hCGRP α , sCT or rat amylin (0.04-20 μ g/10 μ l) or saline were performed through a 30 gauge injection needle connected to a 50 μ l Hamilton syringe by a polypropylene tubing (PE-10). Following the injection, about 1 min was allowed before retrieval of the injection needle to avoid reflux into the guide cannulae. After experiments, rats were sacrificed and the brain was removed, snap-frozen in 2-methylbutane (-40°C) and sectioned to verify the location of the cannula.

7.3.4 Data analysis

In animals treated with d-amphetamine, the number of photocell interruptions (activity counts) were recorded automatically for a total of 3 hours. The data were tabulated as total photocell counts for 2 hrs, i.e. between 30 min and 150 min of recording. The first 30 min of recording was not included because of animal manipulations and to allow for drug interactions. Spontaneous locomotor activity was recorded for 150 min and data were tabulated as total photocell counts between 30 and 120 min. A minimum of 6 rats were tested at each dose. Data from all experiments were analyzed using Sigmastat (Jandel Scientific, CA). Analysis of variance was performed to compare various groups with control. The 95% level of confidence was accepted as statistically significant in all analysis.

7.4 Results

7.4.1 Effects on amphetamine-induced locomotion

Over 2300 activity counts were recorded within 2 hours following a single subcutaneous injection of d-amphetamine (2 mg/kg) in rats (Fig 7.1) compared to less than 100 activity counts for the saline-treated rats. Intracerebroventricular injection of sCT (0.08 μ g) induced substantial decrease in amphetamine-induced locomotion. Maximal effects were observed at 0.16 μ g at which dose, sCT reduced the amphetamine-induced locomotion by about 40% (Fig 7.1). Rat amylin was also potent with significant effects observed at 0.625 μ g and a maximal effect ($\sim$ 40%) seen at 1.25 μ g (Fig 7.1). Finally, hCGRP α was approximately forty and four times less potent in decreasing amphetamine-induced locomotion than sCT and amylin, respectively, as a minimal dose of 2.5 μ g was required to significantly reduce the amphetamine-induced locomotion (Fig 7.1). No further decrease in locomotor activity was observed with doses up to 20 μ g for any of the peptides tested.

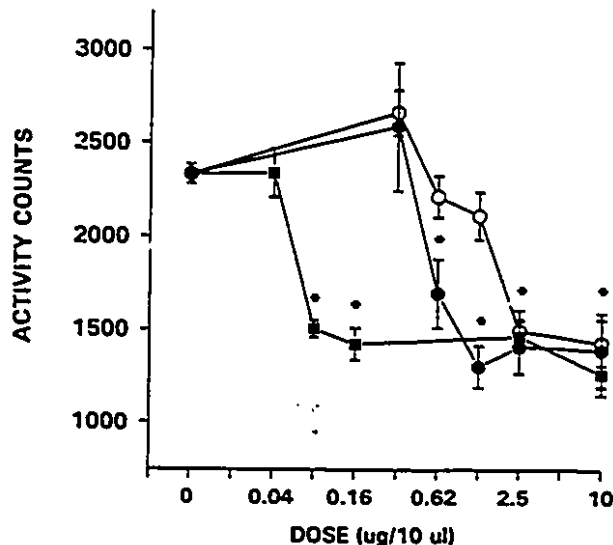


Figure 7.1 Dose dependent effects of intracerebroventricular injections of salmon calcitonin (■), amylin (●) or hCGRP α (○) on amphetamine-induced locomotion. D-amphetamine (2 mg/kg) was injected subcutaneously 15 min prior to the injection of the peptide. Data were tabulated for 2 hours, starting 30 min after the d-amphetamine peripheral injections. A minimum of 6 rats were tested at each dose and data are presented as mean $\pm$ S.E.M. * P <0.05 from amphetamine-treated animals.

7.4.2 Effects on spontaneous locomotion

Total activity counts (~1150) were tabulated for 90 min. The subcutaneous injection of bicuculline methiodide (GABA_A antagonist, 0.5 mg/kg) had no significant effect on the recorded spontaneous motor activity (Fig 7.2). Intracerebroventricular injection of sCT (0.08 µg), rat amylin (0.62 µg) or hCGRPα (1.25 µg) all reduced spontaneous motor activity by as much as 70% (Fig 7.2). Peripheral injection of bicuculline methiodide 15 min prior to the central administration of peptides, significantly blocked the effect induced by hCGRPα but not those of sCT or amylin (Fig 7.2).

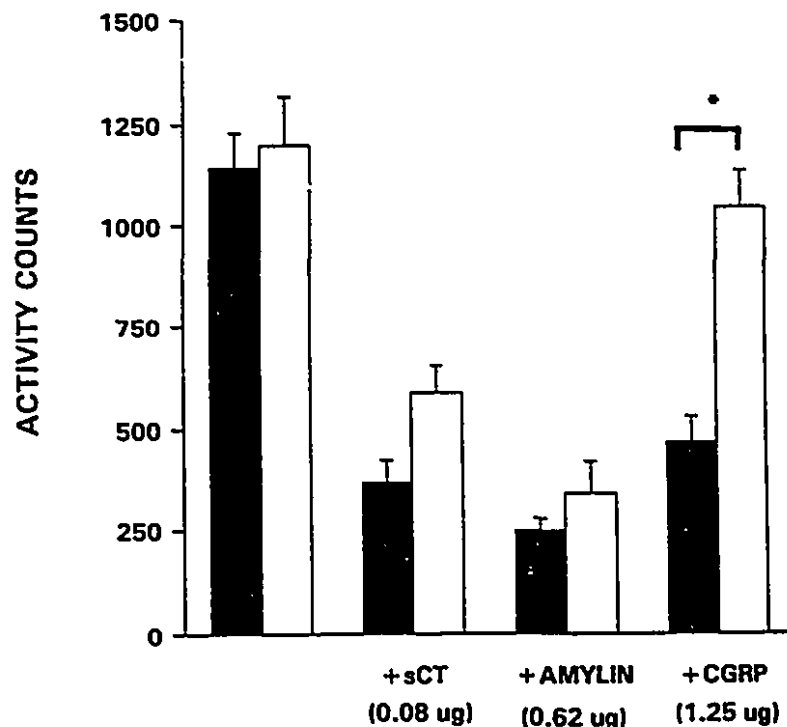


Figure 7.2 Effects of intracerebroventricular injections of salmon calcitonin, amylin or hCGRPα on spontaneous locomotor activity. Rats were kept in an inverse light-dark cycle room for a minimum of two weeks prior to experiments. Bicuculline methiodide (0.5 mg/kg; opened bars) or saline (300 µl; filled bars) were injected subcutaneously 15 min prior to the central administration of peptides. Activity counts were tabulated for 90 min starting 30 min after the injection of bicuculline or saline. A minimum of 6 rats were tested at each dose and data are presented as mean ± S.E.M. *P<0.05 from bicuculline-treated animals.

7.5 Discussion

hCGRP α , sCT and amylin decreased, in a dose-dependent manner, spontaneous and amphetamine-induced locomotion. Similar maximal effects were observed with all three peptides but sCT was the most potent homologue followed by amylin and hCGRP α , respectively. Interestingly, peripheral injection of bicuculline methiodide, a GABA $_A$ receptor antagonist, selectively blocked the effects of hCGRP α on spontaneous locomotion without significantly altering those of sCT and amylin. The mechanism(s) involved in the decrease in locomotor activity induced by these peptides is unknown. The similar effects of all three peptides on amphetamine-induced locomotion, albeit with different potencies, may indicate a common mechanism of action and the interaction with a single class of receptors localize in the nucleus accumbens. On the other hand, the selective blockade by bicuculline methiodide of the effects of hCGRP α on spontaneous locomotion suggests a different mechanism of action for hCGRP α - vs. amylin- and sCT-modulated locomotor activities.

The dopaminergic innervation of the basal ganglia plays a major role in amphetamine-induced locomotion as it is largely mediated by an increased release of dopamine from the presynaptic dopaminergic nerve terminals of the nucleus accumbens (Creese and Iversen, 1975; Pijnenburg *et al.* 1976; Makanjuola *et al.* 1980; for review, Seiden *et al.* 1993). In agreement with this hypothesis, the destruction of the dopaminergic innervation of the nucleus accumbens has been shown to inhibit the amphetamine-induced response (Kelly *et al.* 1975; Joyce and Koob, 1981). In addition, several other neurotransmitter systems are most likely involved in the modulation of locomotor activities, including the cholinergic, GABAergic, glutamatergic and enkephalinergic innervations of the basal ganglia (for review, Angulo and McEwen, 1994). A decrease in the stimulation of the GABA $_A$ receptors of the ventral pallidum appears to be a critical component in motor activity elicited by dopaminergic drugs upon their injection in the nucleus accumbens (Mogenson and Nielsen, 1983; Swerdlow *et al.* 1986; Shreve and Uretsky, 1988; Klitenick *et al.* 1992; Churchill *et al.* 1991). This is in keeping with the data reported in the present study. In contrast to GABA $_A$ receptors, the direct application of GABA $_B$ receptor agonists or antagonists elicited little and no effects on these nucleus accumbens-related dopaminergic behaviors (Wong *et al.* 1991). Furthermore, a major projection from the nucleus accumbens to the ventral pallidum has been reported to be both GABAergic and enkephalinergic (Fonnum *et al.* 1978; Zham *et al.* 1985) and the amphetamine-induced locomotion can indeed be reversed by the

administration of naloxone, an opioid antagonist, or GABA antagonists (Austin and Kalivas, 1989; Kalivas *et al.* 1991; Angulo and McEwen, 1994).

Central administration of hCGRP α induced a decrease in both spontaneous and amphetamine-induced locomotion. Previous reports have shown that CGRP α also produces catalepsy and hypomotility (Jolicœur *et al.* 1992; Clementi *et al.* 1992). Although these behaviors are generally modulated by the dopaminergic innervation of the basal ganglia, no significant change in the levels of dopamine or its metabolite DOPAC has been observed in the striatum following the central administration of CGRP α (Clementi *et al.* 1992; Drumheller *et al.* 1992). In addition, neurochemical lesion experiments do not support the existence of a direct interaction between CGRP receptors and dopaminergic nerve terminals in the nucleus accumbens as unilateral 6-hydroxydopamine injection in the ventral tegmental area has been reported to not significantly alter [125 I]CGRP binding in the nucleus accumbens (Masuo *et al.* 1991). In contrast, significant decrease in [125 I]CGRP binding has been observed following quinolinic acid lesions of the nucleus accumbens suggesting that the CGRP receptors are located on intrinsic neurons of the accumbens and not on dopaminergic nerve terminals of the mesoaccumbens projection (Masuo *et al.* 1991). Accordingly, hCGRP α -induced decreased locomotion is unlikely through a direct action on dopaminergic nerve terminal activities. In fact, the effects of hCGRP α on spontaneous locomotion are apparently mediated through an action on the GABAergic neurons by inducing the release of this inhibitory transmitter as a GABA $_A$ receptor antagonist blocked the action of hCGRP α . In contrast to CGRP effects on locomotion, the anti-amnesic action of CGRP is not modified by a GABA $_A$ receptor blocker suggesting that not all central effects of this peptide are associated to a modulation of the GABAergic tone (Kovács and Telegdy, 1992; Kovács and Telegdy, 1994).

Salmon calcitonin was most potent in decreasing spontaneous and amphetamine-induced locomotor activities. Earlier studies have already reported on the decrease in amphetamine-induced locomotion by sCT (Twery *et al.* 1983; De Beaurepaire and Freed, 1987b). The nucleus accumbens and the hypothalamus have been identified as key structures in the sCT-induced decrease in locomotion (De Beaurepaire and Freed, 1987b). However, a direct action of sCT on dopaminergic neurons appears unlikely as this peptide did not elicit significant behaviors when injected in the striatum, an area enriched with high levels of dopamine (De Beaurepaire and Freed, 1987b). Moreover, similar to [125 I]CGRP binding, [125 I]sCT labeling was not altered in the nucleus accumbens following the injection of 6-hydroxydopamine in the ventral tegmental area, suggesting that [125 I]sCT binding sites are not located on dopaminergic terminals (Masuo *et al.* 1991). However, a

treatment with the GABA_A receptor antagonist failed to alter the action of sCT. Thus, the mechanism by which sCT mediates its locomotor effects remains to be established.

Amylin was less potent than sCT (~ 8 fold) but more than hCGRP α (~4 fold) in decreasing amphetamine-induced locomotion. Thus far, only low levels of amylin-like immunoreactive material have been detected in the brain (Chance *et al.* 1991). Technical difficulties might account for these results as various groups including ours were able to detect specific binding sites for [¹²⁵I]amylin in the rat brain (Beaumont *et al.* 1993; van Rossum *et al.* 1994); sCT demonstrating high affinity for these amylin binding sites (Beaumont *et al.* 1993; van Rossum *et al.* 1994). The nature of the endogenous ligand(s) for these amylin/sCT sites thus remains to be clarified and may be related to the sCT-like peptide(s) recently isolated from the CNS (Sexton and Hilton, 1992). Moreover, amylin has also been reported to be a potent modulator of food intake following central or peripheral injections (Chance *et al.* 1991; Chance *et al.* 1993). The intrahypothalamic injection of amylin has been shown to induce increase in dopamine and serotonin turnovers in various brain areas including the nucleus accumbens, striatum and hypothalamus (Chance *et al.* 1991). On the other hand, the increase in serotonin and dopamine metabolism may be related to an increased transport of the precursors tyrosine and tryptophan across the blood-brain barrier (Chance *et al.* 1992b). Hence, to our knowledge, no clear evidence is presently available to support a direct interaction between amylin and dopaminergic innervation. The mechanisms by which amylin decreased amphetamine-induced and spontaneous locomotor behaviors are thus still unclear but likely do not involve a direct interaction with GABAergic innervation in the basal ganglia as bicuculline failed to alter the effects of amylin on locomotor activity.

Further experiments will be essential to delineate the precise mechanisms by which these three peptides modulate locomotion. The potential effect of selective antagonists for various dopamine, glutamate and opioid receptors would certainly be of interest. Moreover, intra-accumbens injections of each peptide would facilitate a clear interpretation of the data. In summary, central administration of hCGRP α , sCT and amylin all decreased both amphetamine-induced and spontaneous locomotion. The mechanisms by which these peptide homologues induce their effects is still unclear. However, hCGRP α likely interacts via a GABAergic pathway to decrease spontaneous locomotor activity. Taken together, these data hence support the existence of functionally distinct binding sites for hCGRP α , sCT and amylin in the rat brain modulating locomotor behaviors.

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CHAPTER 8

Conclusion

McGill University (1994)

8.1 CGRP and related peptides

A widespread variety of peripheral and central biological actions have been reported for the various members of the CGRP/calcitonin family of peptides, ranging from peripheral vasodilatation to anorexia, as reviewed in Chapter 1. These effects are mediated through specific receptor sites. Relatively little is known about the biochemistry and topography of these receptor sites as thus far, only calcitonin receptors have been cloned. Moreover, the precise nature of the endogenous ligand(s) for some of these receptors (especially amylin and salmon calcitonin) remains to be fully clarified.

As summarized in section 1.2 of Chapter 1, the distributions of CGRP mRNA and CGRP-like immunoreactivity in the brain are well established and globally fairly correlated. The few apparent mismatches observed between the distribution of the peptide and binding sites in certain brain areas (e.g. cerebellum) may suggest an important role for CGRP during development of the central nervous system as transient expression of the peptide was observed at neonatal stages (Morara *et al.* 1989; Provini *et al.* 1992). Technical limitations including low detectable amounts due to fast turnover rate of the mRNA transcript and/or of the peptide might account for some of the mismatches observed between the CGRP mRNA and peptide distributions such as reported in the hypothalamus (Kruger *et al.* 1988). We have shown that CGRP can also act on amylin receptor sites although with significantly lower potency than the prototypic ligand(s) (van Rossum *et al.* 1994b). Similarly, CGRP revealed low affinity for calcitonin binding sites (Albrandt *et al.* 1993; Sexton *et al.* 1993). It is thus unlikely that CGRP would act as the endogenous ligand for either the amylin or calcitonin binding sites.

Based on the data presented in the present thesis as well as from the literature, a summary of the pharmacological profile of putative CGRP and related peptides receptor subtypes is provided in Table 8.1. Clearly, the ultimate pharmacological characterization of putative CGRP receptor subtypes will follow their isolation, purification and cloning. However, in parallel to these molecular approaches, future research should focus on the

identification of new tissue preparations enriched with a single class of the putative CGRP receptor subtypes. The guinea pig urinary bladder and a stable human adrenocarcinoma cell line (Col-29) may represent improved tissue preparations to study the CGRP₂ receptor subtype as they were recently reported to be insensitive (up to 1-3 μ M) to the antagonistic properties of the fragment CGRP₈₋₃₇ (Giuliani *et al.* 1992; Cox and Tough, 1994).

Table 8.1 Summary of the pharmacological profile of putative CGRP and related peptides receptor subtypes

	CGRP ₁	CGRP ₂	Atypical	Amylin	ADM
Selective agonist	none	[Cys(ACM) ^{2,7}]hCGRP α [Cys(ACM) ^{2,7} ,Ala ²⁰]- hCGRP α	hCGRP α sCT amylin	amylin (?)	ADM (?)
Selective antagonist	hCGRP ₈₋₃₇ [Ala ²⁰]hCGRP ₈₋₃₇ hCGRP ₁₂₋₃₇	none	none	AC187	ADM ₂₂₋₅₂ (?)
Prototypical assay	atrium spleen	vas deferens liver urinary bladder Col-29	nucleus accumbens	skeletal muscle	vascular smooth muscle

Based on data presented in this thesis and in the literature

As summarized by Quirion *et al.* 1992, our group was able to provide functional evidence for the existence of CGRP receptor subtypes both in peripheral tissue preparations and in the brain. However, the pharmacological selectivity of these CGRP fragments and analogs seems to be limited to their biological activities as little preferential binding affinity was observed for these same analogs in the various tissue preparations studied (van Rossum *et al.* 1994a). Indeed, these findings could support the existence of different binding domains on the various receptor subtypes for the fragment hCGRP₈₋₃₇ as the latter revealed high binding affinity for both CGRP₁ and CGRP₂

subtypes whereas being functionally active only on the CGRP₁ sites (van Rossum *et al* 1994a). The development of more specific peptide and non-peptide analogs is thus also crucial for the successful characterization of CGRP receptor subtypes. In that regard, CGRP analogs with a substitution of the glycine residue in position 14 by an aspartate are currently under evaluation. This substitution should allow for prolonged biological activities such as previously reported in pre-osteoblast cell lines (Thiebaud *et al.* 1991). The increased potency may also result into a higher specificity of the various analogs for a given receptor type. Moreover, from the initial evaluation of a series of novel analogs with modifications of residues 13 to 23, it appears that the substitution of the glycine residue by an alanine in position 20 in the fragment hCGRP₈₋₃₇ ([Ala²⁰]hCGRP₈₋₃₇) as well as in the linear analogue [Cys(ACM)^{2,7}]hCGRP α ([Cys(ACM)^{2,7},Ala²⁰] hCGRP α) demonstrated increased selectivity for the CGRP₁ and CGRP₂ receptor subtype, respectively (Lavallée *et al.*, 1994). The binding profile of these novel analogs is thus certainly worth investigating and should provide new insights with respect to binding requirements of the various CGRP receptor subtypes.

The respective distribution of amylin mRNA and immunoreactive material in the brain is not yet well defined. At the recent 2nd international symposium on CGRP and related peptides (August 1994, Montréal), Skofitsch *et al* (1994) presented for the first time a map of the widespread distribution of amylin-like immunoreactivity in the rat brain. Although these data are most interesting, the specificity of the polyclonal antisera used in these experiments was not fully established raising possible doubts as to the genuine amylin-like nature of the observed staining. Further studies will thus be required to rule out potential candidates for cross-reactivity with the amylin probe such as sCT-like peptide especially since sCT revealed high affinity for the amylin binding sites in the rat brain (see below). Purification and sequencing of amylin-like peptides present in the rat brain could also be performed from brain extracts from various nuclei that revealed high amounts of amylin binding sites including the nucleus accumbens, several nuclei of the hypothalamus, dorsal raphe and locus coeruleus. Following initial separation by, for example, ion exchange chromatography, the various collected fractions could then be tested for amylin-like activity such as glucose uptake as well as increase in cAMP in

skeletal muscle preparations as those receptors are coupled to adenylate cyclase (Deems *et al.* 1991). If any fraction revealed positive for amylin-like activity, further purification by HPLC and sequencing would be most appropriate to uncover the identity of the peptide(s).

We have recently described the distribution and binding profile of specific amylin binding sites in the rat brain (van Rossum *et al.* 1994b). The ligand selectivity profile of these sites apparently differ from all other binding sites reported thus far, including those for CGRP. Interestingly, salmon calcitonin revealed high affinity for the amylin binding sites present in the rat brain. Furthermore, rat amylin was recently reported to be equipotent to salmon calcitonin to stimulate the production of cAMP in a cell line transfected with the cloned porcine calcitonin receptors (Sexton *et al.* 1994). On the other hand, amylin revealed low affinity for all other forms of the calcitonin receptors cloned thus far. Hence, these reports support the existence of calcitonin binding sites different from amylin binding sites. However, it still remain unclear if the amylin binding sites present in the rat brain correspond to a sub-population of the calcitonin receptors as salmon calcitonin also revealed high affinity for these amylin binding sites. These similarities between some amylin and calcitonin binding sites thus leave open the question about the nature of the endogenous ligand(s) acting with high affinity on the amylin binding sites in the central nervous system. Potential candidates include the recently isolated sCT-like molecule(s) from the rat brain (Sexton and Hilton, 1992). However, further studies are required to confirm this hypothesis.

The development of potent antagonist specific for amylin such as the new analogue, ac-³⁰Asn, ³²Tyr, sCT₈₋₃₂ (AC187, Beaumont *et al.* 1994) should also help in providing evidence in regard to the uniqueness and functional role of the amylin receptors. The inhibition by amylin of insulin-stimulated glucose incorporation into the rat soleus muscle glycogen was shown to be potently antagonized by AC187 followed by sCT₈₋₃₂ and CGRP₈₋₃₇, but with much lower potency (Beaumont *et al.* 1994). New pharmacological tools such as AC187 should thus be most helpful to determine the effects of amylin that are mediated through amylin receptors vs. those likely related to actions at CGRP or calcitonin receptors. Moreover, the activity profile of this new

amylin antagonist on the central actions of amylin such as the decrease in amphetamine-induced and spontaneous locomotions (Chapter 7), would be most essential to shed light on the nature of amylin binding sites in the brain. Similarly, the activity profiles of the fragments sCT₈₋₃₂ and CGRP₈₋₃₇ on the effects induced by the respective native peptides (i.e. sCT and CGRP) as well as amylin on locomotion would also be worth investigating to characterize further the nature of receptors involved in these behaviors. Furthermore, the modulation by dopaminergic, cholinergic, GABAergic and glutamergic agonists/antagonists of the effects of CGRP and related peptides on locomotion would also be most appropriate. Indeed, these neurotransmitters were all reported to play a role in locomotor behaviors (Angulo and McEwen, 1994). As described in Chapter 7 of the present thesis, we have thus far only investigated the effects of a GABA_A antagonist, bicuculline, as CGRP and sCT-like peptides have been previously reported *not* to directly modulate mesolimbic dopaminergic transmission (De Beaurepaire and Freed, 1987; Masuo *et al.* 1991; Clementi *et al.* 1992). Indeed, the GABA_A antagonist could selectively block the effects induced by CGRP on spontaneous locomotion. The decrease induced by CGRP on spontaneous locomotion is thus apparently linked to a modulation of the GABAergic neurons possibly by increasing the release of GABA as the latter usually acts as an inhibitory transmitter. Under our experimental conditions, amylin and sCT do not seem to directly interact with the GABAergic neurons. However, a wider range of concentrations of the various peptides as well as of the GABA_A/GABA_B antagonists should be tested. Different routes of injection should also be studied: for example, central vs. peripheral injections and icv vs. intra-accumbens should be examined in order to precisely establish the mechanism(s) of action of CGRP-like peptides on locomotor activity.

Both salmon and human calcitonin-like immunoreactivity as well as calcitonin binding sites have been identified in the rat brain (Cooper *et al.* 1980; Fischer *et al.* 1981; Skofitsch and Jacobowitz, 1992; Shah *et al.* 1993). Indeed, salmon calcitonin-like peptides have been detected in both human (Fischer *et al.* 1983) and rat brain (Sexton and Hilton, 1992) but their full characterization and sequencing are still awaited. However, it is already well established that salmon calcitonin is usually significantly more

potent than human calcitonin in eliciting various central and peripheral biological actions in the rat as extensively described in Chapter 7. Moreover, as summarized in section 1.4 of Chapter 1, several calcitonin receptors have been cloned from different tissues including the kidney and the brain. Their primary amino acid sequences correlate with the receptor topography of members of the G protein-coupled receptor super-family: the greatest sequence similarity being observed with secretin, vasoactive intestinal peptide and parathyroid hormone receptors (Lin *et al.* 1991; Ishihara *et al.* 1991; 1992; Juppner *et al.* 1991). Up to now, two receptors with high affinity for salmon calcitonin have been cloned from the rat brain, namely the C1a and C1b receptor subtypes. Neither CGRP nor amylin demonstrated high affinity for these calcitonin receptor subtypes (Sexton *et al.* 1993). Therefore, these calcitonin receptors do not share the ligand binding profile of the atypical binding sites present in the nucleus accumbens described in Chapters 2 and 4. Several lines of evidence support that CGRP receptors (CGRP₁, CGRP₂ and CGRP/salmon calcitonin/amylin-sensitive sites) also belong to the G protein-coupled receptor super-family. Indeed, [¹²⁵I]CGRP binding was sensitive to the presence of GTP and/or analogs in tissue preparations enriched with the various CGRP receptor subtypes (van Rossum *et al.* 1993). In addition, [¹²⁵I]CGRP binding in skeletal muscle, lung and cardiac myocytes was also reported to be sensitive to the presence of GTP nucleotides, as summarized in Chapter 6. Hence, CGRP receptors most likely belong to the G protein-coupled receptor super-family. However, cloning is awaited to confirm this hypothesis.

As reviewed in Chapter 5 of the present thesis, very little is currently known in regard to the tissue distribution, receptor sites and biological effects of adrenomedullin as its isolation was reported just about a year ago. From our study, it would appear that adrenomedullin possesses rather low affinity for both CGRP and amylin binding sites in the rat brain (van Rossum *et al.* 1995). Therefore, it is unlikely that adrenomedullin acts as an endogenous ligand on these binding sites suggesting the existence of an additional class of adrenomedullin-preferring receptors. In agreement with this hypothesis, recent reports support the existence of specific high affinity adrenomedullin binding sites in cultured rat vascular smooth muscle cells (Eguchi *et al.* 1994a). However, the use of

radiolabelled adrenomedullin as ligand will be required to fully establish the existence of this unique receptor class in the rat brain. Finally, Eguchi *et al.* (1994b) recently reported the antagonistic properties of the C-terminal fragment hADM₂₂₋₅₂. Although the potency of this new pharmacological tool is rather low (micromolar), it might become useful to differentiate between the CGRP vs. adrenomedullin receptors-mediated biological effects. In addition, this novel tool could provide the initial template to design new molecules with antagonistic properties for the adrenomedullin vs. CGRP receptor sites.

Various members of the CGRP/calcitonin family of peptides are under investigation for clinical applications or are already in use for treatments of various disorders. As described in section 1.3 of Chapter 1 of the present thesis, CGRP is one of the most potent vasodilator substance known (Brain *et al.* 1985; Girgis *et al.* 1985). Consequently, highly potent non-peptide CGRP antagonist could have great clinical potentials in the treatment of several medical entities including migraine, subarachnoid haemorrhage and ischemia (Johnston *et al.* 1990; Mair *et al.* 1990; Goadsby and Edvinsson, 1993; Franco-Cereceda *et al.* 1993). The synthesis of peptidomimetic analogs would facilitate the clinical use of CGRP and related molecules. Such tools should also prove most useful toward a clearer demonstration of the physiological implications of CGRP in the organism. Moreover, efforts should be made toward the design of subtype-specific peptidomimetic analogs. This aspect becomes especially relevant in order to limit potential side effects induced by the activation of various CGRP receptors e.g. hyperthermia, tachycardia and hypoactivity (Fisher *et al.* 1983; Jolicœur *et al.* 1992; Clementi *et al.* 1992). Ideally, by the use of subtype-selective analogs, one might be able to avoid some of the possibly undesired actions induced by CGRP.

Salmon calcitonin has been used for more than a decade now in the treatment of severe osteoporosis, Paget's disease and hypercalcemia (Carstens and Feinblatt, 1991). As observed in the rat, lower doses of salmon than human calcitonin were required to induce similar beneficial effects in human therapy. The side-effect profile of salmon calcitonin was thus shown to be favorable in comparison to its human homologue. The synthesis of new salmon calcitonin analogs with increased bioavailability as well as

improved dosage formulations could render this peptide more convenient to use in the clinic (Carstens and Feinblatt, 1991). However, caution should also be taken with respect to small sCT-like molecules that could cross the blood brain barrier especially since sCT revealed to be *most* potent in inducing a variety of central effects including anorexia and hypoactivity (Freed *et al.* 1979; Twery *et al.* 1983).

The potential use of amylin antagonists in the treatment of non-insulin-dependent diabetes is under intensive research (Cooper, 1994; Young *et al.* 1994). Indeed, the actions of amylin on muscle glycogen metabolism and on endogenous glucose production may contribute to the glucose intolerance observed in this pathology (Young *et al.* 1994). The development of highly specific antagonists such as AC187 is thus critical to delineate the physiological role(s) of amylin and its potential significance in the treatment of some forms of diabetes. Moreover, the nature of the endogenous ligand of the amylin binding sites present in the brain remains to be clarified. However, following its central administration, amylin was reported to increase dopamine and serotonin turnover as well as to induce amnesia and anorexia (Chance *et al.* 1991; Flood and Morley, 1992). Moreover, high concentrations (micromolar) of amylin were found to be toxic to primary hippocampal cultures (May *et al.* 1993). Finally, it is unknown if the amylin binding sites that mediate the peripheral actions of amylin differ from the ones located in brain. Nevertheless, the affinity of novel amylin analogs should be evaluated in both peripheral and central tissues to prevent potential harmful central side effects.

Taken together, future research aimed at the development of subtype-specific peptidomimetic analogs would facilitate the clinical use as well as delineate the pathophysiological implications of CGRP and related peptides in the organism. In regards to receptors, the respective cloning of the CGRP, amylin and possibly adrenomedullin receptor proteins is anxiously awaited and should greatly help to provide key insights as to CGRP and related peptides' pharmacology.

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APPENDIX I

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